Evaluation of the ESP Culture System II for Testing Susceptibilities of *Mycobacterium tuberculosis* Isolates to Four Primary Antituberculous Drugs

JOHN S. BERGMANN AND GAIL L. WOODS*

Department of Pathology, University of Texas Medical Branch, Galveston, Texas 77555-0740.

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The reliability of the ESP Culture System II (herein referred to as ESP II) for testing susceptibilities of *Mycobacterium tuberculosis* isolates to isoniazid, rifampin, ethambutol, and streptomycin was evaluated by comparing results to those of the method of proportion (MOP), which was considered the reference method, for 20 clinical isolates and 30 challenge strains provided by the Centers for Disease Control and Prevention (CDC). Clinical isolates also were tested with the BACTEC TB 460 system; these results agreed with those obtained by the MOP for all isolates and all drugs, except the high concentration of isoniazid, for which agreement was 95%. After resolution of discrepancies, levels of agreement between ESP II and MOP for the clinical isolates were 95 and 100%, respectively, for the low and high concentrations of isoniazid, 100% for rifampin and ethambutol, and 95% for streptomycin. For the 30 challenge isolates, ESP II results for both concentrations of isoniazid agreed with the expected results in all cases, whereas agreement was 93% for both rifampin and streptomycin and 90% for ethambutol. All discrepancies with the CDC isolates were due to failure of ESP II to correctly classify resistant strains. By testing isolates yielding discrepant ethambutol and streptomycin results with a lower concentration of both drugs in the ESP II system, agreement increased to 93% for ethambutol and 100% for streptomycin. For the clinical isolates, the times to an ESP II result of susceptible (means ± standard errors of the means) were 8.47 ± 0.12 days (range, 7 to 10 days) and 8.73 ± 0.29 days (range, 5 to 11 days) when the inoculum was prepared from a McFarland equivalent and from a seed bottle, respectively. The time to an ESP II result of resistant varied by drug and method of inoculum preparation, ranging from 5.50 ± 0.22 days for ethambutol with the inoculum prepared from a McFarland standard to 8.0 days for ethambutol with the inoculum prepared from a seed bottle. These data suggest that the ESP II system is a rapid and reliable method for testing susceptibilities of *M. tuberculosis* isolates to isoniazid and rifampin. Performance, however, may be suboptimal for ethambutol and streptomycin. Testing additional ethambutol-resistant and streptomycin-resistant strains with two concentrations of both drugs is necessary.

Tuberculosis remains an important public health problem in the United States today, despite its declining incidence over the past several years. One aspect of tuberculosis control is treatment with effective antituberculous drugs. To help ensure appropriate therapy early in the course of the disease, experts at the Centers for Disease Control and Prevention (CDC) recommend that for isolates of *Mycobacterium tuberculosis* complex (MTBC) susceptibility test results be available an average of 28 to 30 days from receipt of a specimen in the laboratory (7). Currently in the United States, susceptibility testing of MTBC is performed by using either the method of proportion, which is considered the reference method, or the BACTEC TB 460 system (Becton Dickinson, Sparks, Md.). Of these two methods only the BACTEC TB 460 system has the potential to consistently meet the suggested turnaround time.

The ESP Culture System II (AccuMed International, Westlake, Ohio [formerly Difco]), a fully automated continuously monitoring system for growth and detection of mycobacteria, has been available for commercial use for a few years (9). As for the ESP blood culture system, the technology of the ESP II is based on detection of pressure changes (i.e., either gas production or gas consumption due to microbial growth) within the headspace above the broth culture medium in a sealed bottle. A special detection algorithm has been developed for the very slowly growing mycobacteria, in addition to the detection algorithm used with the ESP blood culture system. Recently, a method for testing susceptibilities of isolates of MTBC to isoniazid, rifampin, ethambutol, and streptomycin was developed for the ESP II system (1, 3, 5, 6). The purpose of this study was to evaluate the reliability of the ESP II system for testing susceptibilities of MTBC isolates to these four drugs.

MATERIALS AND METHODS

**MTBC isolates.** Twenty isolates of MTBC identified with a DNA probe (AccuProbe; Gen Probe, Inc., San Diego, Calif.) from 20 patients at the University of Texas Medical Branch (UTMB), Galveston, were tested by using ESP II, method of proportion, and BACTEC TB 460. To further evaluate the reliability of the ESP II method, the CDC provided 30 MTBC challenge isolates with known susceptibilities to antituberculous drugs (based on results of the method of proportion), which were tested by ESP II alone. Testing personnel at UTMB were unaware of the expected results for these 30 isolates until all testing was completed. As per the manufacturer’s protocol, the control strains of MTBC included ATCC 25618 (susceptible to all drugs tested), ATCC 35831 (resistant to streptomycin), ATCC 35838 (resistant to rifampin), ATCC 35822 (resistant to isoniazid), and ATCC 35837 (resistant to ethambutol), which with the exception of ATCC 35831 are those suggested by the National Committee for Clinical Laboratory Standards for testing MTBC by using BACTEC TB 460 and the method of proportion (4). All isolates were subcultured on Löwenstein-Jensen slants and tested within 6 weeks.

**Preparation of inoculum.** Colonies from a Löwenstein-Jensen slant were scraped and transferred to a sterile tube containing 4.0 ml of saline and 8 to 10 glass beads. The suspension was agitated with a vortex mixer for 1 to 2 min and left undisturbed for 20 min. The supernatant was transferred to another sterile tube and allowed to settle undisturbed for 15 min. The supernatant was removed and adjusted with saline to a density equal to a 1.0 McFarland standard. This
suspension was the standard inoculum for testing by using ESP II, BACTEC TB 460, and method of proportion. In addition, for the ESP II system only, preparation of the inoculum from a seed bottle was evaluated, as follows. A drug-free ESP II bottle containing 1.0 ml of ESP II growth supplement and 1.0 ml of sterile water was inoculated with 0.5 ml of the 1.0 McFarland-equivalent suspension and then placed in the ESP II instrument. On the day that this seed bottle showed evidence of growth, as indicated by a signal from the ESP II instrument, or within 2 days after signalling (during which time the bottle remained in the ESP II instrument), an aliquot of the broth was removed and used as the inoculum for susceptibility testing as described in the following paragraph.

**ESP II susceptibility testing.** Isoniazid, rifampin, ethambutol, and streptomycin were provided by AccuMed International, rehydrated with distilled water, filter sterilized (0.45-μm-pore-size filter), aliquoted, and stored at −20°C until the day of use. Susceptibility testing was performed according to the manufacturer’s recommendations. For each isolate tested, six ESP II bottles were inoculated; one drug-free growth control (GC), one each with rifampin, ethambutol, and streptomycin, and two with isoniazid. Briefly, 1.0 ml of ESP II Myco growth supplement containing 7.5% (wt/vol) bovine serum albumin, 3.0% (wt/vol) dextrose, 0.0009% (vol/vol) oleic acid, 72 U of catalase per ml, and 1.25% (wt/vol) sodium chloride was added to each ESP II bottle. An appropriately labeled ESP II bottles, 1.0 ml of each drug solution was added according to the manufacturer’s protocol, giving final concentrations of 0.1 and 0.4 μg/ml for isoniazid, 1.0 μg/ml for rifampin, and 8.0 μg/ml for both ethambutol and streptomycin. These values were selected by AccuMed based on results of experiments they had previously conducted to determine what final drug concentrations in the ESP II system correlated with the critical concentrations used with the method of proportion. The GC bottle received 1.0 ml of distilled water. Each ESP II bottle was then inoculated with 0.5 ml of a 1:10 dilution (in sterile saline) of the McFarland-equivalent inoculum or with 0.5 ml of a 1:10 dilution of broth from the seed bottle. A connector was placed on each bottle, and the bottles were loaded into the ESP II instrument. To test for sterility, a blood agar plate was inoculated with 0.5 ml of the inoculum and incubated at 37°C for 48 h.

An isolate was considered susceptible to a drug if there was no growth in the drug-containing bottle or if the time to detection of growth in the drug-containing bottle was greater than the time to detection of growth in the GC bottle (rounded to the nearest whole number) plus 3 days. An isolate was considered resistant to a drug if the time to detection of growth in the drug-containing bottle was less than the time to detection of growth in the GC bottle (rounded to the nearest whole number) plus 3 days. To confirm that the positive signal was due to growth of resistant MTBC and not to bacterial contamination, a blood agar plate was inoculated with 0.5 ml of broth from each positive ESP II bottle, incubated at 37°C, and examined for bacterial colonies at 48 h. According to the manufacturer’s protocol, if the GC is not positive by day 10, the run must be considered invalid, and testing of all drugs must be repeated.

**BACTEC TB 460 susceptibility testing.** Anti-tuberculous chemical testing with BACTEC TB 460 was performed and the results were interpreted according to the manufacturer’s instructions, as described in detail elsewhere (2). The final concentrations of the drugs tested were 0.1 and 0.4 μg of isoniazid per ml, 2.0 μg of rifampin per ml, 2.5 and 7.5 μg of ethambutol per ml, and 6.0 μg of streptomycin per ml.

**Method of proportion.** The method of proportion was performed and the results were interpreted according to standard procedure (2), using a 1.2 dilution of the standard inoculum and Middlebrook 7H10 agar (Difco Laboratories, Ann Arbor, Mich.) containing isoniazid (final concentrations, 0.2 and 1.0 μg/ml), rifampin (final concentration, 1.0 μg/ml), ethambutol (final concentrations, 5.0 and 10 μg/ml), and streptomycin (final concentrations, 2.0 and 10 μg/ml), or none of these drugs.

**Discrepancy testing.** In all cases the method of proportion was considered the reference method. For the clinical isolates, if the results of one or more susceptibility test methods disagreed, the tests involving the drug and method(s) were repeated in-house and the isolate was sent to two reference laboratories for testing by the method of proportion. For the challenge isolates, only the ESP II method was repeated if the initial result did not agree with the expected result. Selected isolates were also tested with ESP II at 5.0 μg of ethambutol per ml or 2.0 μg of streptomycin per ml, in addition to the higher concentrations of each agent.

**Statistical analysis.** The difference in mean time to susceptibility test results (from the time of inoculation) between ESP II and BACTEC TB 460 was analyzed by Student’s t test.

**RESULTS**

**Initial susceptibility test results for the 20 clinical MTBC isolates are summarized in Table 1.** In all cases the GC was positive on day 2. ESP II results for rifampin (17 susceptible and 3 resistant) and ethambutol (all 20 susceptible), using both methods of inoculum preparation, agreed with those of BACTEC TB 460 and the method of proportion for all isolates. For the high concentrations of isoniazid tested, results obtained by all three methods agreed, including both methods of inoculum preparation for ESP II, for 19 isolates (18 susceptible and 1 resistant); 1 isolate was found to be susceptible by ESP II and the method of proportion but resistant by BACTEC TB 460.

For the low concentrations of isoniazid tested, using both types of inoculum preparation for ESP II, there was initial agreement among the three susceptibility testing methods for 17 clinical isolates (15 susceptible and 2 resistant). One isolate yielding discordant results was identified as resistant by the method of proportion only. Retesting of this isolate by all methods in-house and testing by the method of proportion in reference laboratories showed it to be susceptible; thus, the initial ESP II results for both methods of inoculum preparation were correct for 18 (90%) isolates. For both methods of inoculum preparation, the remaining two isolates were identified as susceptible by ESP II and resistant by the method of proportion and BACTEC TB 460. After retesting these isolates in-house, only the ESP II results changed (from susceptible to resistant) for 1 isolate by each method of inoculum preparation. Thus, the agreement between ESP II and the method of proportion increased to 95% for each method of inoculum preparation.

For streptomycin, results by ESP II with both methods of inoculum preparation, BACTEC TB 460, and the method of proportion agreed for 19 (95%) isolates (18 susceptible and 1 resistant). The remaining isolates were identified as susceptible by ESP II (with both methods of inoculum preparation) but resistant by BACTEC TB 460 and the method of proportion. When retested using two concentrations of streptomycin with the ESP II system, the latter isolate was identified as resistant by BACTEC TB 460 and method of proportion, susceptible by ESP II to 8.0 μg/ml and resistant by ESP II to 2.0 μg/ml (with both methods of inoculum preparation for both concentrations).

With the ESP II system, the mean times (± standard errors

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**Table 1. Resolved susceptibility test results for clinical isolates of MTBC by method of proportion, BACTEC TB 460, and ESP II**

<table>
<thead>
<tr>
<th>Drug and result</th>
<th>MOP</th>
<th>BACTEC</th>
<th>ESP II</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Isoniazid (low)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Susceptible</td>
<td>16</td>
<td>16</td>
<td>18</td>
</tr>
<tr>
<td>Resistant</td>
<td>4</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td><strong>Isoniazid (high)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Susceptible</td>
<td>19</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>Resistant</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td><strong>Rifampin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Susceptible</td>
<td>17</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>Resistant</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td><strong>Ethambutol</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Susceptible</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Resistant</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Streptomycin</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Susceptible</td>
<td>18</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>Resistant</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
of the means) to a susceptible result (i.e., the time to detection of growth in the GC bottle plus 3 days) were 7.50 (±0.25) days (range, 6 to 10 days) and 9.10 (±0.27) days (range, 6 to 13 days) when the inoculum was prepared from a McFarland equivalent and from a seed bottle, respectively. When retesting due to initial contamination (four isolates) or a faulty connector (one isolate) was included in the analysis, these times increased to 8.95 (±0.76) days (range, 8 to 14 days) and 9.30 (±0.36) days (range, 5 to 11 days), respectively. The mean times to a resistant result when the inoculum was prepared from a McFarland equivalent and from a seed bottle, respectively, were 6.50 (±1.50) days (range, 5 to 8 days) and 10.00 days for 0.1 μg of isoniazid per ml, 6.00 and 7.00 days for 0.4 μg of isoniazid per ml, 7.00 (±1.00) days (range, 6 to 8 days) and 9.00 (±1.53) days (range, 7 to 12 days) for rifampin, and 8.00 and 9.00 days for streptomycin. When retesting due to contamination was included in the analysis, the time to a resistant result increased only for rifampin (i.e., 9.00 [±2.08] days [range, 6 to 13 days]) when the inoculum was prepared from a McFarland equivalent. With the BACTEC TB 460 system, the mean times to a susceptible and a resistant result were 7.30 (±0.27) days (range, 6 to 10 days) and 7.50 (±0.87) days (range, 6 to 10 days), respectively. The difference in time to a result with ESP II and BACTEC TB 460 was not significant.

Table 2 summarizes the initial ESP II results for the 30 CDC MTBC challenge isolates. For 1 isolate the GC was not positive by day 10, necessitating retesting of that isolate with all drugs. At the low concentration of isoniazid tested, ESP II results (inoculum prepared from a McFarland equivalent) agreed with those provided by CDC for all 30 isolates (20 resistant and 10 susceptible). With the inoculum prepared from a seed bottle, there was total agreement among susceptible isolates and for 19 of the 20 resistant isolates at the low concentration of isoniazid (total agreement, 97%). At the high concentration of isoniazid, there was complete agreement between ESP II (both methods of inoculum preparation) and CDC results (15 susceptible and 15 resistant).

With rifampin, ESP II results for the McFarland-equivalent inoculum agreed with those of the CDC for all 19 susceptible isolates and for 9 (82%) of the 11 resistant isolates (overall agreement, 93%). Upon retesting of the two isolates yielding discrepant rifampin results, both were identified as susceptible by ESP II. With the seed bottle inoculum, ESP II and CDC results were concordant for all 11 rifampin-resistant isolates and for 18 of the 19 (95%) susceptible isolates (overall agreement, 97%). When the isolate yielding discrepant rifampin results was retested by ESP II, it was identified as susceptible, as expected.

ESP II and CDC ethambutol results agreed for the 26 susceptible isolates but only for 1 of the 4 (25%) resistant isolates when the inoculum was prepared from a McFarland equivalent (overall agreement, 90%). The only difference in ESP II ethambutol results, when the inoculum was prepared from a seed bottle, was failure to detect any of the four resistant isolates (overall agreement, 87%). Upon retesting of these four ethambutol-resistant isolates with the ESP II system, using 5.0 μg of ethambutol per ml rather than 8.0 μg/ml, two were identified as resistant and two were identified as susceptible with the McFarland-equivalent inoculum, whereas one was identified as resistant and three were identified as susceptible with the seed bottle inoculum. With streptomycin, ESP II results for both methods of inoculum preparation were concordant with the CDC results for the 25 susceptible isolates and 3 (60%) of the 5 resistant isolates (overall agreement, 93%). When the two isolates with discrepant streptomycin results were retested by ESP II, using 2.0 μg of streptomycin per ml rather than 8.0 μg/ml, both were identified as resistant with the McFarland-equivalent inoculum, but only one was identified as resistant with the seed bottle inoculum.

For the 30 challenge isolates, the mean time (± standard error of the mean) to a result of susceptible to any drug by ESP II was 8.47 (±0.12) days (range, 7 to 10 days) when the inoculum was prepared from a McFarland standard and 8.73 (±0.29) days (range, 6 to 11 days) when the inoculum was prepared from a seed bottle. The mean time to a resistant result with the inoculum prepared from a McFarland standard and from a seed bottle, respectively, were 6.22 (±0.15) days (range, 6 to 7 days) and 7.18 (±0.33) days (range, 6 to 9 days) for rifampin, 5.95 (±0.22) days (range, 4 to 7 days) and 6.47 (±0.36) days (range, 4 to 11 days) for the low concentration of isoniazid, 5.93 (±0.28) days (range, 5 to 9 days) and 6.07 (±0.18) days (range, 5 to 7 days) for the high concentration of isoniazid, 6.33 (±0.33) days (range, 6 to 7 days) and 6.25 (±0.25) days (range, 6 to 7 days) for streptomycin, and 6.00 and 8.0 days for ethambutol.

**DISCUSSION**

We evaluated the reliability of the ESP II system (using two methods of inoculum preparation) for determining susceptibilities of MTBC isolates to four of the primary antituberculous drugs by testing clinical isolates, most of which were susceptible to all agents, and challenge strains provided by the CDC, over half of which were resistant to one or more drugs, and comparing results to those obtained by the method of proportion (considered the reference method). Overall, for the 50 clinical and challenge isolates, performance of ESP II was the best (i.e., 100% agreement with the method of proportion with both types of inoculum preparation) for the high concentration
of isoniazid, which is the concentration used by clinicians as a base for decisions concerning resistance and administration of the drug. For rifampin, which is the most important drug in the antituberculous regimen, ESP II incorrectly classified two resistant isolates as susceptible with the inoculum prepared from a McFarland equivalent (96% overall agreement with the method of proportion, with two false-susceptible results) and overcalled resistance with the inoculum prepared from a seed bottle (98% overall agreement, with one false-resistant result). The latter discrepancy was resolved by retesting. The two isolates yielding false-susceptible rifampin results would have been identified as resistant, thus resulting in 100% agreement, if the criterion for resistance by ESP II were changed to the following: the time to detection of growth in the drug-containing bottle is less than the time to detection of growth in the GC bottle (rounded to the nearest whole number) plus 4 days (rather than 3 days, as is the manufacturer’s recommendation).

For the low concentration of isoniazid, the ESP II system undercalled resistance with both methods of inoculum preparation. Two clinical isolates yielded false-susceptible results with the inoculum prepared from a McFarland equivalent, as did three isolates (two clinical and one challenge [correctly identified as resistant on retesting]) with the inoculum prepared from a seed bottle. If the criterion for resistance had been modified as described above, one isolate would have correctly been identified as resistant with each method of inoculum preparation, decreasing the number of false-susceptible results to one with each method of inoculum preparation.

A weakness of our study is the inclusion of small numbers of isolates resistant to streptomycin and ethambutol. Clinical isolates at UTMB are rarely resistant to the primary antituberculous agents, and of the CDC challenge strains, only five were resistant to streptomycin and four were resistant to ethambutol. Consequently, our evaluation of the ESP II system for these two drugs was suboptimal. In general, ESP II undercalled resistance to both ethambutol and streptomycin with both methods of inoculum preparation. Moreover, it appears that to ensure detection of resistance to these two agents, two concentrations of each drug must be tested. By doing so for selected isolates in our study, overall agreement with ethambutol increased from 94% (three false-susceptible results) to 96% (two false-susceptible results) with the inoculum prepared from a McFarland standard and from 92% (four false-susceptible results) to 94% (three false-susceptible results) with the inoculum prepared from a seed bottle. If, in addition, the criterion for resistance were changed as described above, agreement would increase to 98% with both methods of inoculum preparation. By testing two concentrations of streptomycin, overall agreement increased from 94 to 100% with the inoculum prepared from a McFarland equivalent and from 94 to 98% with the inoculum prepared from a seed bottle.

With regard to time to reporting susceptibility test results, the performance of ESP II was comparable to that of BACTEC TB 460. Based on data from an evaluation of the ESP II system for growth and detection of mycobacteria (9), the use of ESP II for mycobacterial culture and susceptibility testing of Mycobacterium tuberculosis would meet the goals recommended by the CDC for reporting results of these mycobacterial tests (7).

ESP II has several advantages to offer. It is less labor-intensive than BACTEC TB 460. Inoculated bottles are placed only once in the ESP II instrument, whereas BACTEC TB 460 bottles are incubated off line in an incubator and then loaded and unloaded several times during the total incubation period. Scheduling the setup and the reading of bottles to coincide with the staffing of the laboratory is not an issue with the ESP II system as it is with BACTEC TB 460. The ESP II data management system simplifies the tracking of results. ESP II is nonradiometric, thus eliminating all issues associated with the use and disposal of radioactive material. Moreover, cross-contamination of bottles is not possible with the ESP II system; however, it has been a problem with BACTEC TB 460 (8).

In summary, our data indicate that the ESP II system is a rapid and reliable method for testing susceptibilities of MTBC isolates to isoniazid and rifampin. To ensure detection of resistance, we recommend that the criterion for considering an isolate resistant be modified to the following: the time to detection of growth in the drug-containing bottle is less than the time to detection of growth in the GC bottle plus 4 days (rather than 3 days). By making this minor alteration, eight isolates in our study would have been correctly identified as resistant, rather than falsely susceptible, and no susceptible isolate (clinical or CDC) would have been incorrectly classified as resistant to any drug. With regard to the performance of ESP II with ethambutol and streptomycin, our study was suboptimal given the small numbers of isolates that were resistant to these two drugs. It appears that to guarantee a correct classification of resistance, in addition to using our suggested modified definition of resistant, two concentrations of both ethambutol and streptomycin should be tested. However, because not all isolates were evaluated with both concentrations, we do not know if ESP II would yield false-resistant results for the lower concentrations of these two drugs. Further evaluation of the ESP II system with ethambutol and streptomycin, testing additional ethambutol-resistant and streptomycin-resistant strains and both concentrations of the two drugs, is necessary.

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