

Quality Control of Individual Components Used in Middlebrook 7H10 Medium for Mycobacterial Susceptibility Testing

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The acceptability of different lots of commercial components which constitute our basal medium for susceptibility testing of mycobacteria was evaluated. The basal medium consisted of Middlebrook 7H10 agar supplemented with 10% oleic acid-albumin-dextrose-catalase and 0.5% glycerol. Studies were performed by using three separate microbiologic assays, and results were compared with parallel tests on previously standardized and acceptable lots of media. Components were rejected if comparison with standardized medium showed a major change in growth support or susceptibility status of any reference strain to any antimicrobial agent tested. Of the components tested in such a manner, 7 of 23 (30%) lots of 10% oleic acid-albumin-dextrose-catalase, 2 of 13 (15%) lots of Middlebrook 7H10 agar, and 0 of 5 lots of glycerol were found to be unacceptable. This study demonstrates that individual lots of components of this basal medium may vary significantly in their suitability for susceptibility testing, and failure to detect such variation may dramatically affect susceptibility profiles.

Since the advent of effective antimycobacterial agents for the treatment of disease produced by the *Mycobacterium tuberculosis* complex, clinicians have been concerned about treatment failures. In spite of modern multiple-drug regimens, a small proportion of patients have persistent or recurrent disease because of the emergence of organisms that are resistant to antimicrobial agents (11). Such organisms can also infect other individuals, causing primary drug resistance (13). Therefore, drug susceptibility tests are performed to provide guidance in the selection of appropriate antimicrobial agents and to monitor the spread of resistant organisms. The procedures used in those tests must be standardized to produce consistent results, so that changes seen over time can be properly interpreted for individual patient management and for public health concerns.

In the United States the susceptibility status of slow-growing mycobacteria is commonly determined by comparing growth in drug-containing medium against growth in drug-free medium (7). Using agar dilution or disk diffusion techniques, antimicrobial agents are incorporated into Middlebrook 7H10 agar supplemented with glycerol and a solution of oleic acid-albumin-dextrose-catalase (OADC) (5, 12). In our early investigations, new lots of 7H10 agar base or supplements occasionally adversely affected the reproducibility of susceptibility test results. Since then, it has been our practice to monitor the incorporation of a new lot of any component into our standardized susceptibility procedures by performing parallel microbiologic assays. The results of these quality control studies over a 15-year period are the basis of this report.

MATERIALS AND METHODS

Strains. Reference cultures used in the quality control of medium components (7H10 agar, glycerol, OADC, drug solutions, and disks) for susceptibility studies on mycobacteria are listed in Table 1. *M. tuberculosis* H37Rv (ATCC 9360) was obtained from the American Type Culture Collection (Rockville, Md.). All other isolates were submitted by

county and city public health laboratories in California. The isolates used in the following assays were chosen for their reproducible antibiograms; those used in assays on media containing drugs at or near diagnostic testing concentrations showed partial resistance during diagnostic testing or during searches for appropriate indicator strains among species not associated with disease. Individual stocks of these reference strains were maintained in Dubos-Tween-albumin broth at -70°C .

Preparation of reference cultures. Reference cultures were grown in Dubos-Tween-albumin broth for 2 to 3 weeks or until turbidity reached a no. 1 McFarland standard. Broths were then vortexed (Deluxe Mixer; Baxter Scientific, Hayward, Calif.) for 5 min prior to filtration through Whatman no. 42 filter paper. Filtrates were stored at -70°C in 1-ml amounts. Samples of the frozen suspensions were thawed and plated onto standardized medium to determine which dilution would subsequently yield approximately 100 to 200 CFU from a 0.1-ml volume.

Basal medium for susceptibility studies. The basal medium used in susceptibility tests and quality control studies consisted of 7H10 agar supplemented with 10% OADC and 0.5% glycerol (final concentration; vol/vol). Medium was dispensed in 5-ml quantities into individual sectors of a Felsen quadrant plate (X plate; Falcon; Becton Dickinson Labware, Oxnard, Calif.). Quadrant I contained only the basal medium, while quadrants II through IV contained basal medium supplemented with a single antimicrobial agent either in a disk or in solution. Following solidification, plates were incubated at 35°C for 24 h to dry the agar surface and allow diffusion from any antimicrobial agent-impregnated disks. Plates for quality control studies were either used immediately after drying or held for a maximum of 14 days at 4°C prior to use. Inoculated plates were incubated in 10% CO_2 in a mylar bag for 3 weeks at 35°C before a final reading was taken.

Antimicrobial agents. Antimicrobial agents were combined with basal medium by mixing solutions into molten agar or by diffusion from antimicrobial agent-impregnated paper disks embedded in plated medium (8). Solutions of powdered

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TABLE 1. Mycobacterial strains used in quality control assays in this study

Strain	Assay ^a
<i>Mycobacterium avium</i> 848	SCA
<i>Mycobacterium avium</i> 1833	SCA
<i>Mycobacterium avium</i> MSP26	DPA
<i>Mycobacterium gastris</i> MSP18	DPA
<i>Mycobacterium gordonae</i> 2180	SCA
<i>Mycobacterium kansasii</i> MSP3	DPA
<i>Mycobacterium kansasii</i> 2545	SCA
<i>Mycobacterium kansasii</i> 2768	SCA
<i>Mycobacterium scrofulaceum</i> MSP10	DPA
<i>Mycobacterium scrofulaceum</i> MSP27	DPA
<i>Mycobacterium tuberculosis</i> H37Rv (ATCC 9360)	CRA, DPA, SCA
<i>Mycobacterium tuberculosis</i> LA40	CRA
<i>Mycobacterium tuberculosis</i> 4973	SCA
<i>Mycobacterium tuberculosis</i> 3462	SCA
<i>Mycobacterium tuberculosis</i> MSP25	SCA, DPA
<i>Mycobacterium tuberculosis</i> 2401	SCA
<i>Mycobacterium xenopi</i> MSP20	DPA

^a Abbreviations: CRA, comparative resistance assay; SCA, standard concentration assay; DPA, disk potency assay.

or crystalline antimicrobial agents were sterilized by filtration and sealed in serum bottles. All glassware used throughout preparation and storage were specially rinsed and soaked in hot distilled water. Solutions for large-volume drug media were prepared as concentrated aqueous solutions which were stored at 4°C and used during a 12-month period in a routine ratio of 1 ml/liter of medium; these solutions were 0.02 and 0.1% isoniazid, 0.2% *p*-aminosalicylic acid, and 0.2 and 1.0% streptomycin. Ethambutol in 0.5% aqueous solution was used at 1 and 2 ml/liter. Solutions for infrequently used drug media were prepared for the usual addition of 1 ml/100 ml of medium and were stored at -20°C in single-use vials; these solutions were 0.05% kanamycin and 0.1% capreomycin. Ethionamide was dissolved in ethylene glycol as a 0.5% solution, stored at 4°C, and added to medium in a routine ratio of 1 ml/liter of medium to minimize the volume of ethylene glycol incorporated into the final product. Samples of all of the solutions described above that were stored as indicated for 1 year showed comparable activities when tested in parallel with fresh solutions. Further details of our experience with drug stability will be presented in a separate report. Rifampin was solubilized in *N,N*-dimethylformamide as a 5% solution and diluted to 0.01% in distilled water at 50°C immediately before incorporation into medium in routine ratios of 1:100 and 5:100. All of the routine ratios of drug solutions to the medium specified above reflect usage in diagnostic testing, i.e., 0.2 and 1 µg of isoniazid per ml, 2 µg of *p*-aminosalicylic acid per ml, 2 and 10 µg of streptomycin per ml, 5 and 10 µg of ethambutol per ml, 5 µg of kanamycin per ml, 10 µg of capreomycin per ml, 5 µg of ethionamide per ml, and 1 and 5 µg of rifampin per ml. Those ratios were adjusted, when necessary, to accommodate the various concentrations desired in the assays described below.

Antimicrobial disks (Sensi-Discs for Use in Culture Media; BBL Microbiology Systems, Cockeysville, Md.) were stored at -20°C and brought to room temperature before they were opened. Unused disks in thawed containers were stored at 4°C for 1 month or less. Disks designated INH-1, INH-5, PAS-10, S-10, S-50, EM-25, EM-50, RA-5, RA-25, and EA-25 were embedded in 5 ml of medium in quadrant plates. Diffusion of 1 µg of isoniazid from an INH-1 disk into 5 ml of medium resulted in a nominal concentration of 0.2 µg

of isoniazid per ml. Medium prepared with a disk embedded in the center of a quadrant of medium and preincubated as described above produces the same results as conventional medium (5). Similarly, the other commercially available disks result in medium containing 1 µg of isoniazid per ml, 2 µg of *p*-aminosalicylic acid per ml, 2 and 10 µg of streptomycin per ml, 1 and 5 µg of rifampin per ml, and 5 µg of ethionamide per ml.

Quality control assays. Three different assays were used to determine the suitability of new lots of components for routine use in susceptibility tests. The three assay systems covered different concerns related to drug susceptibility testing: the stability and interaction with medium of antimycobacterial drugs, antimicrobial disk potency, and checking of media prepared with routine drug concentrations for diagnostic testing. The following features were common to all three assays. (i) When a new component was to be tested, the previously accepted components (standardized medium) and the same components, except for substitution of the new lot (test medium), were prepared concurrently as parallel basal media; (ii) media were distributed into quadrant plates so that each plate had one quadrant of drug-free basal medium and three quadrants of basal medium containing drugs; (iii) dilutions of test strains with predetermined titers were inoculated onto the surfaces of the media in 0.1-ml amounts per quadrant; and (iv) after incubation as described above, the new lot of component was considered to be acceptable if similar numbers and sizes of colonies were observed on all parallel quadrants of test and standardized media, which would indicate that the test lot of basal medium was comparable to the standardized medium and that no unusual inhibitory or drug-binding products were present.

The first assay, termed the comparative resistance assay, was originally developed to monitor the stability of drug solutions and of drug activity when combined in medium. Three twofold dilutions of each drug in basal medium were adjusted so that the middle concentration (1×) permitted minimal growth of at least one of the reference strains on standardized medium. A drug level of half of that concentration (0.5×) was also used to determine whether a new component would produce excessive inhibition when combined with that drug. If growth on the test medium containing a 0.5× concentration of drug was comparable to growth on the standardized medium with a 1× concentration of drug, the new component was responsible for an apparent doubling of drug activity, which could conveniently be recorded as an erroneous 50% decrease in the MIC for that reference strain. The third concentration of drug was doubled (2×) for the converse reason. If the test lot required twice as much drug to suppress growth to the 1× concentration level, the drug-binding effect could be described as increasing the MIC by 100%. This assay was particularly sensitive to the effect of components on drugs, because the drugs were combined with medium in very low concentrations to accommodate the low MICs for the two *M. tuberculosis* reference strains used in this assay (drug-susceptible stock strains H37Rv and fastidious catalase-negative LA40, an isoniazid-resistant clinical isolate which was susceptible to other drugs). For example, H37Rv was used to test isoniazid media at concentrations of 0.1, 0.5, and 0.025 µg of isoniazid per ml, the concentrations most likely to reveal differences by permitting the growth of a larger or smaller proportion of the inoculum on media with at least one of the drug concentrations. Problems with distribution of inoculum and reproducibility of surface plate counts were ameliorated

TABLE 2. Characteristics of rejected lots of individual components for complete 7H10 basal medium

Product and manufacturer	Assay failed ^a	Reason for rejection ^b	Drugs bound ^c	Strain affected
OADC				
Difco	SCA	RCS		<i>M. tuberculosis</i> 3462 <i>M. kansasii</i> 2768
Scott	SCA	RCS		<i>M. tuberculosis</i> 3462 <i>M. kansasii</i> 2768
Bakte Bennett	SCA	DB, RCS	EMB, RMP, ETA	<i>M. tuberculosis</i> 3462 <i>M. tuberculosis</i> H37Rv <i>M. kansasii</i> 2545 <i>M. gordonae</i> 2180
Bakte Bennett	SCA	RCS		<i>M. tuberculosis</i> 3462 <i>M. kansasii</i> 2768 <i>M. gordonae</i> 2180
GIBCO	DPA	DB	KM, CM, SM	<i>M. kansasii</i> MSP28 <i>M. scrofulaceum</i> MSP10 <i>M. xenopi</i> MSP20
Bakte Bennett	CRA, SCA	DB, RCS	ETA, RMP, CM, INH, EMB	<i>M. tuberculosis</i> 3462 <i>M. kansasii</i> 2768 <i>M. kansasii</i> 2545 <i>M. gordonae</i> 2180
Scott	CRA, SCA	DB, RCS	EMB, KM, INH, PAS	<i>M. kansasii</i> 2768 <i>M. kansasii</i> 2545 <i>M. gordonae</i> 2180
7H10				
Difco	CRA, SCA	DB, opacity	CM, ETA	<i>M. tuberculosis</i> H37Rv <i>M. tuberculosis</i> LA40
Difco	CRA	DB	SM, CM	<i>M. tuberculosis</i> H37Rv <i>M. tuberculosis</i> LA40

^a Abbreviations: SCA, standard concentration assay; DPA, disk potency assay; CRA, comparative resistance assay.

^b Abbreviations: DB, drug binding; RCS, reduced colony size.

^c Abbreviations: EMB, ethambutol; RMP, rifampin; ETA, ethionamide; KM, kanamycin; CM, capreomycin; SM, streptomycin; INH, isoniazid; PAS, *p*-aminosalicylic acid.

by inoculating H37Rv onto triplicate plates and LA40 onto duplicate plates.

The second procedure, termed the disk potency assay, was developed to monitor the potency of antimycobacterial disks. Drug solutions were used to prepare media at 67, 100, and 150% of the drug concentration nominally supplied by the disks being tested. The range corresponds to the limits of acceptable potency of susceptibility test disks (± 1 standard deviation) established by the Food and Drug Administration (3). To check the disks, results from the media containing disks were compared with results from conventional media containing 67 to 150% drug concentrations by using the same basal medium for both. To check new components of the medium, 67 and 150% media were prepared with solutions, and 100% media were prepared with disks in both standardized and test media. Four or more *Mycobacterium* isolates chosen to cover the range of susceptibility and resistance to each drug were inoculated onto the plates containing that drug. Plates were incubated as described above, and the numbers and sizes of colonies were compared as described for the comparative resistance assay.

In the third assay, termed the standard concentration assay, each antimicrobial agent was incorporated at the concentrations used in routine media for diagnostic testing. In this assay the antibiograms of a battery of *Mycobacterium*

isolates were evaluated in both the standardized and test lots of susceptibility medium. Again, growth on the test medium was compared with that on the standardized medium to evaluate the acceptability of the new lot. This procedure only became possible after the accumulation of strains which would produce a different amount of growth on routine diagnostic susceptibility medium if the drug activity was diminished or enhanced by interaction with a new component.

Rejection criteria. Both sizes and number of colonies were considered in assessing the suitability of a new component for incorporation into standardized media. Colony sizes were graded as large, medium, small, and pinpoint; a reproducible change in two grades of colony size (e.g., from medium to pinpoint) was considered significant when standardized and test media in parallel quadrants were compared. Similarly, an average of a 20% or greater difference in the numbers of colonies was considered significant. In practice, minimum differences of two grades of size were often accompanied by at least a 20% change in numbers of colonies and vice versa, which made the observable differences more striking. When a significant difference was detected, fresh media were prepared for another comparison by using the same or another assay procedure, whichever

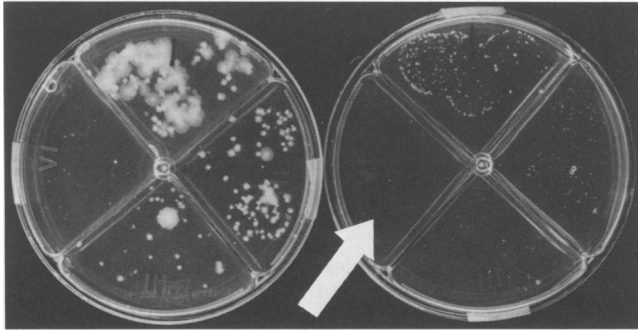


FIG. 1. Reduction of colony size by a new component and the resulting false susceptibility to drug apparent in the comparative resistance assay. The standardized medium on the left contains a quadrant without drug and three quadrants with increasing concentrations of ethionamide. The test medium on the right was prepared identically to the standardized medium and in parallel, except that a new lot of OADC was substituted. All quadrants were inoculated with 0.1 ml of a suspension of *M. tuberculosis*. The arrow indicates the quadrant in which colony size was reduced beyond visibility.

seemed most appropriate; if the difference was confirmed, the component was rejected.

RESULTS

Over a 15-year period, 41 lots (OADC, $n = 23$; 7H10 base, $n = 13$; glycerol, $n = 5$) of individual medium components were evaluated for their suitability for use in susceptibility testing. By using the guidelines outlined above, nine lots (22%) were found to be unacceptable for one or more reasons (Table 2). Of the lots rejected, 7 were OADC (30% of the 23 OADC lots tested). A total of 2 of 13 lots of base and 0 of the 5 lots of glycerol tested during this study period were rejected. The most frequently encountered problems were reductions in the colony sizes of reference strains (Fig. 1) and the variable binding of antimicrobial agents, which led to the observation of either false susceptibility or false resistance (Fig. 2).

The criteria used in the rejection of specific lots of OADC and 7H10 agar are listed in Table 2. Six of the nine lots caused a marked reduction in the colony size of one or more reference cultures; six lots caused an alteration in the activity of one or more antimycobacterial agents with established MICs for control stains. Three lots, all OADC, produced both changes when compared with standardized basal medium.

DISCUSSION

Our early investigations on antimycobacterial drug stability revealed that apparent changes in drug activity are often more closely associated with new lots of components in the medium than with length or conditions of storage. Certain lots of 7H10 agar, OADC, and glycerol were found to produce results at variance with that for the control lot; therefore, they were rejected if they produced reduced colony sizes or numbers on drug-free medium or were diverted to the production of primary isolation media if they simply interfered with antimicrobial activity. Medium components were occasionally exchanged with cooperating laboratories; certain components were found to contribute to interlaboratory discrepancies. In one such exchange, base and OADC were each partially responsible for reportable

differences. It is not surprising that variation in components could exhibit the broad range of effects reported here since other investigators have reported problems with a single drug (1, 2, 4), and similar effects have been noted in susceptibility testing systems for other bacteria (6, 9, 10).

The component with the greatest proportion of rejected lots was OADC, which, coincidentally, is purchased most often because it is a fluid and requires refrigeration, and its shelf life expires 2 years from the date of preparation. The effect of OADC on drug susceptibility studies was not related to a single manufacturer, control strain, or drug (Table 2). Four different manufacturers of OADC supplied one or more unacceptable lots of this supplement. Similarly, eight isolates, representing five different species of mycobacteria, and all of the eight antimicrobial agents involved in these susceptibility tests were found to be affected by one or more unacceptable OADC lots. In one instance, the addition of OADC to the 7H10 base produced a noticeable opacity in the medium which interfered with reading. Variability in the reasons for rejection suggests that either different components within OADC may be individually or collectively causing problems or other contaminants associated with the production of this supplement may be at fault. Further work in determining which ingredients and combinations produce the observed variations and how the variations could be eliminated is warranted.

The valuable characteristics of mycobacterial susceptibility tests on 7H10 basal medium include an acceptable correlation of test results with clinical progress in an established system familiar to clinicians, the medium's ease of preparation, and the transparency of the medium, which facilitates microscopic examination of small colonies. The problems described above do not diminish these values, but point out that the testing of new components is required to ensure a continuity of reliable results.

In summary, the results of this study provide evidence that medium ingredients may adversely affect the susceptibility test results of individual *Mycobacterium* isolates. In order to provide clinicians with reliable test results, old and new lots of medium components must give comparable results.

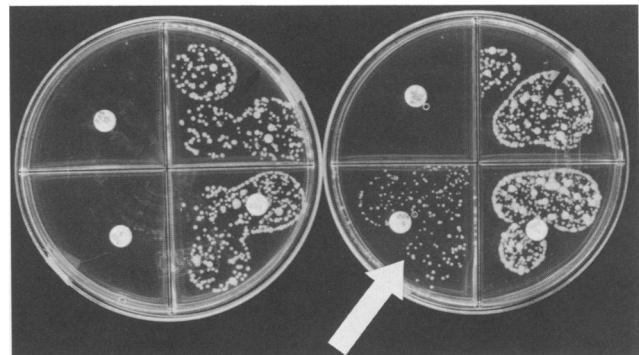


FIG. 2. Drug binding by a new component and the resulting false resistance to drug apparent in the standard concentration assay. The standardized medium on the left contains a quadrant without drug and three quadrants with drug supplied by disks. The test medium on the right was prepared identically to the standardized medium and in parallel, except that a new lot of OADC was substituted. All quadrants were inoculated with 0.1 ml of a suspension of *Mycobacterium scrofulaceum*. The arrow indicates the quadrant in which drug binding by OADC permitted growth of drug-susceptible organisms.

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