Direct colorimetric assay for rapid detection of rifampin-resistant Mycobacterium tuberculosis

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Direct Colorimetric Assay for Rapid Detection of Rifampin-Resistant
*Mycobacterium tuberculosis*

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The colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was standard-
ized for direct detection of rifampin-resistant *Mycobacterium tuberculosis* in sputum samples. The sensitivity
and specificity of the direct MTT assay matched those of the standard indirect susceptibility assay on 7H10
medium, and interpretable results were obtained for 98.5% of the samples within 2 weeks. Traditional methods
of in vitro drug susceptibility testing are time consuming and laborious. Susceptibility tests on clinical isolates
require 6 to 9 weeks, and tests conducted directly on smear-positive samples take about 3 weeks (International
Union Against Tuberculosis and Lung Disease, The public health service national tuberculosis reference
laboratory and the national laboratory network. Minimum requirements, role and operation in a low-income
level III laboratory, Centers for Disease Control and Prevention, Atlanta, Ga., 1985). More-rapid methods are
available but are very expensive for routine use under program conditions in countries with high levels of
tuberculosis endemcity.

A colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) was previously stan-
dardized and evaluated using a BACTEC radiometric method as a “gold standard” for indirect detection of rifampin resis-
tance (1, 8). MTT is a yellow tetrazolium salt that is converted into blue formazan by dehydrogenases of live cells (7). The
amount of blue or purple formazan formation is proportional to the number of live mycobacteria in a sample (8). The results
of the MTT assay matched fully with the results obtained using the BACTEC method (1).

This study was conducted with the objectives of standardiz-
ing the MTT assay for the detection of rifampin resistance
directly on smear-positive sputum samples and evaluating the
assay against the traditional method as the gold standard.

**Standardization and evaluation.** Sputum samples from 16
new smear-positive cases of pulmonary tuberculosis were used
to standardize the assay. Sputum samples from 74 smear-posi-
tive retreatment cases were pelleted after digestion and de-
contamination with 4% NaOH (5). The pellets were neutral-
ized and resuspended in 3 ml of sterile 7H9 broth each. An
aliquot of 500 μl was added to tubes containing 3 ml of 7H9
Middlebrook broth supplemented with 10% oleic-acid-albu-
min-dextrose-catalase, glycerol (0.05%), and PANTA (Becton
Dickinson, Paramus, N.J.) (75 μl). Rifampin (Sigma) at a final
concentration of 2 μg/ml was added to some of the tubes. The
experimental approach is summarized in Fig. 1. All tubes were
incubated at 37°C until the day of the MTT assay.

**MTT assay.** The MTT assay was done each week for 3
weeks. Contamination was checked by growing subcultures on
nutrient agar medium. Each week, an MTT assay was done
using one bacterial control tube with no rifampin and another
tube with bacteria and rifampin. The assay was done as de-
scribed previously (1). Relative optical density (OD) unit
(RODU) values were calculated for each sample by dividing
the OD value of the rifampin-containing tube by the OD value
of the drug-free control. Resistance was defined as RODU >
0.5, and susceptibility was defined as RODU < 0.2. RODU
values obtained each week for samples containing susceptible
isolates were compared (using the Mann-Whitney U test) with
those of samples containing resistant isolates. The lowest OD
value which was considered indicative of growth was deter-
moved by growing a subculture with an aliquot of vortexed
broth medium every week before the MTT assay. The lowest
OD value with a positive culture result was 0.10; therefore, the
results were considered interpretable when the OD value of
the control was ≥0.1.

**Standard sensitivity testing.** Standard biochemical tests
were used to identify all isolates as *Mycobacterium tuberculosi*
(5). A proportion method (5) using Middlebrook 7H10 me-
dium was used as a reference method for rifampin suscepti-
ability testing. Reference *M. tuberculosis* strains ATCC 35836
(rifampin susceptible) and ATCC 35838 (rifampin resistant)
were used as controls.

Among the 74 samples used to evaluate the MTT assay, 5
(6.8%) were excluded, 3 because the OD values of control
tubes remained below 0.1 and 2 because there was no growth
on Löwenstein-Jensen medium. Of the remaining 69 samples,
5 (7.2%) were contaminated; however, for each of the five
samples there was at least one noncontaminated interpretable

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result among the results of the three sets of experiments prepared for the 3 weeks. The contamination rates of 18 samples tested in the presence of PANTA (Becton Dickinson) and in the presence of a much cheaper but similar antibiotic cocktail prepared in-house were the same. There was no contamination in the presence of Löwenstein-Jensen medium.

Table 1 shows the number of interpretable results obtained in each week. In the first week, 43 of 68 (63%) of the samples gave interpretable results; the number of samples with interpretable results grew in the second (98.5%) and third (100%) weeks. The MTT assay identified 8 of 69 (11.6%) samples as containing rifampin-resistant M. tuberculosis and 61 of 69 (88.4%) as containing rifampin-susceptible M. tuberculosis. The susceptibility (sensitivity and specificity) results obtained with MTT concurred fully with findings obtained using the standard assay on 7H10 agar medium. Figure 2 shows that the RODU values of samples containing susceptible bacteria remained below 0.2 in all weeks of experiments and that the RODU values of samples containing resistant bacteria were above 0.5. The differences in the RODU values of samples

![Flow chart of MTT assay](image)

**FIG. 1.** Flow chart of MTT assay. Abbreviations: BC, bacterial control without rifampin; R, tube with 2 μg of rifampin/ml; C, control without bacteria. LJ, Löwenstein-Jensen.

**TABLE 1.** Contamination rate and time of interpretation of a direct MTT assay for detection of rifampin-resistant M. tuberculosis

<table>
<thead>
<tr>
<th>Assay week</th>
<th>No. of contaminated samples/total no. of samples tested (%)</th>
<th>No. of interpretable samples/total no. of samples tested (%)</th>
<th>No. of samples giving the indicated assay result$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MTT 7H10 (indirect)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>S R</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>43/68 (63)</td>
<td>37 6</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>64/65 (98.5)</td>
<td>56 8</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>66/66 (100)</td>
<td>58 8</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>69/69 (100)</td>
<td>61 8 61 8</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Samples were inoculated in duplicates into drug-free broth medium. The change in OD was measured with the MTT assay (1) at 1 and 2 weeks after inoculation.

$^b$ S, susceptible; R, resistant.

**FIG. 2.** Growth patterns of rifampin-susceptible ($n = 37$ samples in the first week, 56 in the second week, and 58 in the third week) and rifampin-resistant ($n = 6$ in the first week and 8 in the subsequent weeks) strains of M. tuberculosis as reflected by RODU values (means ± standard errors) [$\text{RODU} = \frac{\text{OD}_{570} \text{ of rifampin-containing medium}}{\text{OD}_{570} \text{ of drug-free medium}}$]. The difference in RODU values in each week was statistically significant ($P < 0.01$ [Mann-Whitney test]).
containing susceptible and resistant isolates were statistically significant for each week \( P < 0.01 \).

Our findings show that a direct assay based on a tetracycline salt significantly reduces the time required to obtain reliable susceptibility results. The standard direct methods of drug sensitivity testing on solid medium take 3 to 4 weeks (5, 6), and with these conventional methods, there is in addition a need to prepare appropriate dilutions of a specimen as determined on the basis of smear grading (5). Our application of a direct MTT assay is not dependent on smear grading and shortens the turnaround time. Other direct rapid methods (such as the BACTEC 460 system and the mycobacterial growth indicator tube system) have a turnaround time ranging from 9 to 12 days (3, 6). However, they are very expensive for routine use in most countries in which tuberculosis is endemic.

Rifampin resistance is a strong predictor of the presence of multidrug-resistant tuberculosis (2). Therefore, the results of our study focusing on the direct detection of rifampin-resistant \( M. \) tuberculosis indicate the potential of this simple and inexpensive assay for control programs in countries with high levels of tuberculosis endemicity. The same assay could theoretically be used to rapidly screen for resistance to other antituberculosis drugs. Our preliminary findings indicate that the same assay could be used for reliable and rapid detection of isoniazid resistance. This new method should be evaluated under program conditions in a region with a high level of tuberculosis endemicity and optimized for program use.

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REFERENCES


