Determination of Drug Resistance of *Mycobacterium tuberculosis* Cultures

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A total of 3,303 strains of *Mycobacterium tuberculosis* were tested for sensitivity to streptomycin (SM), isoniazid (INH), and *p*-aminosalicylic acid (PAS) by the Steenken modified minimal inhibitory concentration (MIC) test. A simultaneous double blind comparison was carried out on 277 selected strains by the Steenken MIC test and the Canetti proportion method. Agreement between the results for the two tests was 82% for SM, 95% for INH, and 89% for PAS. A small number of strains appeared to be sensitive when tested by one method but resistant by the other. MIC determinations were carried out on 83 strains by using Steenken-Smith, Lowenstein-Jensen, and Middlebrook 7H10 media containing a more extended range of concentrations of the test drugs. The MIC values for both SM and dihydrostreptomycin increased on Steenken-Smith medium compared with the other two. INH did not show any medium effect, whereas PAS showed increased MIC values in 7H10 agar. The significance of the comparisons of the MIC values on the various media is discussed in terms of possible changes in the drug sensitivity testing methods used at present in this laboratory.

It is generally agreed that mycobacterial drug sensitivity tests are subject to a number of variables (including the culture medium, inoculum size, and critical drug concentrations), all of which may affect the apparent level of drug resistance (2). In the past, resistance to streptomycin (SM), isoniazid (INH), and *p*-aminosalicylic acid (PAS) has been routinely determined at the Trudeau Institute laboratory by the Steenken modified minimal inhibitory concentration (MIC) method (4). For a number of reasons, an eventual change to the Canetti proportion assay method was considered probable. However, the effect of a number of variables on the apparent levels of drug resistance shown by the two tests had first to be established. An extensive double blind comparison of the two test methods with cultures sent to the Trudeau Institute as part of the U.S. Public Health Service (USPHS) drug sensitivity testing program was therefore initiated. The results of these tests constitute the basis of the present paper.

MATERIALS AND METHODS

Organisms. *M. tuberculosis* H$_3$R$_6$ (TMC 102) was maintained on Steenken-Smith (S & S) egg slants and subcultured at 14-day intervals. *M. tuberculosis* strains were forwarded to the Trudeau Institute from participating hospitals as part of the drug sensitivity testing program carried out under the auspices of the USPHS. All cultures were transferred to American Trudeau Society (ATS) egg medium and checked for purity. After 10 to 14 days, growth was homogenized thoroughly in 1% gelatin in 0.067 M phosphate buffer (pH 6.8) and standardized photometrically before dilution and immediate use (4).

Drug sensitivity tests. Both the Steenken modified MIC test and the Canetti proportion tests were carried out in plastic trays. The Steenken test was carried out on S & S egg medium containing 10 and 30 µg of SM per ml; 0.2, 1.0, and 5.0 µg of INH per ml; 5 and 10 µg of PAS per ml. The tests were inoculated with approximately 10$^6$ viable bacteria, and density of growth was estimated by an arbitrary scale after 4 weeks of incubation at 37 C. The Canetti test was carried out on Lowenstein-Jensen (L-J) egg medium with dihydrostreptomycin (DSM) at 4 and 10 µg per ml, 0.2 and 1 µg of INH per ml, and 0.5 and 1.0 µg of PAS per ml. These tests were inoculated with 5 × 10$^8$ viable organisms, and the number of colonies developing after 6 weeks of incubation at 37 C were counted. The detailed methods, together with the criteria for resistance, were reported earlier (4).

Double blind comparisons by the Steenken and Canetti methods were simultaneously carried out by using a freshly prepared bacterial suspension. After 1 week of incubation, the identifying numbers were removed from both tests by an individual unconnected with the study, who then replaced the originals with random numbers supplied from the Research Section, USPHS, Rockville, Md. After further incubation, the tests were read and the results for both tests were forwarded to Rockville, Md. The code was then broken.
and the incidence of drug resistance in both tests was compared. Cultures showing variations in resistance to one or more drugs (together with an unknown number of controls which showed full agreement between the two methods) were then retested, a fresh ATS culture being used to prepare the inoculum.

Media. S & S and L-J egg media were prepared as described earlier (4). A final concentration of 0.02% lacmoid was added to assist the visualization of colonies.

Middlebrook 7H10 agar was prepared from dehydrated granules (Difco), sterilized by autoclaving at 121 C for 20 min, cooled to 48 C, and then enriched with 10% OADC (v/v). The agar was aseptically dispensed into 10-cm plastic quadrant plates (Falcon Industries) and stored at 4 C in plastic bags until required.

Extended MIC determinations. S & S, L-J, and 7H10 agar media were enriched with SM or DSM base in concentrations ranging from 0.5 to 30 µg per ml; INH from 0.05 to 10.0 µg per ml, and PAS from 0.1 to 10.0 µg per ml. The four sets of media were inoculated simultaneously with approximately 10^6 organisms, and the tests were incubated at 37 C for 28 days. Growth in drug-free and drug-containing media was graded by the arbitrary standards used for the Steenken test (4).

RESULTS

Comparison of Steenken and Canetti assays. During 1967 and 1968, a total of 3,517 cultures of M. tuberculosis were received at the Trudeau Institute from participating hospitals. A total of 214 cultures were identified as atypical mycobacteria and were discarded from the comparison.

A simultaneous double blind comparison of the drug sensitivity by the Steenken and Canetti methods was carried out on 277 strains of M. tuberculosis, about 100 of which were fully sensitive to all three test drugs. All strains showing apparent resistance to one or more drugs by one method, although still apparently sensitive by the others, were retested.

When the results for the first Steenken assay were compared with those for the second, 86% of the SM, 91% of the INH, and 88% of the PAS replicate assays showed complete agreement as to sensitivity or resistance. The corresponding figures for the two Canetti tests were 78% for DSM, 96% for INH, and 94% for PAS. Thus, the reproducibility of the assay results was good for both INH and PAS although not quite as high for the SM or DSM assays.

The level of agreement between the two methods with respect to resistance against all four test drugs was then examined (Table 1). Eighty-two per cent of the cultures tested were found to be either fully sensitive or resistant to SM, by both sets of criteria. Of the 156 strains rated as resistant by the Steenken method, only 127 appeared to be resistant in the Canetti test, leaving 29 as being SM sensitive according to Canetti’s criteria. A total of 14 cultures were rated sensitive to INH in the Canetti test but resistant in the Steenken test or vice versa. A further 26 strains were rated resistant to PAS by the Canetti test but not by the Steenken, whereas only three of the Steenken PAS-resistant strains appeared to be sensitive in the corresponding Canetti tests (Table 1).

A total of 98 strains were retested. Of these, 23 out of 25 cultures rated as sensitive to SM by both methods in the first comparison gave identical assessments on retest. However, of the 47 strains considered to be resistant by both methods in the first test, only 37 gave an identical assessment on retesting. Ten cultures appeared to be SM resistant in both Steenken tests but sensitive in both Canetti tests. Thirty-three strains gave various assessments of SM resistance in the two tests. Of these, nine showed a shift towards increased resistance in both assays, whereas eight appeared to be sensitive to SM in the second comparison. Only eight aberrant strains were observed in the corresponding INH comparisons.

| Table 1. Simultaneous assessment of drug sensitivity of 277 cultures of Mycobacterium tuberculosis by the two methods |
|---------------------------------------------------------------|
| Steenken MIC* | Canetti proportion |
| Sensitive | PR | Resistant |
| Sensitive | PR | Resistant >1% |
| Streptomycin (3 µg) Dihydrostreptomycin (4 µg) | | |
| 105 | — | — | 99 | 6 |
| — | 16 | 11 | 5 |
| — | 156 (38.5%) | 29 | 127 |
| — | — | — | — |
| — | 139 | 138 (50%) |
| Isoniazid (0.2 µg) | Isoniazid (0.2 µg) |
| — | 82 | — | 80 | 2 |
| — | 195 (70.5%) | 12 | 183 |
| — | — | — | 92 | 185 (67%) |
| 95% agreement | |
| p-Aminosalicylic (5 µg) p-Aminosalicylic (0.5 µg) | | |
| — | 204 | — | 178 | 26 |
| — | 71 (25.6%) | 3 | 68 |
| — | — | — | 181 | 94 (34.0%) |
| 89% agreement | |

* Sensitive, 0 to 1+ growth; PR, partially resistant, 1+; resistant, 2+ to 4+. MIC, minimal inhibitory concentration.
A total of 14 strains showed variations in PAS sensitivity in the two tests. These changes may merely reflect an improved adaptation by the tubercle bacilli to the culture media so that the amount of growth by the organism in the critical drug tube increased, giving the appearance of a change in the level of drug resistance expressed by the organism in one or other assay system. Although only a small segment of the total cultures was affected, it was likely that several factors could be responsible for this variation. Both the Steenken and Canetti tests employ a strictly limited number of drug concentrations. Under these circumstances, the effect of nutritional and environmental factors on the reproducibility of the two assays is difficult to assess. Further tests with a more extended range of drug concentrations in several different media were carried out.

**Effect of culture media on MIC values for the four test drugs: streptomycin.** The MIC values for SM dispensed in S & S medium were consistently higher than in L-J medium (Fig. 1). Because of this trend, 21 strains appeared to be drug resistant on S & S medium but were still sensitive on the L-J medium. None of the strains was rated SM resistant on the L-J medium but sensitive on S & S medium.

Comparison of the MIC values on L-J medium with those on 7H10 agar indicated that 16 of the strains were resistant to SM in the agar medium but not when L-J medium was used (Fig. 1). On the other hand, five of the test strains were rated SM resistant on L-J but not on 7H10 agar. The scattergrams in Fig. 1 suggest that 7H10 agar and L-J medium were better matched than the S & S and L-J media. This was confirmed when the MIC values on S & S medium were compared with those for 7H10 agar; 28 strains appeared to be resistant to SM on the S & S medium but sensitive in the 7H10 assays. However, seven other strains showed the reverse trend.

**Dihydrostreptomycin.** The corresponding studies with DSM indicated that the MIC values were again increased on the S & S medium (Fig. 2).
Only 14 of 83 cultures were rated resistant to DSM on S & S and sensitive on L-J. Three other strains showed the reverse trend.

The corresponding comparison of the MIC values on L-J and 7H10 agar indicated better agreement for these two media. Ten strains appeared to be resistant to DSM in L-J but not in 7H10 agar, and three showed the reverse trend. When S & S medium was compared with 7H10 agar, the MIC values were displaced considerably towards the S & S side; this was reflected by the fact that 23 of the test organisms were rated DSM resistant on this medium but sensitive on the 7H10 agar.

Isoniazid. The scattergrams shown in Fig. 3 indicate that the MIC values for INH in both S & S and L-J media fell about the line of equivalence. Only three strains appeared to be INH resistant on S & S medium and sensitive on L-J medium. Two strains showed the opposite effect. However, the MIC values were slightly higher on 7H10 agar than in the egg media so that six strains appeared to be INH resistant on the 7H10 agar but sensitive on the other media. A further point of interest was the large number of strains showing maximum INH resistance on the three media. Previous experience indicated that many wild strains of M. tuberculosis giving 4+ growth in the presence of 5 µg of INH were resistant to 50 µg of INH per ml or higher (Montalbine and Smith, unpublished data). There seemed little point to extending the present range of drug concentrations merely to obtain an exact MIC for these cultures, however.

p-Aminosalicylic acid. As with INH, the MIC values for all 83 cultures tested against PAS tended to fall into two sharply divided groups, regardless of the culture medium used (Fig. 4). However, tests carried out on 7H10 agar resulted in consistently higher MIC values than on either L-J or S & S media. The Canetti test recommends 0.5 µg of PAS as the critical drug concentration, whereas the Steenken test used 5 µg per ml. By using these criteria, 12 strains were rated as PAS resistant on L-J medium (Canetti) but sensitive on the S & S (Steenken). Comparison of S & S medium and 7H10 by using the 5 µg per ml cut-off point rated 13 strains as PAS resistant on 7H10 agar and sensitive on the S & S medium.

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**Fig. 2.** Comparison of the MIC values for dihydrostreptomycin on the three test media. See legend to Fig. 1 for further details.
Fig. 3. Comparison of the MIC values for isoniazid on the three test media.

Fig. 4. Comparison of the MIC values for p-aminosalicylic acid on the three test media.
Comparisons of the MIC values for the four drugs tested in the three media are represented in Fig. 5 as the ratios between the MIC values on one medium compared to that observed on one of the other two. The INH ratios indicate that the MIC values for this drug are not appreciably affected by the culture medium. However, the corresponding PAS data clearly showed a considerable skewing in the 7H10 agar, whereas both the SM and DSM data showed a corresponding displacement to the side of the S & S medium.

**DISCUSSION**

The notorious variability in the drug sensitivity assessments of *M. tuberculosis* strains tested in different laboratories led to the setting up of a number of central testing laboratories in different parts of the world (7). The Trudeau laboratory was so designated by the USPHS. Published data from a number of laboratories suggest that (despite considerable technical variations in the methods used) a statistically similar assessment of the incidence of drug resistance is achieved with respect to most *M. tuberculosis* cultures (3, 6). In the present study, an extensive double-blind comparison involving two test methods and three different media was carried out in the hope that some of the subjective factors involved in the choice of an optimal assay system might be overcome. Since it seems almost impossible to remain nonpartisan on the issue of the best drug sensitivity test method (1), the present results were analyzed in such a way that the observers were ignorant of the results for the individual tests. The most remarkable fact emerging from this comparison was that an almost 98% concurrence to the overall drug sensitivity or resistance was observed for the 3,303 strains of *M. tuberculosis* originally included in this study. Among the selected strains subjected to the double comparison, there was 80% agreement regarding SM resistance, 95% for INH, and 89% for PAS resistance. In a similar type of comparison of the Canetti and the MRC type of MIC test, the corresponding figures were 89% agreement for SM, 98% for INH, and 98% for PAS (5). However, the organisms used in the latter study included a high proportion of fully sensitive strains which probably accounted for the higher level of agreement obtained.

For some time, the advisability of changing the Steenken protocol to use L-J egg medium instead of S & S medium has been under consideration. Some wild strains of *M. tuberculosis* grow more slowly on S & S than on L-J egg medium. Furthermore, the viability of most wild strains of tubercle bacilli remains higher on L-J medium than it does on S & S medium. This has some practical importance since a fresh inoculum for use in a retest can be prepared directly from the control culture when L-J, but not when S & S medium, is used in the assay. However, the data in Fig. 5 indicate that a change from S & S to L-J medium would result in increased MIC values for SM for many organisms. This could lead to an apparently higher proportion of resistant cultures in future tests. Neither the INH nor PAS titrations seem to be affected by a change to L-J medium although a change to 7H10 agar could well affect the PAS titrations.

Neither the Steenken nor the Canetti test methods demonstrated a clear advantage as the best means of determining the level of drug sensitivity. The method to be used routinely in any drug sensitivity testing program therefore remains a matter of personal preference rather than one.
of clear scientific superiority by one test over the other (2). In view of the extensive body of information already in existence from the Steenken modified MIC tests carried out over the past 20 years, together with the considerable advantage to be gained from the continued use of a well tried and familiar technique, little advantage would be gained from any immediate change in the method used by this laboratory for the USPHS Drug Sensitivity Testing Program.

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