A New Genetic Assay for Rifampicin Susceptibility of
*Mycobacterium tuberculosis*

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Abstract

A new method for testing rifampicin (RFP) susceptibility of *Mycobacterium tuberculosis* was developed. This method is based on detection of the internal sequence derived from 71-kDa heat shock protein mRNA in tubercle bacilli heat-treated in the presence of RFP. The target sequence was amplified by reverse transcription and PCR, followed by agarose gel electrophoretic analysis. No amplification occurred in one RFP-susceptible strain by exposure to 45°C for 45 min in Middlebrook 7H9 broth containing RFP (10 μg/ml) after overnight incubation at 37°C. On the other hand, an amplified 275-bp product was obtained from the RFP-resistant strain MY-129. In a subsequent trial using 65 clinical isolates, this method defined their RFP susceptibility levels as well as the verification of the MICs obtained by the conventional agar dilution method, with the exception of one RFP-susceptible strain. Thus, this method provides a rapid and practical system to determine RFP susceptibility in *M. tuberculosis*.

Introduction

Rifampicin (RFP) has been one of the most useful drugs in addition to isoniazid for chemotherapy of tuberculosis. However, multidrug-resistant strains of *Mycobacterium tuberculosis* have been found with increasing frequency in clinical specimens. Thus, the simple use of RFP and some other drugs for treatment of tuberculosis may sometimes be ineffective and the numbers of patients requiring more appropriate treatment have been increasing.

The conventional method for culturing mycobacteria requires a long period, i.e. 4 to 8 weeks for isolation, and an additional 3 to 4 weeks for drug susceptibility testing. Therefore, several rapid detection methods have been developed and accepted as clinical diagnostic techniques for tuberculosis. However, a rapid method for drug susceptibility testing, one of the most important goals, remains to be established.

Patel et al.2) reported that 71-kDa heat shock protein (HSP) mRNA of *M. leprae* disappeared in heat-sterilized cells, and the viability of the organism could be determined by detection of this molecule as a marker. They also reported that 71-kDa HSP mRNA transcript levels of *M. bovis* BCG pretreated with RFP were reduced by 90% by heat treatment3).

These findings suggested the possibility of a new assay method for RFP susceptibility of *M.
**A New Rifampicin Susceptibility Test**

**tuberculosis.** Therefore, using this 71-kDa HSP mRNA, we developed a more practical method for rapid susceptibility testing of RFP.

**Materials and Methods**

**Organisms and bacteriological procedures.** *M. tuberculosis* H37Ra and *M. bovis* BCG were used as RFP-susceptible strains. An RFP-resistant strain of *M. tuberculosis*, MY-129, was selected from clinical isolates. Sixty-five clinical isolates were also evaluated by our method. These organisms were maintained in Middlebrook 7H9 broth at 37°C.

The MIC was determined by the twofold agar dilution method, in which RFP concentration ranged from 64 to 0.0625 μg/ml. Samples (5 μl) of the test bacterial suspension, adjusted to a concentration of about 10⁶ colony-forming units (cfu)/ml, were spotted onto plates.

The rate of RFP resistance a strain was determined by the standard proportional method.

In these studies, Middlebrook 7H10 agar medium was used. The plates were examined for colony formation after 3 weeks of incubation at 37°C.

**Extraction of mRNA.** Suspensions (100 μl) of the cultured organisms were transferred into microfuge tubes containing 800 mg of glass beads (φ1 mm). A volume of 300 μl of ISOGEN-LS® (Nippon Gene Co., Toyama, Japan), a modified acid guanidinium thiocyanate-phenol-chloroform reagent, was then added to each tube which was then mixed vigorously for 15 min, and 100 μl of chloroform was added. After being mixed for 15 sec, the mixture was centrifuged at 10000 ×g for 15 min at 4°C. A 200 μl portion of the upper phase was transferred to another tube, and then an equal volume of 2-propanol and 1 μl of 20 mg/ml glycogen were added. The mixture was kept at room temperature for 5 min, then centrifuged at 10000 ×g for 10 min. After the supernatant was decanted, the tube was rinsed with 800 μl of 75% ethanol and centrifuged again at 10000 ×g for 5 min. The tubes were then left to dry by evaporation for 30 min on a clean bench after aspiration. The dried precipitate was dissolved in 20 μl of distilled water treated with diethyl pyrocarbonate (DEPC) containing 35 U of RNase inhibitor. The mixture was digested with DNase to remove chromosomal DNA by the method of Grillo and Margolis. Briefly, the mixture was incubated with 18 U of DNase for 1 hour at 37°C, then purified with TE-saturated phenol/chloroform/isoamylalcohol (25/24/1, v/v/v) and the remaining RNA was precipitated with 2-propanol. The 75% ethanol-washed precipitate was dried and dissolved with 10 μl of DEPC-water.

**Reverse transcription and PCR.** Reverse transcription reaction mixture consisted of 10 mM Tris-HCl (pH 8.3), 90 mM KCl, 200 μM each dATP, dTTP, dCTP, dGTP, 1 mM MnCl₂, 800 nM 71-kDa HSP antisense primer (5’ACCGCGGCATCAACCTTGTT3’), 2.5 U of rTth DNA polymerase (Perkin-Elmer Co.), and 2 μl of RNA sample, and was adjusted to a total volume of 10 μl. The mixture was overlaid with 1 drop of mineral oil to prevent evaporation, and incubated at 70°C for 15 min for cDNA synthesis. For PCR, 40 μl of chelating solution consisting of 5% glycerol, 10 mM Tris-HCl pH 8.3, 0.1 M KCl, 750 μM ethylene glycol-bis (β-aminoethyl ether) N, N, N’-tetraacetic acid, 0.05% Tween 20, 2.5 mM MgCl₂, and 200 nM sense primer (5’ATTGTGCACGTCACCGCCAA3’) was added to the cDNA mixture. The PCR profile was 40 cycles of 1 min each at 94, 62 and 72°C, with a final extension for 5 min at 72°C. Ten μl samples of the PCR products were analyzed by electrophoresis in 2% agarose gels with ethidium bromide staining to detect amplification of the 275-bp fragment target sequence.

To confirm complete digestion of chromosomal DNA, a negative control sample mixture, which was kept on ice during the cDNA synthesis step, was prepared for each sample.

**A genetic method for RFP susceptibility testing.** A broth culture was adjusted to a turbidity of McFarland No. 1. A 1:100 volume of the suspension was inoculated into 7H9 broth containing RFP.
at 10 μg/ml (RFP-broth). After incubation at 37°C for one day, the culture was heated at 45°C for 45 min. A broth culture without RFP was prepared at the initial step and stored at −70°C. This culture, as a positive control, was tested simultaneously with the RFP-broth culture. The 71-kDa HSP mRNA was extracted from these samples and detected as described above. Amplification of the 275-bp fragment from RFP-broth culture was regarded as an indication of RFP-resistance.

Results

Detection of 71-kDa HSP mRNA at different proportions in RFP-resistant organisms. As shown in Fig. 1, the 275-bp fragment of 71-kDa HSP mRNA of M. tuberculosis H37Ra disappeared

![Agarose gel electrophoretic analysis of PCR products obtained from samples in which the proportion of resistant cells was adjusted to 0, 1, 10, or 100%. A suspension of M. tuberculosis MY-129 (RFP-resistant) was mixed with a suspension of H37Ra (RFP-susceptible) to obtain the indicated proportions (v/v). The suspensions of MY-129 and H37Ra concentrations were 3.3 × 10⁷ and 2.3 × 10⁷ CFU/ml, respectively. C, positive control; C', positive control without reverse transcription; S, RFP-broth; S', RFP-broth without reverse transcription; M, DNA molecular size marker (ϕX174 restricted with HaeIII)](image)

<table>
<thead>
<tr>
<th>Proportion of resistance</th>
<th>0%</th>
<th>1%</th>
<th>10%</th>
<th>100%</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>C'</td>
<td>S</td>
<td>C</td>
<td>C'</td>
</tr>
</tbody>
</table>

Table 1 Results of the new method using 65 M. tuberculosis isolates resistant to rifampicin at different MICs

<table>
<thead>
<tr>
<th>MIC (μg/ml)</th>
<th>No. of strains</th>
<th>The new method (275-bp fragment)</th>
<th>% resistance and No. of strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.125</td>
<td>24</td>
<td>0 24</td>
<td>100% (1)</td>
</tr>
<tr>
<td>0.25</td>
<td>14</td>
<td>0 14</td>
<td>100% (2)</td>
</tr>
<tr>
<td>0.5</td>
<td>4</td>
<td>1 3</td>
<td>100% (1), 90% (4), 80% (1), 70% (1), 50% (1), 30% (1)</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>1 1 0</td>
<td>100% (1)</td>
</tr>
<tr>
<td>16</td>
<td>2</td>
<td>2 2 0</td>
<td>100% (2)</td>
</tr>
<tr>
<td>32</td>
<td>2</td>
<td>2 2 0</td>
<td>100% (2), 90% (4)</td>
</tr>
<tr>
<td>64</td>
<td>2</td>
<td>2 2 0</td>
<td>100% (2), 90% (4), 80% (1)</td>
</tr>
<tr>
<td>&gt;64</td>
<td>16</td>
<td>16 0</td>
<td>100% (2), 90% (4), 80% (1), 70% (1)</td>
</tr>
</tbody>
</table>

aProportion of resistance at RFP, 1 μg/ml. The figures in parentheses represent the numbers of strains.

bWeak amplification was observed
during incubation and heat treatment in RFP-broth. In the case of the RFP-resistant strain *M. tuberculosis* MY-129, no changes were observed. The present method allowed detection of resistant organisms at 1% in a sample, although the intensity of the target band in agarose gels was weak compared with controls. Resistant organisms could be detected clearly when present at 10%.

**RFP susceptibility testing and correlation with conventional tests.** As shown in Table 1, the target sequence was not detected in 41 RFP-susceptible strains (MIC, <1 μg/ml). One RFP-susceptible strain (MIC=0.5 μg/ml) revealed weak amplification. Distinct amplification was observed in every RFP-resistant strain.

**Changes in viable cell count after heat treatment in the presence of RFP.** The number of CFU of *M. bovis* BCG changed as follows (values represent logarithms of CFU/ml averaged over four determinations): the initial value of 6.3 decreased to 4.5 after heat treatment for 45 min at 45°C in RFP-broth, although a change from 6.3 to 6.5 was observed after heat treatment in Middlebrook 7 H9 broth without RFP. The decrement of CFU was small (6.3 changed to 6.0) even in RFP-broth without heat treatment. On the other hand, the initial CFU of one RFP-resistant strain (4.7) did not change even after heat treatment in RFP-broth.

**Discussion**

The rate of appearance of resistance to at least one antituberculosis drug among new cases of tuberculosis has been reported to be about 13%8). Furthermore, multidrug-resistant strains of *M. tuberculosis* have been found with increasing frequency among patients, particularly those infected with HIV or who have risk factors such as previous treatment for tuberculosis1). Therefore, as the necessity for appropriate treatment had been increasing, rapid susceptibility testing is becoming more important for effective therapy.

The mechanisms of resistance to antituberculosis drugs have been studied from the standpoint of gene mutation. In resistant strains, the genes encoding targets were reported to be mutated or to have deletions, i.e. *kat G*9) and *inh A*10) for isoniazid, rRNA”for streptomycin, and the β subunit of RNA polymerase” for RFP. However, these mutations are not the mechanism in every resistant strain. Therefore, clinical applications of these studies are impractical. A more practical approach to detect susceptibility using a mycobacteriophage expressing the firefly luciferase gene in viable *M. tuberculosis* was reported13), but it has not become widely used because of the requirement for particular materials.

In the present study, we developed a practical and simple method for rapid RFP susceptibility testing using 71-kDa HSP mRNA as a marker of viable tubercle bacilli. The levels of this molecule was decreased rapidly to below the limit of detection by a combination of RFP and heat treatment.

The 71-kDa HSP of *M. tuberculosis* is a member of the 70-kDa HSP family, which are produced rapidly in response to various stresses such as a rise in temperature3). Forty-, 65- and 90-kDa proteins have been identified as the main tuberculous heat shock proteins other than the 71-kDa protein, although their functions within cells are still unclear3). Generally, stress proteins are involved in degradation and reactivation of damaged proteins, and are essential for survival under conditions of stress".

RFP inhibits RNA polymerase immediately after penetration into the cell13), and consequently protein biosynthesis is suppressed. A lack of these stress proteins may cause lethal damage to the cell, and then a radical decrease (about by 98%) in the number of viable cells occurs in RFP-susceptible strains, as described in the results section. In addition, generally, prokaryotic mRNA is processed rapidly and its half-life is known to be about few minutes; therefore, it is thought that 45 minutes is sufficient for degradation of the remaining mRNA. It is thought that the 71-kDa HSP mRNA
increased in RFP-resistant strains and decreased in susceptible strains during heat treatment, resulting in the conspicuous difference between the two.

The RFP concentration used in the present method is different from the critical concentration in the standard agar dilution method. The test conditions were established through preliminary studies concerning the drug concentration and incubation period. With a low drug concentration (i.e. 1 μg/ml), incubation for more than four days was required for disappearance of the target fragment of RFP-susceptible strains. On the other hand, this phenomenon occurred rapidly at a high RFP concentration (10 μg/ml), so one-day incubation was thought to be sufficient. Under these conditions, as shown in Table 1, the results with 65 clinical isolates correlated with the conventional resistance criterion, i.e. 1 μg/ml MIC, with the exception of one strain.

It has been suggested that if more than 1% of the test population is resistant to a drug, resistance has developed or is in an advanced stage of development5). Therefore, the inoculum size should be set at the order of 10⁵ CFU in samples, because the detection limit in the present method was about 10⁴ CFU in 100 μl of sample. This may be achieved by inoculation of bacterial suspensions adjusted to a turbidity of McFarland No. 1, into the test broth at 1/50 to 1/100 volume. However, using this inoculum size, 1% of the resistant organisms in a sample may give no more than a weak band in agarose gels as shown in Fig. 1. Nevertheless, increasing the inoculum size is not recommended because of the occurrence of false-positive reactions. As shown in Table 1, more than 10% of the cells in the RFP-resistant strains used in this study were resistant and revealed obvious amplification of the target fragment. However, a weak band was observed in one of the RFP-susceptible strains. This weak amplification would not be distinguishable from that in strains with a low proportion of resistant cells. Therefore, if the intensity of amplification is obviously weaker than the control, judgment of susceptibility should be reserved.

We expect that our method will allow direct susceptibility testing from clinical specimens or semi-direct testing combined with short-period incubation. This method revealed relatively high specificity in our preliminary study. The specificity may be achieved by using a thermostable enzyme with reverse transcriptase activity at relatively high temperatures.

In conclusion, we report here that RFP-susceptibility of M. tuberculosis can be determined without waiting for multiplication of the organism, by using a marker for viability. This method will contribute to the effective treatment of tuberculosis. We are currently developing the procedure for direct testing of clinical specimens and also intend to apply it to other antituberculosis drugs.

Acknowledgments

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A New Rifampicin Susceptibility Test


Mycobacterium tuberculosis のrifampicin感受性を知る新しい遺伝子学的試験法

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要 旨

結核菌のリファンピシン（RFP）感受性試験の新方法を開発。この方法はRFP存在下で加熱処理を施した結核菌菌内71-kDaのヒトショック蛋白mRNAの内部配列を検出すものである。275塩基長の目的配列は逆転写反応の後PCR法で増幅された。PCR産物はアガロースゲル電気泳動とエチジウムブロマイド染色により検出、RFP濃度を10μg/mlに調製したMiddlebrook 7H9 broth中で一晩培養後、45℃45分間の加熱処理を行うと、RFP感受性結核菌H37Ra株からは目的バンドは検出されなかった。一方、耐性菌MY-129株では275bpの増幅が観察された。続いて实施した65株の臨床分離株を被検菌とした試験では、寒天平板希釈法によるMIC値から判定されたRFP感受性の有無と本法の結果は1株も含めて一致した。本法は結核菌のRFP感受性を早期に判定する実際的な方法であると考えられる。

平成7年12月20日