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Evaluation of a Rapid Radiometric Method for Drug Susceptibility Testing of *Mycobacterium tuberculosis*

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A total of 106 isolates of *Mycobacterium tuberculosis* were tested for drug susceptibility by the conventional 7H11 plate method and by a new rapid radiometric method using special 7H12 liquid medium with ^{14}C-labeled substrate. Results obtained by the two methods were compared for rapidity, sensitivity, and specificity of the new test method. There was 98% overall agreement between the results obtained by the two methods. Of a total of 424 drug tests, only 8 drug results did not agree, mostly in the case of streptomycin. This new procedure was found to be rapid, with 87% of the test results reportable within 4 days and 98% reportable within 5 days as compared to the usual 3 weeks required with the conventional indirect susceptibility test method. The results of this preliminary study indicate that the rapid radiometric method seems to have the potential for routine laboratory use and merits further investigations.

Automated and radiometric methods have been employed for culture and antimicrobial susceptibility tests in general bacteriology for more than a decade (3, 7). Cummings and co-workers in 1975 carried out preliminary work to show that the same principle could be utilized to detect growth of *Mycobacterium tuberculosis* (2). They used [*U*-^{14}C]glycerol and [*U*-^{14}C]acetate as the labeled substrate for detection of ^{14}C-labeled CO_2 in the medium. Middlebrook et al. further developed the technique and introduced a special 7H12 medium which contained [1-^{14}C]palmitic acid as the labeled substrate (6). Kertcher and co-workers (4) demonstrated the possibility of using the same principle for testing drug susceptibility of *M. tuberculosis* by adding an anti-tuberculosis drug to a medium which contained [^{14}C]formate as a labeled substrate and detecting the inhibition of radioactive CO_2 production. This method, however, did not offer any quantitative estimation of the proportion of resistant organisms in the test mycobacterial population and could not be readily correlated with the 1% resistance level used in the conventional method (1).

A technique of indirect drug susceptibility testing was developed using 7H12 medium, adjusting the inoculum size in such a way that semiquantitative results could be achieved with the 1% threshold as determinant of resistance. This technique was tried in a cooperative study with the Centers for Disease Control and was found to be comparable to the conventional system when the same suspension was used for conventional and radiometric methods (8) The extent of agreement of results with the two methods was found to be 95%, with a detection time of 2 to 18 days by the rapid radiometric method. However, the suspensions were not fresh, having been stored for a considerable time before they were tested for drug susceptibility by the radiometric procedure. It was felt that even faster results could be obtained if the suspensions were more freshly prepared from clinical isolates, thus having greater viability and more closely approximating the clinical laboratory situation. A study, therefore, was initiated between the State of Maryland Department of Health and Mental Health, Laboratories Administration (DHMH), and the University of Maryland, Division of Experimental Pathology, to compare the results of this radiometric method with those of the standard plate method and evaluate its efficiency when more recently prepared suspensions of mycobacterial isolates were used. This is a report of the results of indirect drug susceptibility only.

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MATERIALS AND METHODS

Cultures. Cultures utilized in this study were grown from specimens received by DHMH for culture of mycobacteria. These isolates were routinely tested for susceptibility to the primary drugs streptomycin (SM), isoniazid (INH), ethambutol (EB), and rifampin (RIF). The isolates were collected and tested over a 3-month period.

Conventional indirect drug susceptibility tests by plate method. The drug susceptibility test procedure employed by the DHMH is an abbreviated version of the proportion method (1, 5). The technique allows one to determine the proportion of the bacterial population that is resistant to the concentration of drugs tested. When 1% or more of microorganisms tested are resistant to the drug, the population is considered resistant to that chemotherapeutic agent. The test drugs were incorporated into 7H11 agar medium.

In the current study, all testing was performed by the indirect method, employing a suspension from primary growth of the clinical specimen. When there were only one or two colonies in the primary culture, a subculture was made. After sufficient growth, this subculture was used for making the suspension. A representative amount of growth was picked from the primary culture by using a sterile applicator stick. This sample was transferred to a sterile screw-capped tube containing six to eight glass beads and 3 ml of Tween-albumin medium. A homogeneous suspension was obtained by placing the tube on a table Vortex mixer for 5 min. After the large particles had settled, the supernatant suspension was pipetted out and adjusted with sterile distilled water to approximate a MacFarland no. 1 turbidity standard. The suspension was divided into two portions. One portion was saved for the rapid radiometric drug susceptibility test; these saved suspensions were stored at room temperature in a wall cabinet and were submitted to the University of Maryland once weekly.

The other portion was used for the plate susceptibility test on the same day. The suspension was diluted $1:10^2$ and $1:10^4$. The higher dilution was inoculated (1 drop) onto the apex corner of each drug and control quadrant. One drop of the lower dilution was inoculated into each of the two outer corners of each quadrant. After the drops had been absorbed into the medium, each plate was sealed in a polyethylene bag and incubated under 5% CO_2 at 35°C. The plates were read and reported after 3 weeks of incubation. Plates were inspected at ×20 to ×40 magnification. The inoculum had been adjusted to contain sufficient colony-forming units so that 50 to 200 colonies grew in the control quadrant. Any resultant growth in a test quadrant was compared with that obtained in the control quadrant. Populations yielding colonies in the drug-containing quadrant in excess of 1% of the control quadrant were reported as resistant to the test drug.

Indirect drug susceptibility by rapid radiometric method. The rapid radiometric drug susceptibility test procedure employed at the University of Maryland is a modified version of the proportion method. The test drugs were incorporated in a 7H12 liquid medium which contained [1-^{14}C]palmitic acid (6). The critical proportion of resistance was evaluated at the 1% level, as described later.

The drug susceptibility test was performed within 24 h of receiving the suspension from DHMH, usually on the same day. All suspensions were assigned a new number. Since opaque egg medium is sometimes picked up during the scraping of the culture and makes the suspension turbid and thus misleading, a smear was made and stained by the Ziehl-Neelsen method to check the density of acid-fast bacteria present in the suspension. Some suspensions, which were contaminated or which had very few or no bacilli on smear, failed to give useful results by either of the two methods and were not included in this study.

The 7H12 medium (Johnston Laboratories, catalog no. 12A) was used as the culture medium. A 2-ml quantity of this medium in a 20-ml bottle contained 2 μCi of ^{14}C-labeled substrate.

Stock drug solutions were made in deionized water as follows: SM, 40 and 80 μg/ml; INH, 4 μg/ml; RIF, 20 and 40 μg/ml (dissolved at slightly acidic pH); EB, 100 and 200 μg/ml. These solutions were filter sterilized (Nalgene, 0.2-μm filter); the first 30 to 40 ml of filtrate was discarded because of the initial absorption of drug onto the filter. These stock solutions were stored frozen at −70°C in 2- to 3-ml volumes.

Before the drug susceptibility testing, a tube of each stock solution of drug was thawed; 0.1 ml was added to each 7H12 bottle with a tuberculin syringe, one bottle being used for each concentration of the drug. The final concentrations of drugs in the medium are listed in Table 1. Thus, for each test a total of eight bottles were used, including one bottle without drug to serve as a control. With the conventional plate method, the drug concentrations used were those already established in the routine procedure at DHMH. With the radiometric method, two concentrations each of SM, RIF, and EB were used to establish the concentration giving results more comparable to those given by the conventional method.

Inoculation of the medium. Each suspension, after being checked for acid-fast bacilli by stained smear, was further suspended by aspirating and pushing the suspension back and forth in a disposable, plastic tuberculin syringe (with a nondetachable 26-gauge needle and inside a biological safety hood), and 0.1 ml of this suspension was inoculated into each of the drug-containing bottles. For the control inoculum, 0.1 ml of this suspension was added to 9.9 ml of diluent (fatty acid-poor albumin, 0.2%, and Tween 80, 0.02%, in distilled or deionized water, filter sterilized). After thorough mixing, 0.1 ml of this was inoculated into the control bottle. The inoculation of this 1:100 dilution of

TABLE 1. *Drug concentrations used*

Drug	Conventional plate method (μg/ml of 7H11 agar medium)	Rapid radiometric method (μg/ml of 7H12 liquid medium)[a]
SM	2	2
	10	4
INH	0.2	
	1	0.2
RIF	1	1
		2
EB	10	5
		10

[a] Actual final concentrations in the medium were 10% less than stated because 0.1 ml of drug solution and 0.1 ml of bacterial inoculum were added to 2.0 ml of medium in each bottle.

the suspension into the control was done to determine the conventional 1% level of resistance by comparing the growth in the control and drug-containing bottles.

All of the inoculated bottles were incubated at 37 to 38°C and were checked daily with a BACTEC 460 instrument (Johnston Laboratories). A 5% CO_2-in-air mixture was used as the flushing gas for the bottles during each testing.

Reading and interpretation of the results. [1-^{14}C]palmitic acid is metabolized during growth of mycobacteria, and $^{14}CO_2$ is released (6). Since the palmitic acid was labeled, the CO_2 thus evolved was also radioactive. This CO_2 was flushed out of the bottle by the BACTEC instrument, and its radioactivity was recorded in terms of numbers on a scale from 0 to 999, designated in this system as the growth index (GI). The GI was printed on a tape with the rack and bottle number. When an anti-tuberculous drug is present in the medium, growth is inhibited if the mycobacteria are drug susceptible, resulting in the suppression of $^{14}CO_2$ production. Preliminary studies have indicated that the GI correlates well with the resistance or susceptibility of the test organisms to a mycobactericidal drug, and that, during the first few days at least, the output of $^{14}CO_2$ is proportional to the size of the inoculum in 7H12 without drug.

The bottles were read daily. When the control GI reading was 30 or more, the results were interpreted by calculating the increase in GI from the previous day. If the daily increase of GI of the control was greater than the daily GI increase of the drug bottle, the test mycobacteria were reported as susceptible to that drug. If the daily increase of GI of the control was less than that of the drug bottle, the organisms were reported as resistant to that drug. Since the size of the inoculum in the control was 1/100 of the inoculum for the drug bottle, the critical proportion for resistance could thus be ascertained at the 1% level by comparing the growth rate of the control and drug bottles.

In Fig. 1 a typical growth pattern of an INH-resistant strain of *M. tuberculosis* is shown as obtained by the radiometric drug susceptibility method.

All results with coded cultures were reported to the DHMH Laboratories as soon as they were ready.

Comparison of the two methods. The specimen numbers were decoded after the conventional drug results were finalized and reported, and the results by both methods were compared. Specimens on which complete results were not available by either of the methods, due to contamination or loss of viability, were not included in this analysis. Results on mycobacteria other than *M. tuberculosis* were also not included in this study.

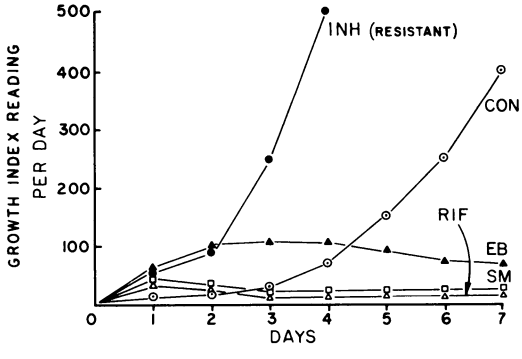

FIG. 1. *Radiometric drug susceptibility test pattern of an INH-resistant strain of M. tuberculosis. CON, Control.*

RESULTS

A total of 133 cultures were processed. Of these, 16 were found to be mycobacteria other than *M. tuberculosis*, and 11 specimens were not included because the results were not available due to contamination or nonviability. Thus 106 specimens of *M. tuberculosis* were analyzed in this study.

Drug susceptibility results obtained by the two methods are summarized in Table 2. Overall there was 2% disagreement between the two methods. Of the eight cultures for which disagreements were noted, six were observed to be resistant by the conventional method and susceptible by the radiometric method. Only two strains were found to be resistant by the radiometric method whereas the conventional method indicated them to be susceptible. Table 3 presents the detailed analysis of these results. With SM the radiometric method identified 10 strains as resistant at 2 μg/ml and 5 strains as resistant at 4 μg/ml, whereas the conventional method identified 13 strains as resistant at 2 μg/ml and 11 strains as resistant at 10 μg/ml. However, results of only a 2-μg/ml concentration of SM are compared in the table. With INH, RIF, and EB there were 16, 5, and 3 resistant strains, respectively, by the radiometric method, and 17, 6, and 2 resistant by the conventional method.

Table 4 presents the sensitivity, specificity, and predictive values of the radiometric method. This comparative analysis indicates that there is a good specificity (ability to detect susceptibility) with the new method. There are good predictive values of resistance and susceptibility as compared with the reference method, except for EB, which showed a low predictive value of resistance. As far as the sensitivity (ability to detect resistance) of this new technique is concerned, the values are better for EB, INH, and RIF than for SM.

TABLE 2. *Overall comparison of drug susceptibility results with M. tuberculosis by the two methods*

No. of specimens	Number of results in:	
	Agreement	Disagreement
95	380	0
11	36	8

TABLE 3. *Analysis of susceptible (S) and resistant (R) strains encountered by the two methods*

Result with conventional method	No. of strains with radiometric method result							
	SM (2 μg)		INH (0.2 μg)		RIF (1 μg)		EB (10 μg)	
	S	R	S	R	S	R	S	R
S	93	0	88	1	100	0	103	1
R	3	10	2	15	1	5	0	2

TABLE 4. *Analysis of sensitivity, specificity, and predictive values of the radiometric method as compared with the reference method*

Drug	Concn (μg/ml)	Parameter[a]			
		Sensitivity	Specificity	Predictive value	
				Resistance	Susceptibility
SM	2	0.77	1.0	1.0	0.97
INH	0.2	0.88	0.99	0.94	0.98
RIF	1	0.83	1.0	1.0	0.99
EB	10	1.0	0.99	0.67	1.0

[a] Values determined as follows: sensitivity, D/(C + D); specificity, A/(A + B); predictive value (resistance), D/(B + D); predictive value (susceptibility), A/(A + C), where A is when the test strain was found susceptible by both methods; B is when the test strain was found susceptible by the reference method and resistant by the radiometric method; C is when the test strain was found resistant by the reference method and susceptible by the radiometric method; and D is when the test strain was found resistant by both methods.

TABLE 5. *Number of days required for reportable results by radiometric method*

No. of days	No. of specimens reported	Cumulative %
3	24	23
4	68	87
5	12	98
6	1	99
7	1	100

The range of time required for completion of drug susceptibility testing by the radiometric method was 3 to 7 days with an average of 3.9 days (Table 5); 87% of the specimens were reportable within 4 days, and 98% were reportable within 5 days. The readings by the conventional method were routinely taken after 21 days of incubation.

DISCUSSION

Conventional methods for drug susceptibility testing of mycobacteria are well standardized and widely used, but the main disadvantage of this method is the long waiting period before results can be obtained. The indirect drug susceptibility testing method takes about 3 weeks. This long waiting period minimizes the usefulness of the drug susceptibility results for practicing physicians.

There are two important factors to be evaluated in considering any newly proposed drug susceptibility testing method. First, its results should be comparable in accuracy with the conventional system; second, it should be significantly faster. In this study, the radiometric method meets both of these requirements.

The time period required by the radiometric method for indirect drug susceptibility was 3 to 7 days. In general, 87% of the results were reportable in 4 days. However, the time required by this method depended upon the nature of the inoculum and its standardization. If the inoculum was too light, or of low viability, it took more than 4 days to obtain reportable results. On the other hand, if the inoculum was too heavy the GI of the control was more than 30 within 2 to 3 days, but the results could not be interpreted clearly unless the bottles were further incubated to observe an actual decline of the GI in the drug-containing bottles. The indication of resistance to a drug could be observed much earlier than susceptibility; since the inoculum in the drug bottle was heavy (100 times that of the control), the release of labeled CO_2 occurred more rapidly if there was no inhibition by the drug.

It was found in this trial with different concentrations of drugs that more appropriate concentrations to be used in the radiometric method were as follows: SM, 2μg/ml; INH, 0.2 μg/ml; RIF, 1 μg/ml; and EB, 10 μg/ml. Results obtained with these concentrations were very clear cut and quite similar to those from the conventional plate method. Moreover, some of the specimens which had low viability were subcultured in 7H12 medium, and, when the GI, measured daily, read about 900, this liquid culture, after being well suspended, was used for carrying out the radiometric drug susceptiblity assay. Results were satisfactory as compared with the conventional method.

It was observed that this approach could also be followed in case of a positive 7H12 medium bottle when this medium was used for the pri-

mary isolation. The details of this study will be reported later.

The disagreement in the results of the two methods was found to be 2%. Many SM-resistant cases were found to have a low proportion of resistant organisms, and four of these strains were found to be susceptible to 4 μg/ml, though resistant to 2 μg/ml, by the radiometric method. With INH, RIF, and EB, disagreements between the two methods were clear cut. It is important to emphasize that in nearly all of the instances of disagreement the conventional method reported resistance whereas the radiometric method reported susceptibility. Disagreements of this type can often be attributed to imperfect dispersion of the bacteria in the inoculum, which is critical for plate counting methods but not for metabolic methods. Moreover, it is possible that the radiometric method is more sensitive, because in liquid medium there is more cell-to-drug contact, and, due to shorter incubation time, there is less loss of potency of the test drug in the medium. However, this possibility must be investigated further.

This study supports our hypothesis that susceptibility test results could be reported more quickly, with improved specificity, sensitivity, and predictive values, using fresh clinical isolates, rather than old stock strains. Although some of our suspensions were stored for up to a week at room temperature, these gave much better results than the old refrigerated specimens tested in the earlier study. This work also indicates that the radiometric method of drug susceptibility has great potential: it is time saving, and the results correlate well with the conventional method used in most laboratories. Further trials in different laboratories on a larger scale are needed to evaluate cost, convenience, and practicality of this radiometric method for routine clinical use.

This report includes indirect drug susceptibility test results of *M. tuberculosis* only. Studies on direct susceptibility testing and on susceptibility testing of mycobacteria other than *M. tuberculosis* are under way and will be reported later.

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