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Comparison of the Performances of Two In-House Rapid Methods for Antitubercular Drug Susceptibility Testing

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Received 20 July 2008/Returned for modification 3 September 2008/Accepted 11 November 2008

Resistance to rifampin (rifampicin), isoniazid, and streptomycin of 69 Mycobacterium tuberculosis isolates was analyzed by an in-house method based on mycobacteriophage D29 and a colorimetric micromethod. Both methods showed sensitivity and specificity values ranging from 93% to 100%. These simple methods offer an option for drug resistance assessment of M. tuberculosis.

The quest for rapid, simple methods that could replace both the lengthy, conventional bacteriological culture and the more sophisticated and expensive methods by shortening the time for determination of drug sensitivity of Mycobacterium tuberculosis clinical isolates led to techniques based on the replication of mycobacteriophages. One such method, the phage replication-based assay (PhaB), is based on the lytic mycobacteriophage D29 and uses phage replication as a marker of the presence of viable cells of the tubercle bacilli (13). Although the performance of PhaB for clinical samples is hampered by undetermined factors—the detection threshold is rather high (10⁴ bacilli/ml) (18)—this method has been tested with great success at reference laboratories in Latin America, Africa, and India for determination of susceptibility to rifampin (rifampicin) (RIF) (9, 10, 19, 22, 24) and isoniazid (INH) (2, 3). In Argentina, only the national reference laboratory (National Institute of Microbiology Dr. Carlos Malbrán) has implemented the in-house method, using it with good results for analysis of resistance to RIF (20). Since there are very few reports on the utilization of PhaB for the examination of resistance to other antitubercular drugs (14, 23) and since we believe that such a simple and inexpensive method should be tested globally, we decided to study the feasibility of using PhaB for the determination of resistance to RIF, INH, and streptomycin (STR) in our mycobacteriology laboratory. This facility is located in an academic setting and provides microbiological services to a local hospital of medium complexity in a heavily populated urban area.

Sixty-nine clinical isolates of M. tuberculosis (30 from reference centers in Argentina and 39 from our culture collection) as well as the control strains M. tuberculosis H37Rv and Mycobacterium bovis BCG Pasteur 11732 were included in this study. The strains were numbered coded, and the operators had no information on the resistance status. Mycobacterium smegmatis mc²155 was used as host strain for the propagation and detection of phage D29. Propagation and titration of stocks of D29 (kind gift from G. Hatfull) were performed as described elsewhere (5, 13, 23).

Mycobacterial suspensions were made by dispersing freshly grown cells taken from Lowenstein-Jensen slants with glass beads and a few drops of saline in screw-cap tubes. From these tubes, a variable amount was added to screw-cap test tubes containing 7H9 broth medium so that the final turbidity (judged by naked eye) was comparable to that of the a McFarland standard of 1. The preparation of indicator plates was performed as described by Gali et al. (7).

The D29-based assay was performed as described by Wilson et al. (23) except for minor modifications described below. Drug treatments were as follows: RIF, 24-h exposure time at 1 and 10 μg/ml; STR, 48-h exposure time at 2 and 8 μg/ml; INH, 72-h exposure time at 1 and 2 μg/ml. After the addition of the virucide (ferrous ammonium sulfate, to a final concentration of 30 mM [15]) and twofold dilution with 500 μl phage buffer (13), 20-μl aliquots of each sample (control and the two drug concentrations tested) were distributed with disposable plastic loops on indicator plates divided into three sectors. Positive (M. tuberculosis H37Rv and M. bovis BCG, used as the test strain) and negative (no mycobacteria) controls were included. Plates were visually inspected after 24 to 36 h of incubation at 37°C.

Seeking alternative fast and simple methods for determination of drug susceptibility, we also performed a resazurin microtiter plate assay (REMA) on the isolates. This indicator-based method allows for detection of cell viability scored as a color change in a microtiter plate format and has been used with different indicators (1, 4, 6, 11, 16). The overall setup of the 96-well microtiter plate for REMA has been described in several publications (1, 11, 16). Positive (no drug) and negative (no mycobacteria) growth wells were included in all the assays. The drugs were tested in the following ranges: RIF, 2 to 0.015 μg/ml; INH, 1 to 0.007 μg/ml; STR, 8 to 0.06 μg/ml. Growth was evidenced by addition of 20 μl of resazurin (Sigma; 10 mg/ml in water) to one of the growth control wells; if a change of color of the dye—indicative of growth—was seen, 20 μl of resazurin was added to the rest of the wells; otherwise, the plate was incubated for another 24 h and the process was repeated. The final point of the reduction of the dye was scored after 18 h of incubation. The MIC was the lowest drug concentration that produced no growth.

The gold standard proportion method (PM) was carried out on Middlebrook 7H11 agar plates according to NCCLS rec-
programs. Due to the bias in their origin: most of them were used in quality control programs.

Brook 7H9 broth. Aliquots (20 μl) of the 1:100 and 1:10,000 dilutions were inoculated onto plates in the absence or presence of drugs and incubated for 21 days before growth was monitored. The cutoff concentrations were, according to CLSI recommendations (16a; available at http://www.clsi.org/source/orders/free/m24-aa.pdf). The inoculum was prepared as mentioned above starting from a turbidity comparable to a McFarland standard of 1 and then serially diluted with Middlebrook 7H9 broth.

Susceptibility or resistance to the tested drugs was judged by change of color—indicative of growth—in the case of REMA and by growth (more than 1% of the growth obtained in control cultures) in the case of PM, as well as by either the absence of plaques or a large reduction (≥95%) in the number of plaques due to lysis in the presence of the drugs compared to the numbers observed in the case of the control (no drug) plates.

There are relatively few reports on the use of D29 for determination of antitubercular drug susceptibility in spite of the 10 years that have passed since Wilson et al. (23) developed the method. Most of them assessed resistance to a single drug (RIF) in primary cultures. Thus, we decided to test the susceptibility to three drugs, namely, RIF, INH, and STR, by the PhaB method. Our results showed a good agreement between PM, REMA, and PhaB, provided that, for PhaB, incubation times for the test strains and drugs were adequate. Thus, while in our hands 24 h was enough for a clear result when assaying resistance to RIF, longer periods were required for optimal visualization of the results for STR (48 h) and INH (48 to 72 h). In spite of the extra work of plating samples on three consecutive days against a single plating at the third day, we believe that our proposed schedule rapidly pinpoints RIF resistance, which is suggestive of multidrug resistance status due to its frequent link with INH resistance (20, 21). Both methods performed equally well for the three drugs assayed (Table 1), with sensitivity and specificity values between 93.6% and 100% (Table 2). Strains showing discrepant results were analyzed again. Upon repetition (three independent determinations by the three methods) one strain which was initially misclassified as sensitive to RIF by PM was determined to be resistant to this drug by the three methods; thus, both REMA and the D29-based assay correctly classified it the first time it was tested. A second discrepancy was observed with three strains determined to be INH sensitive by PM. In this case, MICs were 0.25 to 0.5 μg/ml. Although low, this level of resistance was accurately detected by REMA and the D29-based assay. STR gave the largest number of discrepancies (two by PhaB and three by REMA); these could not be resolved by repeated analysis of the strains. Nevertheless, these results are encouraging since we correctly assigned resistance to a large number of M. tuberculosis clinical isolates previously analyzed by state and national reference centers with negligible deviations from their results.

The methods tested herein complement each other for the determination of the drug resistance status of clinical M. tuberculosis strains, with PhaB giving results faster but analyzing susceptibility to a fixed drug concentration, unlike REMA, which analyzes a range of concentrations but takes longer before the results are obtained. A common characteristic of both methods is their quick turnaround time compared to that of PM. While drug susceptibility testing by PM starting from primary cultures takes 28 to 42 days in Lowenstein-Jensen culture medium and 21 days when 7H11 medium is used, results may be obtained within 7 to 12 days when vital dyes are used (6, 11, 16). PhaB results can be obtained at 48, 72, and 96 h when susceptibility to RIF, STR, and INH, respectively, is assayed. Thus, PhaB has a very short turnaround time—followed by REMA—compared to standard culture methods. More sophisticated, fast methods such as BacTec take approximately the same time as do microcolorimetric methods, and, since the former methods are not widely available, additional time is required for the reception of results due to administrative delays. Thus, the two methods discussed here offer a clear advantage in turnaround times.

PhaB and REMA are low-cost methods, indicating that they would be affordable for extended use, although a cost analysis shows that PhaB is cheaper ($2/per sample per drug) than REMA ($3.75/per sample per drug). In this regard more exhaustive analysis should be performed due to country-to-country differences in costs of the materials used.

Two recent publications by Kalantry et al. (8) and Pai et al. (17) reported meta-analyses of mycobacteriophage-based methods. These authors conclude that, although phage-based assays have high specificity, they show variable sensitivity and thus cannot replace microscopy and conventional bacteriological culture yet. In a recent paper published by Martin et al. a meta-analysis of microcolorimetric methods was presented.

<table>
<thead>
<tr>
<th>Drug</th>
<th>PM No. of strains with indicated result</th>
<th>PhaB</th>
<th>REMA</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIF</td>
<td>S 44, R 25</td>
<td>S 43, R 26</td>
<td>S 43, R 26</td>
</tr>
<tr>
<td>INH</td>
<td>S 47, R 22</td>
<td>S 44, R 25</td>
<td>S 44, R 25</td>
</tr>
<tr>
<td>STR</td>
<td>S 50, R 19</td>
<td>S 48, R 21</td>
<td>S 47, R 22</td>
</tr>
</tbody>
</table>

*The extremely high proportion of resistant strains determined in this study is due to the bias in their origin: most of them were used in quality control programs.*

*Statistical calculations were performed with Epidat 3.0, free software available at http://www.sergas.es/MostrarContidos_Portais.aspx?IdPaxina=50100.*

<table>
<thead>
<tr>
<th>Test method and drug</th>
<th>Sensitivity (%) (95% CI)</th>
<th>Specificity (%) (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PhaB</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIF</td>
<td>97.7 (86.2–99.9)</td>
<td>100 (84–100)</td>
</tr>
<tr>
<td>INH</td>
<td>93.61 (81.43–98.33)</td>
<td>100 (81.50–100)</td>
</tr>
<tr>
<td>STR</td>
<td>96 (85.14–99.30)</td>
<td>100 (97.07–100)</td>
</tr>
<tr>
<td>REMA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RIF</td>
<td>100 (89.3–100)</td>
<td>97.7 (79.76–99.8)</td>
</tr>
<tr>
<td>INH</td>
<td>93.61 (81.43–98.33)</td>
<td>100 (81.5–100)</td>
</tr>
<tr>
<td>STR</td>
<td>93.61 (82.45–98.43)</td>
<td>100 (79.07–100)</td>
</tr>
</tbody>
</table>

*Statistical calculations were performed with Epidat 3.0, free software available at http://www.sergas.es/MostrarContidos_Portais.aspx?IdPaxina=50100.*

*CI, confidence interval.*
(12). Their conclusions supported the use of these highly sensitive and specific (ranging between 89% and 100%) methods for the rapid detection of multidrug-resistant tuberculosis. Altogether, the relevant literature points out that it would be highly advisable to carry out more evaluations for both PhaB and REMA to help validate these methodologies.

The tested methods are relatively easy to perform, do not rely on expensive supplies or equipment, and do not require excessive technical skills, strongly suggesting that they are useful tools for rapid identification of resistant and multidrug-resistant *M. tuberculosis* strains even in low-complexity clinical settings such as this one. Finally, the rapid screening for drug resistance at local laboratories would reduce the workload at more specialized institutions such as the National Reference Center for Tuberculosis; at the same time, the fast availability of the results will identify multidrug-resistant strains and allow the establishment of an appropriate therapeutic treatment.

This work was supported by grants from Sustainable Science Institute (United States) and Secretaría de Ciencia y Técnica, Universidad Nacional de Rosario, to H.R.M., and fellowships to A.I.D.L.I. and E.J.S. through a Fogarty International Center/NIH grant, the AIDS International Training and Research Program (grant 5D43 TW0010137), and the Fogarty International Programme. H.R.M. is an independent researcher from the Research Council of the National University of Rosario.

We thank S. Pezzotto (Instituto de Inmunología, Facultad de Ciencias Médicas, Universidad Nacional de Rosario) for advice on use of Epipat 3.0 software and the anonymous reviewers of the manuscript for valuable comments.

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