Detection of *Mycobacterium tuberculosis* resistance mutations to rifampin and isoniazid by real-time PCR

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Abstract

**Objective:** The objective of our study was to evaluate the use of a real-time polymerase chain reaction (PCR)-based technique for the prediction of phenotypic resistance of *Mycobacterium tuberculosis*. **Materials and Methods:** We tested 67 *M tuberculosis* strains (26 drug resistant and 41 drug susceptible) using a method recommended for the LightCycler platform. The susceptibility testing was performed by the absolute concentration method. For rifampin resistance, two regions of the *rpoB* gene were targeted, while for identification of isoniazid resistance, we searched for mutations in *katG* and *inhA* genes. **Results:** The sensitivity and specificity of this method for rapid detection of mutations for isoniazid resistance were 96% (95% CI: 88% to 100%) and 95% (95% CI: 89% to 100%), respectively. For detection of rifampin resistance, the sensitivity and specificity were 92% (95% CI: 81% to 100%) and 74% (95% CI: 61% to 87%), respectively. The main isoniazid resistance mechanism identified in our isolates is related to changes in the *katG* gene that encodes catalase. We found that for rifampin resistance the concordance between the predicted and observed phenotype was less than satisfactory. **Conclusions:** Using this method, the best accuracy for genotyping compared with phenotypic resistance testing was obtained for detecting isoniazid resistance mutations. Although real-time PCR assay may be a valuable diagnostic tool, it is not yet completely satisfactory for detection of drug resistance mutations in *M tuberculosis*. 
**Keywords:** *Mycobacterium tuberculosis*, real-time PCR, resistance

**Introduction**

Tuberculosis (TB) remains a major global health problem despite the availability of effective antituberculosis therapy for over 50 years. The World Health Organization (WHO) estimates that approximately one-third of the global community is infected with *Mycobacterium tuberculosis*. According to WHO data, with regard to infection rates, Romania is among the top five countries from the European region, with high notification rates both for new and relapse cases of TB; more than 25000 new and relapse cases are recorded every year.\(^1\)\(^2\)

Since the early 1990s, an alarming trend and a growing source of public health concern has been the emergence of resistance to multiple drugs. Multidrug resistance (MDR) is defined as resistance to at least isoniazid (INH) and rifampicin (RMP). Although it remains unclear whether the drug-resistant strains are less transmissible than the susceptible strains,\(^3\) infection-control precautions need to be maintained, since patients with drug-resistant TB are likely to remain infectious for long periods. Thus, the public health consequences of drug-resistant tuberculosis might be more serious than those of drug-susceptible disease.

The prevalence of *M tuberculosis* (MTB) drug resistance in Romania was recently evaluated by a national survey performed between 2003-2004. This showed that 3.6% of the strains isolated from newly diagnosed patients and 8.6% from relapse cases were resistant to one antituberculosis drug (INH).\(^2\) Moreover, the results of this study indicate that MDR was observed in 2.9% of the MTB strains from newly diagnosed patients and in 11% of those isolated from relapse cases. Taking into account that in Romania more than 25000 TB cases (new cases and relapses) are reported each year, we can estimate that more than 1100 patients are infected with MDR-TB strains.

The need to limit the transmission of drug-resistant strains and to reduce the time between diagnosis and effective therapy requires rapid identification of resistance. Classical phenotypic determination of resistance may take up to 10 weeks after referral of a sample to the laboratory. Nucleic acid amplification assays can greatly shorten the detection time. Due to this major advantage, in the last few years, a lot of effort has been invested in designing performance protocols for genotyping MTB strains. Real-time PCR came to be the main approach because of its special features: high sensitivity and specificity as well as speed, with no need for any post-PCR sample manipulation. The results from fundamental research (such as the sequencing of the complete MTB genome) were used to design specific primers and probes that would allow the identification of gene mutations associated with drug resistance in MTB.

It is known that RMP interferes with RNA synthesis by binding to bacterial RNA polymerase. Resistance to RMP is conferred by mutations resulting in at least eight amino acids substitutions in the *rpoB* subunit of RNA polymerase. Mutations in a limited region of *rpoB* have been found in >95% of RMP-resistant clinical isolates of MTB and has been shown to result in high-level resistance (MIC >32 μg/mL) to RMP and cross-resistance to all rifamycins.\(^4\)

INH acts by inhibiting an oxygen-sensitive pathway in the mycolic acid biosynthesis of the cell wall. At least four genes have been described to be involved in resistance to isoniazid: the katG gene, which encodes a catalase; the *inhA* gene, whose product is a target for INH; and the *oxyR* gene and the neighboring *aphC* gene, as well as their intergenic region.\(^5\) Several real-time
PCR-based methods targeting these specific genomic regions have been described. The purpose of the present study was to evaluate the LightCycler instrument in the detection of these mutations associated with resistant MTB strains isolated from Romanian patients.

**Materials and Methods**

**Strains and resistance testing**

Forty-one susceptible and twenty-six resistant clinical isolates of MTB (23 resistant to both INH and RMP, 1 mono-RMP resistant, and 2 resistant to INH only) from 62 different patients were studied. The susceptibility testing was performed by the absolute concentration method (Meisser). This method is based on the comparison between the growth of mycobacteria on drug-free medium with that of growth on drug-containing media (antituberculosis drugs incorporated in the medium at different concentrations) 21 days after inoculation with a standardized inoculum. Two critical concentrations were used for every tested drug: 0.2 μg/mL and 1 μg/mL for INH and 20 μg/mL and 40 μg/mL for RMP. According to this method, resistance to a drug is defined by the growth of more than 20 colonies on drug-containing media (INH 1 μg/mL, RMP 40 μg/mL).

**Extraction of mycobacterial DNA**

We extracted MTB DNA by the thermal lysis procedure in the presence of Chelex 100 (Amersham Pharmacia Biotech, Uppsala, Sweden). Briefly, we obtained one loopful of bacteria scraped from Löwenstein-Jensen solid medium and suspended it in 100 μL sterile water; the same volume of Chelex 10% suspension was added and the mixture was incubated for 45 minutes at 45°C and 5 minutes at 100°C. The samples were centrifuged at 12000 g for 5 minutes and the supernatant was used in the subsequent steps of the experiment.

**Real-time PCR using the LightCycler**

The MTB drug-resistance genotyping was performed by adapting a previously described protocol. The method published by Torres et al. was designed as a single-tube method capable of detecting RMP and INH resistance mutations; one set of primers and two fluorescently labeled hybridization probes were used for each targeted region. One set of primers and two sets of probes (rpoB1 and rpoB2) that targeted the rpoB gene were used for detection of RMP resistance and one set of primers and probes each for the katG and inhA genes in order to test for INH resistance. All primers and probes were synthesized by TIB Molbiol (DNA Synthesis Service; Roche Diagnostics, Berlin, Germany). The real-time PCR was followed by melting curve analysis, both performed on the LightCycler instrument (Roche Diagnostics, Mannheim, Germany). We used the same PCR conditions (components concentration, cycling, and melting programs) as previously described, but we added 10 more cycles of amplification to the 35 recommended. We included into each experimental run one negative control (the DNA template was replaced with PCR-grade water) and one positive control (the DNA template was isolated from *M. tuberculosis* H37Rv, a strain susceptible to both INH and RMP).

**DNA sequencing**
Direct PCR sequencing was performed with the commercial BigDye terminator DNA sequencing kit (Applied Biosystems, CA, USA), according to the manufacturer's recommendations. Briefly, the extracted DNA was amplified with the same primers used in the real-time PCR. The thermal cycling was performed on a GeneAmp System 9700 (Applied Biosystems, CA, USA) thermal cycler. The resulting PCR product was purified using MicroCon YM-100 concentrators (spin columns) and sequenced bidirectionally using the BigDye terminator chemistry. The capillary electrophoresis was performed on an ABI Prism 3100-Avant genetic analyzer (Applied Biosystems, CA, USA) and the raw analysis of the sequences was made by Sequencing Analysis Software, version 3.7. Finally, the sequences were aligned with BioEdit software (version 5.0.6) (www.mbio.ncsu.edu/BioEdit/bioedit.html) in order to generate an assembled full range sequence, which was then compared with the MTB H37Rv sequence (GenBank Accession No. NC000962).

Statistical analysis

In order to determine the cutoff of Tm changes to predict mutations associated with resistance, we generated ROC curves. The area under the ROC curves was then determined, and the cutoff points were identified to maximize test sensitivity (and thus decrease the false negative rate). To further enhance sensitivity we also assessed in parallel the tests detecting the presence of either rpoB1 or rpoB2 for RMP resistance and the presence of either katG or inhA gene for INH resistance. SPSS 10.0 software (SPSS, Inc., Chicago, IL, USA) was used for the database construction and ROC curves, and CAT maker 1.1 (Centre for Evidence-Based Medicine, Oxford, GB, 2004) to calculate the attributes of the diagnostic tests studied.

Results

During the real-time PCR experiments, the amplification of the DNA template was monitored by continuously measuring the fluorescence level. For samples as well as for the positive control, the fluorescent signals started to rise at a number of cycles, ranging between 20-35. The Tm s for the probes annealed to the PCR product were generated by running the melting analysis program (ramping from 50°C to 85°C with 0.1°C per second) and calculated using the LightCycler software. While running different sets of samples along with the positive control, we observed that the melting temperature (Tm) for the MTB H37Rv was variable, ranging between 70.08°C and 71.26°C. Therefore, for each experimental run we analyzed the changes in Tm for the PCR products derived from our collection of resistant and susceptible MTB clinical isolates as compared with the Tm of the H37Rv tested in the same run rather than using directly the observed Tm s.

We also noticed that the Tm values for the H37Rv strain as well as for other wild-type (wt) field strains tested were lower than expected (70.08°C for H37 as compared with 72.8°C in the original communication). For the resistant strains, in one case (sample 2312), we obtained a lower Tm value than the expected Tm, the δTm was however consistent with AGC > ACC mutation in position 315. In [Figure 1] we have represented the melting profiles for the positive control (H37Rv) and the other seven samples when we analyzed the katG PCR products. It can be seen that sample 2312 has a melting profile different from that of H37Rv; this is in agreement with the phenotypic results, which scored this sample as resistant to INH. The other isolates had the same melting profile as the positive control and were also found to be susceptible to INH by the phenotypic analysis.
The $T_m$ changes for the products derived from our collection of resistant and susceptible MTB clinical isolates as compared with $T_m$ of the susceptible strain varied widely: 0.00-2.29 ($rpoB1$), 0.00-4.63 ($rpoB2$), 0.01-3.32 ($inhA$), and 0.01-5.86 ($katG$).

In order to determine the cutoff of $T_m$ changes predictable for mutations associated with resistance we used the ROC curves. With regard to RMP resistance, the area under the ROC curves (with the 95% confidence intervals) for $rpoB1$ and $rpoB2$ were 0.750 (0.621 to 0.878) and 0.711 (0.584 to 0.839), respectively; for INH resistance, the areas for $katG$ and $inhA$ were 0.935 (0.862 to 1.008) and 0.666 (0.530 to 0.802), respectively. The cutoff points of $T_m$ changes predictable for mutations associated with resistance for $rpoB1$, $rpoB2$, $inhA$, and $katG$ were, respectively, 0.90, 0.95, 1.30, and 1.10.

For INH resistance, the genotyping results correctly matched classical resistance testing in 24 (96%) of 25 isolates. There were two isolates reported as genotypically resistant and phenotypically susceptible [Table 1]. We found that 20 strains had mutations in the $katG$ gene, while only one had mutations in the $inhA$ gene; four strains had mutations in both genes. We can conclude that the main INH resistance mechanism identified in the MTB isolates from Romanian is related to changes in the gene that encodes catalase.

<table>
<thead>
<tr>
<th>Isoniazid genotype</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Resistant</td>
</tr>
<tr>
<td>Resistant</td>
<td>24</td>
</tr>
<tr>
<td>Susceptible</td>
<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25</strong></td>
</tr>
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</table>

**Table 1:** Rapid detection of resistance-associated mutations for INH (presence of either $inhA$ or $katG$) as compared to the INH-resistance phenotype

For RMP our results only partly matched those generated by the conventional testing. There were 11 isolates reported as genotypically resistant while their phenotype was susceptible; however, only two isolates among 24 phenotypically RMP-resistant strains generated a susceptible hybridization pattern [Table 2].

**Figure 1:** Melting profiles for H37Rv and the other seven MTB strains tested
Table 2: Rapid detection of resistance-associated mutations for RMP (presence of either rpoB1 or rpoB2) as compared to the RMP-resistance phenotype

<table>
<thead>
<tr>
<th>Rifampin genotype</th>
<th>Resistant</th>
<th>Susceptible</th>
<th>Total</th>
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<tbody>
<tr>
<td>Rifampin phenotype</td>
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<td></td>
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</tr>
<tr>
<td>Rmp</td>
<td>33</td>
<td>2</td>
<td>35</td>
</tr>
<tr>
<td>Total</td>
<td>24</td>
<td>43</td>
<td>67</td>
</tr>
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</table>

The sensitivity and specificity of the rapid detection of mutations for INH (presence of either inhA or katG) were 96% (95% CI: 88 to 100) and 95% (95% CI: 89 to 100), respectively, with a positive likelihood ratio (LR+) of 20 and a negative likelihood ratio (LR−) of 0.04. For RMP (presence of rpoB1 or rpoB2), the sensitivity and specificity were 92% (95% CI: 81 to 100%) and 74% (95% CI: 61 to 87%), respectively, with LR+ of 3.58 and LR− of 0.10.

Sequencing was performed in order to obtain more information about nucleotide changes in the examined genes so that differences between the results generated with the two resistance techniques could be explained. Therefore, five MTB strains were partially sequenced in the inhA and katG regions and the other six strains in the rpoB gene. Unfortunately, not all the samples with discordant results between genotyping and phenotyping were available for the sequencing experiment. For comparison, we included also samples found sensitive or resistant to INH and RMP by both tests. Nucleotide sequence analysis (three genes targeted) of the strains with discordant results revealed a number of differences from the sequence of H37Rv [Figure 2]. When translated into amino acids, no peptide sequence changes were observed.

Figure 2: Nucleotide alignment (rpoB gene) of H37Rv along with four MTB isolates tested. Samples 2165 and 331 had concordant results by phenotypic and genotypic analysis (2165 susceptible to RMP, 331 resistant to RMP), while 2282 and 3550 had discordant results: sample 2282 phenotypically resistant and genotypically susceptible, sample 3550 phenotypically susceptible and genotypically resistant.

Discussion

There are a variety of methods to determine the susceptibility of MTB to antituberculosis drugs, but none of them is perfect. The objective of our study was to evaluate to what extent differences in sequence between circulating strains might hamper the use of real-time PCR-based techniques for the prediction of phenotypic resistance of MTB strains. We used a technique recommended for the LightCycler platform to analyze 67 sequences from 26 drug-resistant and 41 drug-susceptible MTB strains. Our results suggest that this platform can be used, but there are some limitations. The least important is related to reproducibility. While testing the resistance to INH and RMP with real-time PCR we found that the $T_m$ for the sensitive as well as for the resistant strains varied from one experiment to another due to factors that could not be identified. However, the differences between the $T_m$ s of the resistant and sensitive strains were consistently observed and could be reliably associated with predicted resistance.
Although genotypic assays are very useful for the rapid detection of drug resistance, there are some limitations. First, not all MTB-DR isolates have mutations in the so-called hot spots of the genes associated with resistance. For instance, about 20%-30% of the INH-resistant strains do not have mutations in katG, inhA, kasA, or aphC genes. For that reason, it is very difficult to design a test that could identify all the possible mutations that confer resistance to anti-MTB drugs. This was the case with the MTB isolates from Romanian patients. Here, only two of the main genes involved in conferring resistance to INH were targeted by PCR. We found that for the Romanian strains, targeting katG was adequate to detect INH resistance.

In the analysis of data from other studies, geographical differences in the frequencies of specific mutations are also apparent: the katG gene was mutated at codon 315 in 64% of INH-resistant strains from South Africa and central and western Africa but in only 26% of Singaporean isolates. Furthermore, even the commercial tests for genotyping MTB drug resistance have been reported to have some limitations. A recent study evaluated the results of the two commercially available line probe assays and showed that while the accuracy for RMP resistance was very good, the sensitivity for INH was variable.

We found that for RMP resistance, the concordance between the predicted and observed phenol-type was less than satisfactory. This is not entirely unexpected, because a single mutation, although implicated in resistance, might not be enough to generate a resistant phenotype. Two explanations can account for these observations. The most important is the presence of mutations within the rpoB locus that are not associated with resistance but nevertheless influence the annealing properties of the probes. This is most likely why a significant number of strains were classified as resistant to RMP by genetic analysis and sensitive by phenotypic testing.

Furthermore, when performed sequencing, we observed changes at the nucleotide level that did not affect the amino acid sequence, but could alter the sensitivity of the genotypic test: an extra one or two mismatches could influence the T_m value, which could affect the interpretation of the sensitivity based on hybridization. This was observed for rpoB, with a high number of false positive results (26%). A much smaller number of false negative results has been observed in strains tested for resistance to INH (4%) and RMP (8%); in this case no mutations were found in the target sequence of the tested genes. The molecular determinants of the resistant phenotype are expected to be found elsewhere.

On the other hand, it should be kept in mind that isolates that are susceptible according to molecular assays that target specific mutations may contain other unknown mechanisms of resistance, and these mechanisms will be missed by these techniques.

A much smaller number of strains were reported sensitive by the hybridization analysis and resistant by the phenotypic analysis. The explanation for this is that a small albeit significant number of strains have determinants of resistance outside the area targeted by the assays we used. A similar phenomenon has been reported by others. Another possibility is that changes have occurred in genes whose products participate in antibiotic permeation or metabolism.

In addition, the results of the absolute concentration method used in the phenotypic test are less reliable compared with the proportion method (the most preferred choice). Errors in the susceptibility testing may be related to any of the following: cultures older than 21-30 days, incorrect size of inoculum, incorrect dilution, or errors in incorporation of antibiotics in culture media. This technique should be further evaluated since the circulating strains in different geographical
regions might behave differently when genotypically tested.

This real-time PCR assay could be useful when investigation of drug-resistant TB is mandatory, for example, in cases with a history of one or more previous treatment(s) with several failing, discontinued regimen, or in the situation of exposure to a known source of drug-resistant TB. Although real-time PCR assays may be a valuable diagnostic tool, they are not yet completely satisfactory for MTB drug-resistance detection. Phenotype-based assays will continue to have a place in the clinical mycobacteriology laboratory.

Conclusion

Thus, based on our experience, the real-time PCR assay could be used in clinical practice, albeit with caution, in cases with risk factors for resistance. The results can be used for guiding the initiation of therapy, but the treatment should be adjusted correspondingly as soon as the phenotypic testing results are available. The best accuracy for genotyping compared with phenotypic resistance testing was obtained for detecting INH resistance mutations targeting the katG gene.

References


Molecular Detection of Antimicrobial Resistance

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Utrecht, The Netherlands

2. Fluit2001 (condensed)
Traditional phenotypic determination of resistance may take up to 10 weeks after referral of a sample to the laboratory, but both commercial and in-house amplification assays can greatly improve the detection time. Therefore, it is not surprising that within the past 8 years a multitude of different resistance assays based on molecular techniques were specifically developed for *M. tuberculosis*. However, many laboratories have had trouble with the technical rigor imposed by these assays (222). For review of mycobacterial resistance, see reference 128.

**Rifampin Resistance**

One of the first assays for the detection of rifampin resistance using PCR-SSCP was published by Telenti et al. (336). In a second paper the assay was more extensively evaluated both in a manual format with radioactively labeled amplification products and with 5'-fluorescein-labeled primers for detection on an automated DNA sequencer (337). Evaluation of the results showed that all 17 of the then known mutations in the rpoB gene leading to resistance could be detected. Equally important, the assays could be applied to minimally grown cultures in Bactec 12B medium with a growth index of \( \approx 100 \) or on spuTa with at least 10 organisms per field at a magnification of \( \times 250 \). This clearly established the potential of PCR-SSCP as a powerful technique for the early detection of antimicrobial drug resistance in *M. tuberculosis*. The application of rifampin resistance detection by PCR-SSCP to cerebrospinal fluid specimens from patients with tuberculosis of the central nervous system also yielded excellent results (289).

PCR-SSCP requires careful control over electrophoresis conditions, which is difficult to achieve in many laboratories. This recognition led to a comparison of PCR-SSCP and deoxy fingerprinting (84). Dideoxynucleotide sequencing is in fact an extension of SSCP. After PCR amplification of the gene fragment of interest, a second PCR is performed with a radioactively labeled primer. A dideoxynucleotide is added, which leads to chain termination similar to that obtained in dideoxy sequencing. The products are then analyzed in a similar manner to that in SSCP. Because more fragments are generated, differences between the susceptible and resistance types are more easily obtained in accordance with conventional susceptibility testing and PCR-SSCP. A drawback of this method is its use of a radioactive label. However, by using fluorescent labels, this assay can probably be adapted for use with an automated sequencer.

However, Kim et al. (150) observed that PCR-SSCP reported some isolates as resistant whereas their phenotype was susceptible, but in these isolates the part of the rpoB gene that was amplified contained a silent mutation and a deletion of two amino acids. Apparently, these mutations do not affect the susceptibility to rifampin. These authors therefore concluded that sequencing probably could rule out false-positive results.

Direct testing of a clinical specimen for resistance to rifampin by PCR without prior species determination is believed to be difficult because of the high levels of homology reported between different mycobacterial species, but Whelen et al. (392) devised a rpoB-based seminested amplification which was specific for *M. tuberculosis*. The assay correctly identified 21 of 24 culture-positive specimens, 13 of which were acid-fast smear negative in a panel of 51 clinical specimens. Three specimens
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### FIG. 5. Principle of oligonucleotide array sequencing. Alignment of the overlapping probes reconstructs the complement of the original target (see the text for details).

**ANTIBIOTIC RESISTANCE IN *MYCOBACTERIUM TUBERCULOSIS***

**Introduction**

In the wake of the human immunodeficiency virus epidemic and the breakdown of medical services in several Eastern European countries, the incidence of tuberculosis is rising rapidly. Of note, the treatment of tuberculosis is threatened by the breakdown of medical services in several Eastern European countries, the incidence of tuberculosis is rising rapidly.

The variety of molecular techniques used for diagnostic applications demonstrate that no universal technique exists which is optimal for detection of nucleic acids. The choice of a particular technique is also dependent on the information required or the targets under consideration, but some techniques are more favored than others. New techniques continue to be developed that involve a new approach to amplification, hybridization, formats, and labels (158).
Drug-susceptibility testing in tuberculosis: methods and reliability of results

S.J. Kim

ABSTRACT: The demand for reliable drug-susceptibility testing (DST) increases with the expansion of antituberculosis drug-resistance surveillance, and with the need for an appropriate treatment of multidrug-resistant tuberculosis, whose incidence gradually increases in many parts of the world. However, the reliability of DST results obtained through widely used methods does not meet acceptable levels, except for DST to isoniazid and rifampicin.

In general, susceptibility results are highly predictable, while resistance results show low predictive values when the resistance prevalence is <10%. Poor reliability stems from a weak correlation with clinical response and a low reproducibility due to the poor standardisation of the complex and fragile test procedures. Therefore, in vitro criteria of resistance for susceptibility testing should be carefully determined with representative clinical samples of Mycobacterium tuberculosis isolated from patients never treated with any antituberculosis drug, and from patients having failed treatment with a regimen containing the tested drug; DST should then be carefully standardised to obtain reproducible results.

The critical concentration of some drugs is close to the minimal inhibitory concentration for wild susceptible strains and, thus, drug-susceptibility testing is prone to yield poorly reproducible results. These issues call for physicians’ attention when using the results from drug-susceptibility testing for case management.

KEYWORDS: Drug resistance, drug-susceptibility testing, tuberculosis

In many countries, the wide use of the standard short-course regimen has led to an increasing incidence of multidrug-resistant (MDR) tuberculosis (TB), defined as resistance to at least isoniazid (INH) and rifampicin (RFP) [1–3]. Significant high rates of MDR-TB were observed in some parts of the world, not only among previously treated TB patients, due to poor case management, but also among new cases due to transmission in the community. The situation has turned into a pressing demand for drug-susceptibility testing (DST) in order to accomplish drug-resistance surveillance (DRS), and also to develop efficient regimens for appropriate treatment of individual cases.

As a result of inappropriate and/or inadequate treatment, drug resistance emerges by selective multiplication of resistant mutants within the lesions, in spite of the presence of growth-inhibitory concentrations of a drug. The frequency of drug-resistant mutants and their resistance levels vary depending on the drug and the mutated genes and sites, whose phenotypic expressions include the following: alterations of the binding site of drug-target molecules; loss of enzymes activating drug molecules; permeability changes to the drug, including efflux; and production of drug-inactivating enzymes, such as β-lactamase. There are a variety of methods to determine the susceptibility of Mycobacterium tuberculosis to antituberculosis drugs, but none of them is perfect, and their results do not satisfy clinicians for the effective treatment of TB patients.

Most of the currently used DST methods suffer from low predictability associated with clinical irrelevance of the results and from unacceptable
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KEYWORDS: Drug resistance, drug-susceptibility testing, tuberculosis

In many countries, the wide use of the standard short-course regimen has led to an increasing incidence of multidrug-resistant (MDR) tuberculosis (TB), defined as resistance to at least isoniazid (INH) and rifampicin (RFP) [1–3]. Significant high rates of MDR-TB were observed in some parts of the world, not only among previously treated TB patients, but also among new cases due to transmission in the community. The situation has turned into a pressing demand for drug-susceptibility testing (DST) in order to accomplish drug-resistance surveillance (DRS), and also to develop efficient regimens for appropriate treatment of individual cases.

As a result of inappropriate and/or inadequate treatment, drug resistance emerged by selective multiplication of resistant mutants within the lesions, in spite of the presence of growth-inhibitory concentrations of a drug. The frequency of drug-resistant mutants and their resistance levels vary depending on the drug and the mutated genes and sites, whose phenotypic expressions include the following: alterations of the binding site of drug-target molecules; loss of enzymes activating drug molecules; permeability changes to the drug, including efflux; and production of drug-inactivating enzymes, such as β-lactamase. There are a variety of methods to determine the susceptibility of Mycobacterium tuberculosis to antituberculosis drugs, but none of them is perfect, and their results do not satisfy clinicians for the effective treatment of TB patients.

Most of the currently used DST methods suffer from low predictability associated with clinical irrelevance of the results and from unacceptable...
Contribution of \( \text{kasA} \) Analysis to Detection of Isoniazid-Resistant \( \text{Mycobacterium tuberculosis} \) in Singapore

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Several genes and genomic regions of \( \text{Mycobacterium tuberculosis} \) participate in the development of resistance to isoniazid (INH), a frontline antituberculous drug. Mutations in the catalase-peroxidase gene \( (\text{katG}) \) diminish activation of INH, and structural or promoter mutations of enoyl acyl carrier protein reductase \( (\text{encoded by inhaA}) \) modify the interaction of this drug target with INH. Mutations in the \( \text{oxyR-ahpC} \) intergenic region represent a surrogate marker for \( \text{katG} \) lesions (1, 3–9, 12–18).

Analysis of these regions does not, however, allow identification of all INH-resistant \( \text{M. tuberculosis} \) strains. The recent description of a novel target, ketoacyl acyl carrier protein synthase \( (\text{encoded by kasA}) \), involved in elongation of fatty acids intermediate in the biosynthetic pathway of mycolic acids, opened the possibility for identifying additional INH-resistant organisms (10). The aim of this study was to assess the contribution of \( \text{kasA} \) analysis to the investigation of INH resistance in a large collection of \( \text{M. tuberculosis} \) isolates from Singapore.

All drug-resistant isolates in Singapore are sent to the Central Tuberculosis Laboratory, Department of Pathology, Singapore General Hospital. Consecutive INH-resistant \( \text{M. tuberculosis} \) isolates collected from August 1994 to December 1996 \((n = 160)\) and 32 susceptible controls were included in the study. Drug susceptibility testing was done by the BACTEC 460 radiometric method (Becton Dickinson, Towson, Md.), and the isoniazid concentration was 0.1 \( \mu \text{g/ml} \).

Genotypic analysis by PCR amplification and sequencing targeted the codon 315 region of \( \text{katG} \) (codons 292 to 387) (5) and the promoter regions of \( \text{inhaA} \) and \( \text{ahpC} \) (18). The entire \( \text{kasA} \) gene (GenBank accession no. Z70692) was investigated by amplifying three overlapping fragments with the oligonucleotide primers shown in Table 1.

Among INH-resistant strains, targeted analysis of \( \text{katG} \) identified mutations W300stop \((n = 1)\), S302R \((n = 36)\), S315N \((n = 5)\), and L336R \((n = 2)\) and \( \text{katG} \) deletions in nine strains. To confirm that these deletions were not artifactual, PCR of the \( \text{katG} \) gene with primers to other regions of the gene was done (5). Mutation of \( \text{katG} \) at codon 315 was observed in 41 of 160 (26%) INH-resistant isolates.

Analysis of the \( \text{inhaA} \) promoter identified the following nucleotide substitutions flanking the presumed ribosome binding site: \(-15 \text{C}\rightarrow\text{T} \((n = 43)\) and \(-8 \text{T}\rightarrow\text{A} \((n = 1)\) (numeration according to Ramaswamy and Musser [14]). A novel \( \text{A}\rightarrow\text{T} \) substitution \((n = 1)\) located 92 nucleotides 5’ of the ribosome binding site was also identified.

Analysis of the \( \text{oxyR-ahpC} \) intergenic region identified substitutions at positions \(-46 \text{G}\rightarrow\text{A} \((n = 1)\), \(-30 \text{C}\rightarrow\text{T} \((n = 2)\), \(-12 \text{C}\rightarrow\text{T} \((n = 2)\), and \(-6 \text{G}\rightarrow\text{A} \((n = 1)\) relative to the \( \text{mRNA} \) start site (14). Mutations in the 5’-terminal region of the \( \text{ahpC} \) gene product were observed at P2S \((n = 1)\), associated with deletion of \( \text{katG} \), and T5I \((n = 1)\). Nucleotide substitutions in the defective \( \text{oxyR} \) gene were observed at nucleo-

<table>
<thead>
<tr>
<th>Primer</th>
<th>Description</th>
<th>Sequence</th>
<th>Nucleotides</th>
</tr>
</thead>
<tbody>
<tr>
<td>kasA1S</td>
<td>First fragment, sense</td>
<td>5’CGTTACGGGGCGGCTTGGAGG</td>
<td>30633–30652</td>
</tr>
<tr>
<td>kasA1AS</td>
<td>First fragment, antisense</td>
<td>5’CCGTTCTGGATCGACCTCCG</td>
<td>30983–30964</td>
</tr>
<tr>
<td>kasA2S</td>
<td>Second fragment, sense</td>
<td>5’GGACAGCTATGGGGATCACC</td>
<td>30936–30955</td>
</tr>
<tr>
<td>kasA2AS</td>
<td>Second fragment, antisense</td>
<td>5’ACCCAGAATGCGGGCAACG</td>
<td>31463–31444</td>
</tr>
<tr>
<td>kasA3S</td>
<td>Third fragment, sense</td>
<td>5’GCACGCCAAAGCCCGTGGGC</td>
<td>31418–31437</td>
</tr>
<tr>
<td>kasA3AS</td>
<td>Third fragment, antisense</td>
<td>5’GGGCTTCCGGGACCGCGATG</td>
<td>31940–31921</td>
</tr>
</tbody>
</table>

* The \( \text{M. tuberculosis} \) sequence used to design the primers was obtained from GenBank (accession no. Z70692).

* Corresponding author. Mailing address: Department of Clinical Research, Block 6, Level 6, Singapore General Hospital, Outram Road, Singapore 169608, Singapore. Phone: 65-3213730. Fax: 65-2257796. E-mail: gcrlsg@sgh.gov.sg.
the promoter regions of analyzed may not be universally applicable. The strategy implemented for genotypic strategies for detection of drug resistance in other genes. A particular polymorphism, G312S, was also most (13 of 16) presented mutations associated with resistance successful in Spain (detection of 87% of resistant strains). In 100 of 160 (62.5%) resistant strains, while it proved alterations in 100 of 160 (62.5%) resistant strains, with the exception of one isolate having a point mutation. No alterations were identified in susceptible isolates (Tables 2 and 3). Sixteen resistant isolates presented mutations (R121K | Arg → Lys, AGG → AAG | 1 (0.6)), 269 Gly → Ser, GGT → AGT | 3 (2)), 312b Gly → Ser, GGC → AGC | 11 (7)), and 387a Gly → Asp, GGC → GAC | 1 (0.6) | None). Therefore, information regarding the frequencies and types of mutations or deletions which have been documented in one country or geographical region may not be applicable elsewhere.

Due to the limited performance of the chosen targeted approach to INH resistance, we investigated the contribution of kasA analysis to the overall performance of targeted genotypic detection of INH resistance. Mdluli et al. (10) identified kasA polymorphisms in 4 of 28 (14.3%) INH-resistant isolates (codons 121, 269, 312, and 413) but not among 43 INH-susceptible strains. While kasA polymorphisms (codons 121, 269, 312, and 387) were identified in 10% of INH resistant isolates in the present study, the most frequent substitution (G312S) was also shown to be a frequent polymorphism (19%) among susceptible strains. In this study, mutation of kasA did not represent a frequent event associated with INH resistance, and analysis of this target contributed minimally to the diagnostic strategy.

We acknowledge the National Medical Research Council of Singapore for funding this project.

REFERENCES

Geographical differences in the frequencies of specific mutations are also apparent in analysis of data from other studies: the katG gene was mutated at codon 315 in 64% of INH-resistant strains from South Africa and central and western Africa (4) but in only 26% of Singaporean isolates; mutations in the regulatory region of the inhA gene have been reported in 6.5 to 21.6% of INH-resistant isolates (7, 12–15); and oxyR-ahpC intergenic region substitutions have been reported in 24.2 to 32.9% of INH-resistant isolates (7, 17). Interestingly, investigation of the same set of isolates for rpoB mutations associated with rifampin resistance demonstrated the same prevalence and distribution of specific mutations as are present in other geographical regions (data not shown).

In the case of INH, discrepant results between studies likely reflect different geographical prevalences of specific genotypes. Certainly, the possibility of a limited number of epidemic strains contributing to these differences needs to be assessed. These geographical differences in the prevalences of specific polymorphisms were underscored by our previous report on the katG R463L substitution in Singaporean isolates, where this substitution constitutes a frequent natural polymorphism unrelated to INH resistance (8). Therefore, information regarding the frequencies and types of mutations or deletions which have been documented in one country or geographical region may not be applicable elsewhere.

We acknowledge the Central Tuberculosis Laboratory, Department of Pathology, Singapore General Hospital, for providing isolates.

TABLE 2. Genetic characterizations of 160 INH-resistant isolates and 32 INH-susceptible isolates from Singapore

<table>
<thead>
<tr>
<th>Phenotype (n)</th>
<th>No. (%) with indicated genotypes</th>
<th>Genotypea</th>
<th>codon</th>
<th>amino acid change</th>
<th>Mutation</th>
<th>No. (%) of isolates with indicated mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH-R (160)</td>
<td>36 (23) Mut —e</td>
<td>— —</td>
<td>—</td>
<td>Arg → Lys, AGG → AAG</td>
<td>1 (0.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 (4) Del —</td>
<td>—</td>
<td>—</td>
<td>Gly → Ser, GGT → AGT</td>
<td>3 (2)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>31 (19) Mut —</td>
<td>—</td>
<td>—</td>
<td>Gly → Ser, GGC → AGC</td>
<td>11 (7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3 (2) Mut/Del —</td>
<td>—</td>
<td>—</td>
<td>Gly → Asp, GGC → GAC</td>
<td>1 (0.6)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 (3) Mut/Del —</td>
<td>—</td>
<td>—</td>
<td>None</td>
<td>None</td>
<td>144 (90)</td>
</tr>
<tr>
<td></td>
<td>3 (2) Mut/Del —</td>
<td>—</td>
<td>—</td>
<td>None</td>
<td>None</td>
<td>28 (18)</td>
</tr>
<tr>
<td></td>
<td>5 (3) Mut/Del —</td>
<td>—</td>
<td>—</td>
<td>None</td>
<td>None</td>
<td>28 (18)</td>
</tr>
<tr>
<td></td>
<td>4 (3) Mut —</td>
<td>—</td>
<td>—</td>
<td>None</td>
<td>None</td>
<td>28 (18)</td>
</tr>
<tr>
<td></td>
<td>7 (4) Mut —</td>
<td>—</td>
<td>—</td>
<td>None</td>
<td>None</td>
<td>28 (18)</td>
</tr>
<tr>
<td></td>
<td>1 (0.5) Mut —</td>
<td>—</td>
<td>—</td>
<td>None</td>
<td>None</td>
<td>28 (18)</td>
</tr>
<tr>
<td></td>
<td>57 (36) —</td>
<td>—</td>
<td>—</td>
<td>None</td>
<td>None</td>
<td>28 (18)</td>
</tr>
<tr>
<td>INH-S (32)</td>
<td>25 (78) —</td>
<td>—</td>
<td>—</td>
<td>None</td>
<td>None</td>
<td>28 (18)</td>
</tr>
<tr>
<td></td>
<td>1 (3) —</td>
<td>—</td>
<td>—</td>
<td>None</td>
<td>None</td>
<td>28 (18)</td>
</tr>
<tr>
<td></td>
<td>6 (19) —</td>
<td>—</td>
<td>—</td>
<td>None</td>
<td>None</td>
<td>28 (18)</td>
</tr>
</tbody>
</table>

a INH-R, INH resistant; INH-S, INH susceptible.
b Mut, mutation; Del, deletion.
e —, no detectable mutation.

TABLE 3. Genetic polymorphisms of the kasA gene in INH-resistant and -susceptible clinical isolates of M. tuberculosis

<table>
<thead>
<tr>
<th>Phenotype (n)</th>
<th>Codon</th>
<th>Amino acid change</th>
<th>Mutation</th>
<th>No. (%) of isolates with indicated mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>INH resistant (160)</td>
<td>121a Arg → Lys, AGG → AAG</td>
<td>1 (0.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>269 Gly → Ser, GGT → AGT</td>
<td>3 (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>312b Gly → Ser, GGC → AGC</td>
<td>11 (7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>387a Gly → Asp, GGC → GAC</td>
<td>1 (0.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>None</td>
<td>144 (90)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>None</td>
<td>None</td>
<td>28 (18)</td>
<td></td>
</tr>
</tbody>
</table>

a Novel mutation.
b Polymorphism also present in susceptible isolates.

tides 18 (G→A [n = 2]), 27 (G→T [n = 1]), and 28 (C→A [n = 1]). All oxyR mutations were observed in the presence of other mutations established to be associated with resistance, e.g., katG S315T or an inhA promoter mutation. While an association of ahpC coding region mutations with INH resistance remains plausible, the functional role of oxyR mutations remains doubtful.

Overall, the targeted strategy identified katG mutations in 54 of 160 strains (34%), inhA mutations in 45 strains (28%), and oxyR-ahpC mutations in 12 strains (7.5%) (Table 2). Twenty-three of 160 INH-resistant strains (14%) carried more than one mutation. No alterations were identified in susceptible strains, with the exception of one isolate having a point mutation in the defective oxyR gene (nucleotide 18).

Analysis of kasA identified a number of polymorphic sites both in resistant and susceptible isolates (Tables 2 and 3). Sixteen resistant isolates presented mutations (R121K [n = 1], G269S [n = 3], G312S [n = 11], G387D [n = 1]); however, most (13 of 16) presented mutations associated with resistance in other genes. A particular polymorphism, G312S, was also present in 6 of 32 (19%) susceptible strains.

The present study raises two relevant points for discussion of the implementation of genotypic strategies for detection of drug resistance in M. tuberculosis. First, it demonstrates that targeted approaches that limit the number of genetic regions analyzed may not be universally applicable. The strategy implemented in Singapore (analysis of the codon 315 region and the promoter regions of inhA and oxyR-ahpC) detected mutations in 100 of 160 (62.5%) resistant strains, while it proved successful in Spain (detection of 87% of resistant strains). Additional mutations could be present in katG regions not included in the analysis or in the structural inhA gene or could correspond to unidentified mechanisms of resistance (2, 11, 14).

We acknowledge the National Medical Research Council of Singapore for funding this project.