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# Interlaboratory Drug Susceptibility Testing of *Mycobacterium tuberculosis* by a Radiometric Procedure and Two Conventional Methods

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**A total of 224 recent isolates of *Mycobacterium tuberculosis* from 163 patients selected to have multidrug resistance were tested against streptomycin (SM), isoniazid, rifampin, and ethambutol (EMB) by the rapid radiometric BACTEC method and two conventional proportion methods: the World Health Organization (WHO) method, using Lowenstein-Jensen medium; and the Veterans Administration reference laboratory for mycobacteria (VA) method, using Middlebrook 7H10 agar medium. The results were compared, focusing on the concentrations of the drugs in all three methods. Among the four drugs tested, most of the discrepancies in measured activity were observed with SM and EMB, generally because of differences in the drug concentrations used by the three methods. A 4-μg amount of SM in the BACTEC method was found to be slightly less active than 10 μg in the VA method and significantly more active than 4 μg of dihydrostreptomycin in the WHO method. With EMB, 2.5 μg in BACTEC was similar to 5 μg in the VA method and 2 μg in the WHO method, while 10 μg in the BACTEC method was found to be more active than 10 and 2 μg in the VA and WHO methods, respectively. To attain close agreement, drug concentrations used in the BACTEC method should be carefully selected when a comparison is to be made with any conventional method employed in a laboratory. Standardization of in vitro susceptibility testing is greatly needed to achieve uniformity among the test methods used to evaluate tuberculosis therapeutics.**

The introduction of radiometric techniques in the field of mycobacteriology is a recent development. In 1977, Middlebrook et al. (7) showed that a new liquid medium, 7H12, used with the semiautomated BACTEC system for the detection of mycobacterial growth could have clinical laboratory usefulness. The first evaluation of rapid radiometric drug susceptibility testing of *Mycobacterium tuberculosis* was reported by Snider et al. in 1981 (10). Although the overall agreement at one laboratory was 95% and the predictive values for susceptibility were between 97 and 100%, the predictive values for resistance were considered low for streptomycin and ethambutol. In this study the culture suspensions used for radiometric testing were not freshly prepared, and it was believed that poor viability could have affected the results. Shortly thereafter, Siddiqi et al. (9) reported that freshly prepared suspensions yielded better and faster results. The overall agreement of radiometric results with those obtained by a conventional method was 98%, with specificity, sensitivity, and predictive values for resistance higher than those measured in the earlier study.

The modified proportion method with Middlebrook agar medium has been compared with the radiometric BACTEC procedure in several studies (8, 9, 10). The other proportion method practiced throughout the world uses Lowenstein-Jensen (LJ) medium and has been evaluated (6). It was felt, however, that further evaluations were required with a large number of drug-resistant strains to evaluate the sensitivity and specificity of the radiometric method. This study was also designed to evaluate the different drug concentrations used in these procedures to determine which would better compare with the established critical concentrations for resistance and to develop more uniform and compatible concentrations among these methods.

In the present study, a large number of recent patient isolates of *M. tuberculosis* known to have multidrug resistance of various degrees were selected for testing by radiometric (BACTEC) and the two modified proportion methods: one recommended by the Centers for Disease Control (CDC) and used with some modifications by the Veterans Administration Reference Laboratory for Tuberculosis and Other Mycobacterial Diseases (VA method), and the other recommended by the World Health Organization and used by the Laboratory Centre for Disease Control (LCDC), Canada (WHO method).

## MATERIALS AND METHODS

**Cultures.** A total of 224 cultures of *M. tuberculosis* from 163 patients were selected at the Veterans Administration reference laboratory during routine drug susceptibility testing of fresh isolates. As soon as species identification and drug susceptibility testing results at the laboratory were available, a duplicate LJ culture was mailed to Johnston Laboratories, Inc., Towson, Md., for radiometric (BACTEC) testing. Special care was taken to select cultures with resistance of various degrees to one or more drugs. After making a suspension from the growth for BACTEC susceptibility testing, the same slant was mailed by Johnston Laboratories to the LCDC for testing by the WHO method.

**Drug concentrations used in each method. (i) BACTEC.** Concentrations (in micrograms per milliliter of 7H12 medium) used were: streptomycin (SM), 2; isoniazid (INH), 0.2; rifampin (RIF), 2.0; and ethambutol (EMB), 2.5 and 10.0.

**(ii) VA.** Concentrations (in micrograms per milliliter of

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TABLE 1. Comparison of SM results

| Method (μg/ml) | Determination[a] | No. of indicated determinations by method (μg/ml): BACTEC (4) S | BACTEC (4) R | VA (2) S | VA (2) R | VA (10) S | VA (10) R |
|---|---|---|---|---|---|---|---|
| VA (2) | S | 137 | 0 | | | | |
| | R | 37[b] | 45 | | | | |
| VA (10) | S | 173 | 8[c] | | | | |
| | R | 1[c] | 37 | | | | |
| WHO (4) | S | 130 | 0 | 126 | 4[c] | 130 | 0 |
| | R | 44[b] | 45 | 11[c] | 78 | 51[d] | 38 |

[a] S, Susceptible; R, resistant.
[b] Seventeen borderline cases.
[c] Five borderline cases.
[d] Four borderline cases.

7H10 medium) used were: SM, 2.0 and 10.0; INH, 0.2; RIF, 1.0; and EMB, 5.0 and 10.0.

**(iii) WHO.** Concentrations (in micrograms per milliliter of LJ medium [before inspissation]) used were: dihydrostreptomycin (DSM), 4.0; INH, 0.2; RIF, 40.0; and EMB, 2.0.

**BACTEC method.** Details of the BACTEC radiometric method were described previously (9). Before inoculation of the culture, drug medium was prepared freshly by inoculating 0.1 ml of stock drug solution into a vial of 7H12 (BACTEC 12A) medium. Stock solutions were prepared so that when 0.1 ml was added to 2 ml of 7H12 medium the desired final concentration was achieved. The growth from an LJ slant was carefully scraped and transferred into a glass tube containing a few glass beads in approximately 5 ml of special diluting fluid (0.2% fatty acid-free bovine albumin and 0.02% Tween 80 in deionized water). The growth was homogenized by using a Vortex mixer and then left undisturbed for about 30 to 45 min. The supernatant was carefully aspirated and was adjusted to a turbidity approximately equivalent to that of a McFarland no. 1 standard by adding diluting fluid. A 0.1-ml amount of this suspension was inoculated into each of the drug-containing 7H12 vials, and 0.1 ml of a 1:100 dilution of this suspension was inoculated into each control (without drug) vial. All of the vials were read on a BACTEC 460 instrument (Johnston Laboratories) at time zero. They were then incubated at 37°C and read daily at approximately at the same time until the growth index (GI) of the control reached a level of 30 or higher. The GI indicates the amount of $^{14}CO_2$ produced by the metabolism of $^{14}C$-labeled substrate in BACTEC 12A medium during the growth of mycobacteria. Differences in the GI readings from the previous day (ΔGI) in the control and in the drug-containing vials were compared to interpret the results. If ΔGI of the control vials was greater than the ΔGI of the drug-containing vials, then the culture was judged to be susceptible to the drug, and if ΔGI of the control vials was less than the ΔGI of the drug-containing vials, then the culture was judged to be resistant to the drug. The test was read for 1 or 2 additional days if the ΔGI of the control vial was close to the ΔGI of the drug-containing vial. In such cases, depending upon the increasing or decreasing pattern of the GI reading, the culture was reported as borderline susceptible (0.8 to 1% resistant population) or borderline resistant (1 to 10% resistant population). If the daily GI in the test drug vial reached a level higher than 500 and then began to decline, the culture was considered resistant irrespective of the ΔGI.

**WHO method.** The standard version of the WHO proportion method was perfomed at LCDC, Canada, as described by Canetti et al. (1, 2). Appropriate concentrations of drugs were added to LJ medium before inspissation. This method determines the proportion of the bacterial population which is resistant to a "critical drug concentration." Usually, if the growth on drug-containing medium is 1% or more of the growth on the control medium, that particular drug is not or soon will not be useful in the treatment of a given patient. Critical drug concentrations in this procedure are based on bacteriological criteria as well as on clinical response to antituberculosis chemotherapy.

**VA method.** Details of the VA method have been described earlier (5, 11). Paper disks impregnated with antituberculosis drugs (BBL Microbiology Systems, Cockeysville, Md.) were added to individual quadrants of sterile polystyrene X plates (Becton Dickinson Labware, Oxnard, Calif.). The disks, approximately centered, were submerged in melted Middlebrook 7H10 agar medium enriched with oleic acid-albumin-dextrose-catalase (BBL). Uniform diffusion of the drug in 5 ml of medium per quadrant provided the desired concentrations indicated above for this method. Control quadrants contained 7H10 medium without disks.

Fresh clinical isolates received on LJ medium from Veterans Administration medical facilities were each subcultured to five LJ medium slants prepared in the laboratory from base powder (Difco Laboratories, Detroit, Mich.). At the same time, growth from the original isolates was subcultured in Middlebrook 7H9 broth containing albumin-dextrose-catalase (Difco) and incubated for 6 days at 35 to 37°C with daily shaking by hand to disperse clumps. A portion of the culture was added to a tube containing 7H9 broth medium to obtain a barely turbid suspension (approximate optical density of 0.06 at 580 nm). For inoculation of the drug susceptibility quadrants a modified proportion method was followed (5, 11, 12).

## RESULTS

Results determined by all three methods were available for 219 of the 224 initial cultures. BACTEC results with SM were compared with the VA and WHO methods (Table 1). The results indicated that 2 μg of SM in the VA method did not correlate highly with 4 μg in the BACTEC method. There were 37 (16.9%) specimens which were resistant by the VA method and susceptible by BACTEC. However, when 10 μg in the VA method was compared with 4 μg in BACTEC, there were only nine disagreements (4.1%), eight being susceptible by the VA method only. A 4-μg amount of DSM in the WHO method was found to be significantly less active than 4 μg in BACTEC, and the results were not highly correlated (20.1% disagreements). Between the two conventional methods, 2 and 4 μg in the VA and WHO methods, respectively, compared more closely (6.8% disagreements) than did 10 μg (VA) and 4 μg (WHO), which had 51 (23.3%) disagreements, all resistant by the WHO method.

In comparisons of INH susceptibility, all three methods used the same INH concentrations (Table 2). There were a total of four (1.8%) disagreements between BACTEC and the VA methods, three of which were resistant by the VA method only. There were nine (4.1%) disagreements between the BACTEC and WHO methods, three of which were resistant by the WHO method only and six by BACTEC. Comparing the two conventional methods, there were nine (4.1%) disagreements, seven of which were resistant by the VA method.

Determination of RIF susceptibility by the three methods gave very uniform results (Table 3). There was 100% agreement between the BACTEC and VA methods, while there were four (1.8%) disagreements between the BACTEC and WHO methods. The two conventional methods had four (1.8%) disagreements, three being resistant by the WHO method only and one by the VA method only.

In tests with EMB, two concentrations, one low and one high, were used by the BACTEC and VA methods, while the WHO method used only one concentration. When results with the low BACTEC concentration (2.5 μg) were compared with those with the low VA method concentration (5 μg), a total of nine (4.1%) disagreements were observed, mostly borderline cases, eight being resistant by the VA method only (Table 4). At a higher concentration of 10 μg in each method, the disagreement was the same (4.1%), six being resistant by the VA method only and three by BACTEC only. The BACTEC low concentration (2.5 μg) did not compare as closely with 2 μg in the WHO method; there were 21 (9.6%) disagreements, 15 being resistant by BACTEC only. With the high EMB concentration (10 μg) in BACTEC there were 41 (18.7%) disagreements, all resistant by the WHO method (2 μg). In the comparison of the VA and WHO methods, the low concentration of the VA method (5 μg) compared more closely with the WHO method (2 μg) (26 [11.9%] disagreements, 21 being resistant by the VA method only) than did the high concentration (10 μg) (38 [17.4%] disagreements).

Specificity, sensitivity, and predictive values were calculated for those concentrations which had close agreement in the previous analyses (Table 5). Specificity values were high in most of the comparisons (91 to 100%). However, lower sensitivity values were obtained when the results with 4 μg of SM were compared in the BACTEC and WHO methods as well as when the results with 10 μg of EMB were compared in the BACTEC and VA methods. In many cultures, only a very small proportion of the bacterial population (less than 10%) was shown to be resistant to a test drug, especially in the cases of SM and EMB. These cultures, designated as borderline, caused some problems in the interpretation of results and may have adversely affected the overall sensitivity and predictive values.

Since overall agreement between the BACTEC and WHO methods for SM was low, 13 cultures were randomly selected from those which were judged resistant by the WHO method only. These were tested again by both methods, using SM and DSM (Table 6). It appeared that SM was more active than DSM in BACTEC as well as the WHO method and thus results in more susceptibility in in vitro drug susceptibility testing of *M. tuberculosis* than does DSM.

TABLE 2. Comparison of INH resutls

| Method (μg/ml) | Determination[a] | No. of indicated determinations by method (μg/ml): | | | |
|---|---|---|---|---|---|
| | | BACTEC (0.2) | | VA (0.2) | |
| | | S | R | S | R |
| VA (0.2) | S | 97 | 1 | | |
| | R | 3[b] | 118 | | |
| WHO (0.2) | S | 97 | 6[c] | 96 | 7 |
| | R | 3[c] | 113 | 2 | 114 |

[a] S, Susceptible; R, resistant.
[b] One borderline case.
[c] Two borderline cases.

TABLE 3. Comparison of RIF results

| Method (μg/ml) | Determination[a] | No. of indicated determinations by method (μg/ml): | | | |
|---|---|---|---|---|---|
| | | BACTEC (2) | | VA (1) | |
| | | S | R | S | R |
| VA (1) | S | 160 | 0 | | |
| | R | 0 | 59 | | |
| WHO (40) | S | 159 | 3 | 159 | 3 |
| | R | 1 | 56 | 1 | 56 |

[a] S, Susceptible; R, resistant.

## DISCUSSION

The modified proportion method (VA method) and the WHO proportion method are two widely accepted conventional methods for drug susceptibility testing of *M. tuberculosis* (1, 2, 5, 11, 12). However, in practice, these methods vary greatly in the type of medium used, in drug concentrations in the medium, and in the preparation of drug media. Moreover, a variety of modifications have been adopted by individual institutions. Any newly developed technique has to be compared with conventional methodologies. However, if the conventional methodologies are not well standardized, it becomes a difficult task to test the reliability of the new technique.

The basic procedure for radiometric drug susceptibility testing using the BACTEC instrument has already been established (9, 10). This study was carried out to compare the susceptibility results on specially selected *M. tuberculosis* cultures with multidrug resistance and to establish a comparable drug concentration which could yield uniform results.

Among the four primary antituberculosis drugs tested, the results with SM and with EMB showed some discrepancies. Some of the important factors which may have contributed to these discrepancies are: (i) differences in the active drug concentrations in the medium, (ii) a low proportion of drug-resistant organisms in the test population of a culture, and (iii) substantial differences in the medium used by the three methods. These discrepancies could be reduced by adjusting the concentrations of the test drugs. Proper drug concentrations in a susceptibility test system are a critical issue and should be carefully investigated to yield maximum agreement with a well established and clinically evaluated method. In this study, the same concentrations of INH were used in all three methods and yielded fairly high correlation of results. The concentration of RIF in the WHO method was much higher because of its inactivation in egg-based media, and results with 40 μg/ml in LJ yielded a high correlation with those found with 1 μg/ml in 7H12. However, results obtained with SM and EMB require more extensive evaluation.

It appeared that 4 μg of SM in BACTEC was slightly less active than 10 μg in the VA method since most of the disagreements were found to be resistant by BACTEC only. A slight increase to 6 μg in the SM concentration with the BACTEC method could yield even closer agreement with the 10 μg of the conventional VA method. Moreover, in order to compare results at 2 μg in the VA method, the concentration of SM in 7H12 medium should be lowered. Our preliminary results indicated that 1 μg/ml in 7H12 medium compares well with 2 μg in 7H10 medium. In the WHO method, only one concentration of DSM (4 μg) was used whose results were found to be in agreement with,

TABLE 4. Comparison of EMB results

| Method (μg/ml) | Determination[a] | No. of indicated determinations by method (μg/ml): BACTEC (2.5) S | BACTEC (2.5) R | BACTEC (10) S | BACTEC (10) R | VA (5) S | VA (5) R | VA (10) S | VA (10) R |
|---|---|---|---|---|---|---|---|---|---|
| VA (5) | S | 151 | 1[b] | | | | | | |
| | R | 8[b] | 59 | | | | | | |
| VA (10) | S | | | 203 | 3[c] | | | | |
| | R | | | 6[c] | 7 | | | | |
| WHO (2) | S | 153 | 15[d] | 168 | 0 | 147 | 21[e] | 168 | 0 |
| | R | 6[d] | 45 | 41[b] | 10 | 5[e] | 46 | 38[f] | 13 |

[a] S, Susceptible; R, resistant.
[b] Nine borderline cases.
[c] Five borderline cases.
[d] Eleven borderline cases.
[e] Twelve borderline cases.
[f] Seven borderline cases.

although slightly less active than those of 2 μg of SM in 7H10 medium. The type of SM used also made some difference. When SM and DSM were compared by the WHO and BACTEC methods, it was found that SM at the same concentration was more active in both media and yielded more susceptibility than DSM. Overall, BACTEC GI readings in SM and DSM medium were also indicative of this fact. These results tend to confirm earlier studies showing that the MIC of SM is approximately half the MIC of DSM for *M. tuberculosis* (3). This should be taken into account by adjusting the critical drug concentration when switching from DSM to SM in drug susceptibility tests. DSM has been discontinued for use in treatment for more than a decade. Its use in in vitro drug susceptibility tests, however, has been continued in many parts of the world with egg-based media because it is known to be less susceptible to inactivation than SM (3).

With EMB, variations of results have always been a problem in in vitro susceptibility testing, especially at lower concentrations. Two concentrations of EMB were used in the VA method, and differences in the percentage of resistant cultures against 5 and 10 μg were significant (32 and 6%, respectively). Moreover, many of the cultures were borderline, having 1 to 10% of the population resistant to the drug. When susceptibility tests of some of these cultures were repeated, variable results were obtained at the lower concentration. It appears that 5 μg of EMB in 7H10 may be slightly low, while 10 μg may be too high. Alterations in the concentration of EMB could yield better and more objective results. A single intermediate concentration would be desirable for those who test only one concentration. It appears from these results that 2.5 μg in the BACTEC method is closely comparable with 5 μg in 7H10, while 10 μg in the BACTEC is slightly more active than 10 μg in 7H10 and could be lowered to 7.5 μg for a better comparison with 10 μg in 7H10 medium. The difference in concentrations of EMB in the two methods could be due to some loss of activity in 7H10 medium, which has been reported in the literature (4). Moreover, the disagreements within the two conventional methods, 10 μg in the VA method and 2 μg in the WHO method, are an obvious indication that these concentrations are not comparable. The significance of 10 μg EMB in in vitro susceptibility testing is in need of clarification.

BACTEC is a rapid test and the results are generally reported in 4 to 5 days. In this study the results were reported in an average of 4.9 days, while the conventional results were reported routinely in 3 to 4 weeks. There is very little or no loss of potency of the drugs in BACTEC 12A medium in such a short time; thus, slightly lower concentrations of some drugs may be required. Our preliminary work indicates that EMB is more stable in 7H12 than in

TABLE 5. Specificity, sensitivity, and predictive values of comparisons of methods for drug concentrations which yielded close agreement

| Drug | Method and concn (μg/ml) | Specificity (%) | Sensitivity (%) | Predictive value (%) of determination: Susceptible | Predictive value (%) of determination: Resistant |
|---|---|---|---|---|---|
| SM | BACTEC, 4; VA, 10 | 96 | 97 | 99 | 82 |
| | BACTEC, 4; WHO, 4 | 100 | 51 | 75 | 100 |
| | VA, 2; WHO, 4 | 92 | 95 | 97 | 88 |
| INH | BACTEC, 0.2; VA, 0.2 | 99 | 98 | 97 | 99 |
| | BACTEC, 0.2; WHO, 0.2 | 94 | 97 | 97 | 95 |
| | VA, 0.2; WHO, 0.2 | 98 | 94 | 93 | 98 |
| RIF | BACTEC, 2; VA, 1 | 100 | 100 | 100 | 100 |
| | BACTEC, 2; WHO, 40 | 98 | 98 | 99 | 95 |
| | VA, 1; WHO, 40 | 99 | 95 | 98 | 98 |
| EMB | BACTEC, 2.5; VA, 5 | 99 | 88 | 95 | 98 |
| | BACTEC, 10; VA, 10 | 98 | 54 | 97 | 70 |
| | BACTEC, 2.5; WHO, 2 | 91 | 88 | 96 | 75 |
| | VA, 5; WHO, 2 | 97 | 69 | 88 | 90 |

TABLE 6. Comparison of susceptibilities of 13 cultures to SM and to DSM

| Determination | No. of cultures with reaction to drug as determined by method (μg/ml): | | | |
|---|---|---|---|---|
| | BACTEC (4) | | WHO (4) | |
| | SM | DSM | SM | DSM |
| Susceptible | 9 | 0 | 8 | 0 |
| Resistant | 0 | 11 | 5 | 13 |
| Borderline | 4 | 2 | 0 | 0 |

7H10-7H11 media. Moreover, the MICs of most of the drugs in BACTEC 12A medium are lower than in a solid medium (S. Siddiqi, C. Hwangbo, C. L. Woodley, and R. C. Good, Program Abstr. 24th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1201, 1984). Overall, BACTEC results for *M. tuberculosis* were in high agreement with the conventional results at comparable concentrations. The majority of discrepancies between BACTEC and conventional results were among those cultures which had a low proportion of resistance. These cultures were reported by BACTEC as borderline susceptible or borderline resistant depending upon the GI inhibition pattern. Intralaboratory results on further routine susceptibility testing on a large series of clinical isolates by BACTEC and the conventional methods used at the Veterans Administration reference center and LCDC (the WHO method) will be reported later.

The widely accepted proportion concept considers a culture resistant if 1% or more of the bacterial population of a test culture is found to be resistant. However, variations in results are experienced in those cultures which have a low proportion of resistant organisms in the bacterial population. In such instances, if the test is repeated, results are often variable. Tubercle bacilli, since they are in clumps, are hard to disperse and thus create difficulty in calculating viable counts and establishing a percentage of the resistant population. Reporting could be made simpler and perhaps more reproducible if a range of from 1 to 10% instead of just 1% resistant population was taken into consideration and if susceptibility results were reported in three categories: susceptible (less than 1%), resistant (greater than 10%), and partially resistant (1 to 10%). However, the 1% proportion concept has been widely followed for many years, and any change should be based on a careful evaluation.

In this study the BACTEC results showed close agreement with the conventional findings, especially with the VA proportion method, but we note that drug concentrations used in BACTEC testing must be carefully selected in order to compare results with a particular existing conventional method in use in a laboratory. Different techniques of the conventional method and different concentrations of test drugs have created a complex situation in susceptibility testing in tuberculosis. It is time to concentrate our efforts toward standardization of techniques and drug concentrations in order to bring a uniformity to susceptibility testing. The introduction of the rapid radiometric method should provide the impetus to take the necessary steps to achieve this goal.

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