Modification of the Microtiter Technique for Antimicrobial Drug Susceptibility Testing by Incorporation of Indicators

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A pH indicator and dextrose were incorporated into growth media as a modification of microtiter methods for determining the minimal inhibitory concentration of antimicrobial drugs. This modified method was tested to evaluate the ease of reading end points by changes in the indicator color. Application of the procedure to two media, three indicators, and eight species of bacteria indicated that definitive end points could be reached as a result of indicator color change caused by acid production during bacterial growth. This method is accurate and reproducible. It is a modification which eliminates a need for plating and facilitates the reading of minimal inhibitory concentration end points.

The serial dilution technique, in test tubes, has been a standard method for determining the minimal inhibitory concentration (MIC) of antibacterial drugs (6). Because this method involves a large volume of equipment and extensive preparation and time for completion, microtitration techniques have recently been employed to save time and equipment. Microtitration techniques were first used in hematology and serology (5). The techniques were later applied to work in the bacteriological fields, and procedures for MIC determinations were developed (3).

When applying the microtiter technique to determine the susceptibility of certain organisms to antimicrobial agents in biological fluids, we found the determination of growth end points to be uncertain because of the cloudy or translucent nature of the fluids assayed and the low number of cells in the inoculum. The use of pH indicators (phenol red, bromothymol blue, or bromocresol purple) and glucose in the growth medium was evaluated as a modification of former procedures in an effort to overcome the problem of interpreting growth and to clarify the end point.

MATERIALS AND METHODS

Media. Double-strength Phenol Red Broth Base (Difco), or Mueller-Hinton Broth (BBL), containing a pH indicator was prepared, and 1% glucose (twice concentrated) was added. The indicators used were phenol red (0.018 g/liter), bromothymol blue (0.025 g/liter), or, less frequently, bromocresol purple (0.020 g/liter).

Drug solutions. Stock solutions ranging from 1,000 to 4,000 µg/ml were prepared for the following antimicrobial drugs: trimethoprim [2,4-diamino-5-(3,4,5-trimethoxybenzyl)-pyrimidine], supplied by Hoffman-La Roche, Inc., Nutley, N.J.; diaveridine [2,4-diamino-5-(3,4-dimethoxybenzyl) pyrimidine], supplied by Burroughs Wellcome & Co., Inc., Tuckahoe, N.Y.; sodium methicillin and kanamycin, supplied by Bristol Laboratories, Syracuse, N.Y.; gentamicin, supplied by Schering Corp.; sulfisomadine, supplied by Ciba Pharmaceutical Co., Summit, N.J.; sulfadiazine, supplied by Matheson, Coleman, and Bell; sulfamethoxazole, supplied by Hoffman-La Roche, Inc.; sulfapyridine and sulfamerazine, supplied by Matheson, Coleman, and Bell; sulfacetamide, obtained from B. L. Lemke and Co., Lodi, N.J.; sulfachlorpyridazine, supplied by Mallinckrodt Pharmaceuticals, St. Louis, Mo.; and triple sulfa (USP). Trimethoprim and diaveridine stock solutions were prepared by dissolving the drugs in isethionic acid and diluting them to volume with distilled water. The sulfonamide derivatives were dissolved in sodium hydroxide and diluted to volume with distilled water. Other antibiotic stock solutions were prepared in phosphate buffers, kanamycin and gentamicin at pH 8.0 and methicillin at pH 6.0, or in distilled water as required for their solubility. These stock solutions were diluted in the assay diluent (saline or biological fluids) to give drug standards in the range of the MIC.

Assay organism. The assay organism varied with the antimicrobial agent to be tested or the purpose of the assay. The most common assay organisms used in the studies were Staphylococcus aureus ATCC 6538, ATCC 6538P, B-1, and B-2; Escherichia coli ATCC 9723, ATCC 4352, ATCC 9723P, ATCC 10536, C-5, LJ-2, C-6, E-6, and E-5; Enterobacter (Klebsiella) aerogenes ATCC 13882 and HH; Streptococcus faecalis ATCC 10541; Proteus vulgaris ATCC 8427, TCT-1, B-2, and 5; P. mirabilis ATCC 14153;
P. morgani ATCC 8076a; Pseudomonas aeruginosa ATCC 10145, B-2, G-2, TCT-1, and C-1; and Citrobacter sp. TCT-1 and TCT-2. Other than the ATCC cultures, the strains of organisms listed above were all clinical isolates. The organism was inoculated into Nutrient Broth or Trypticase Soy Broth and incubated at 37°C for 5 to 7 hr to obtain logarithmic growth. This subculture was diluted 1:10 in distilled water, and 0.1 or 0.2 ml was used to inoculate 10 ml of assay medium. The growth medium was inoculated prior to being dispensed into the microtiter wells to eliminate added dilution factors caused by dispensing the bacteria separately.

Assay materials. Disposable, U-bottom microtiter plates (Cooke Engineering Co.), nondisposable polyethylene micro-pipettes, and standard micro-diluters (50-μl, Cooke Engineering Co.) were used in the assay. All materials were sterilized by ethylene oxide except the micro-diluters, which were flame.

MIC assay. The wells in columns 2 to 12 of the microtiter plates were filled with 50 μl of diluent (this diluent varied, being either saline or a biological fluid). Wells in column 1 were filled with 100 μl of antibiotic (antimicrobial) standard. Twofold serial dilutions were made with 50-μl micro-diluters in columns 1 to 10 by use of the standard technique. Column 11 was left free from drug as an organism control and column 12 as a medium control. Inoculated medium, 50 μl, was added to columns 1 to 11, doubling the volume in each well. Uninoculated medium (50 μl) was added to column 12. Each MIC determination was run in triplicate. Plates were sealed with a plastic cover, adhesive or non-adhesive, incubated at 37°C for 15 to 18 hr (overnight), and examined for a color change of the indicator.

Plating. Samples from microtiter wells were aseptically transferred by flame micro-dilutors onto the surface of a Trypticase Soy Agar (TSA) plate. After overnight incubation at 37°C, the plate was examined for bacterial growth.

RESULTS

When medium containing pH indicator was used in microbial microtiter assays rather than Nutrient Broth or an assay medium alone, results were clearly defined and easier to read. The end point was demonstrated by the change of indicator color to bright yellow, resulting from acid production during growth of the assay microorganism. No change in color indicated no growth (no acid production). Transfers onto a TSA plate as a test for growth to indicate MIC correlated with end points determined by color change of the indicator in the wells. These transfers were unsuitable for the assay of bacteriostatic drugs, because bacteria inhibited by the presence of a drug in the wells would grow when removed to a plate. Transfers were done only during preliminary work on the procedure and were not routinely included. Transfer to TSA plates may be necessary, however, when the minimal bactericidal concentration is of interest rather than the bacteriostatic concentration.

MIC determinations were done in triplicate, and the MIC values were reproducible from assay to assay (Table 1). The results of 37 assays run on the antimicrobial drug trimethoprim are shown in Table 1. These assays involved two bacteria, S. aureus ATCC 6588P and E. coli ATCC 10536, and three media, Phenol Red Broth Base, Mueller-Hinton Broth with a phenol red indicator, and Mueller-Hinton Broth with a bromothymol blue indicator. The drug concentrations were from 1.6 to 50 μg/ml. With few exceptions, the MIC varied no more than one well (twofold concentration) for any organism-medium combination. The degree of reproducibility is indicated by the standard deviation. Variation of the concentration of drug standard did not significantly alter the results. Change of the indicator to bromothymol blue in Mueller-Hinton Broth did not change the end point (Table 1). Substituting bromoresol purple for bromothymol blue as the pH indicator caused the end point to change to different value, owing to the greater quantities of acid necessary to change the color.

**Table 1. Results of 37 assays run on separate occasions over a 1-year period, representing the degree of MIC reproducibility**

| Assay no. | Medium | pH indicator | Assay organism          | Conc of trimethoprim (μg/ml) | No. of runs | MIC (μg/ml) |
|-----------|--------|--------------|-------------------------|-----------------------------|-------------|-------------|
| 1–18      | PR     | PR           | S. aureus ATCC 6538P    | 50                          | 53          | 0.26 ± 0.12d|
| 19–23     | MH     | PR           | S. aureus ATCC 6538P    | 10–50                       | 12          | 0.14 ± 0.07 |
| 24–34     | MH     | PR           | E. coli ATCC 10536      | 1.6–50                      | 32          | 0.13 ± 0.04 |
| 35–37     | MH     | BTB          | E. coli ATCC 10536      | 25–50                       | 9           | 0.14 ± 0.04 |

*a* The diluent used in all assays was prostatic fluid.

*b* All media contained glucose (1%). PR = Phenol Red Broth Base; MH = Mueller-Hinton Broth.

*c* PR = phenol red; BTB = bromothymol blue.

*d* Standard deviation.
Table 2. Minimal inhibitory concentration ranges obtained for trimethoprim by three different methods

| Method                  | Assay organism     | Total no. of strains | No. of strains inhibited by various concn of trimethoprima |
|-------------------------|--------------------|----------------------|------------------------------------------------------------|
| *Modified microtiter technique* |                     |                      |                                                            |
|                         | *Proteus vulgaris*  | 4                    | 50 25 12.5 6.3 3.1 1.5 0.8 0.4 0.2 0.1 <0.1               |
|                         | *Escherichia coli*  | 7                    |                                                            |
|                         | *Enterobacter aerogenes* | 2                 | 50 25 12.5 6.3 3.1 1.5 0.8 0.4 0.2 0.1 <0.1               |
|                         | *Staphylococcus aureus* | 4                  |                                                            |
| *Tube dilution method (4)* |                     |                      |                                                            |
|                         | *Proteus sp.*       | 27                   | 50 25 12.5 6.3 3.1 1.5 0.8 0.4 0.2 0.1 <0.1               |
|                         | *E. coli*           | 48                   |                                                            |
|                         | *Klebsiella-Aerobacter* | 23              |                                                            |
| *Plate dilution method (1, 2)* |                     |                      |                                                            |
|                         | *P. vulgaris*       | 10                   | 32 16 8 4 2 1 0.5 0.25 0.12 0.06 0.03                    |
|                         | *E. coli*           | 16                   |                                                            |
|                         | *Klebsiella sp.*    | 10                   |                                                            |
|                         | *S. aureus*         | 18                   |                                                            |
|                         | *P. vulgaris*       | 1                    | 50 25 12.5 6.3 3.1 1.5 0.8 0.4 0.2 0.1 <0.1               |
|                         | *E. coli*           | 1                    |                                                            |
|                         | *K. aerogenes*      | 1                    |                                                            |
|                         | *S. aureus*         | 1                    |                                                            |

a The concentrations of trimethoprim tested (micrograms per milliliter) are shown in boldface type.

Table 2 demonstrates the accuracy of the method by comparing the MIC levels for trimethoprim found by the microtiter method with the use of a pH indicator to the MIC levels found by plate dilution methods reported by Bushby and Hitchings (1) and Darrell, Garrod, and Waterworth (2), and by the tube dilution method reported by Reisberg, Herzog, and Weinstein (4). With four strains of *P. vulgaris*, the MIC values obtained by the present method were within a range of 0.8 to 0.1 µg/ml; values obtained by plate dilution methods (1, 2) ranged from 4.0 to 0.5 µg/ml, and those obtained by a tube dilution method (6) ranged from 12.5 to 0.4 µg/ml. Similar correlations were observed with the other assay bacteria. The MIC of trimethoprim for *E. coli* by the modified microtiter technique was 0.4 to 0.1 µg/ml as compared with 0.2 to 0.03 µg/ml by plate dilution (1, 2) and 0.8 to 0.2 µg/ml by tube dilution (4). The MIC values for *Klebsiella sp.* and *E. aerogenes* were 1.5 to 0.2 µg/ml by microtiter, 16 to 0.06 µg/ml by plate dilution (1, 2), and 50 to 0.4 µg/ml by tube dilution (4). *S. aureus* assays by microtiter with indicator media resulted in MIC values from 0.8 to 0.2 µg/ml; by plate dilution methods, values of 0.5 to 0.12 µg/ml were obtained (1, 2; Table 2).

The modified microtiter method was used with equal success to determine the MIC of the following additional antimicrobials: diaveridine, sodium methicillin, kanamycin, and gentamicin. When the method was applied to sulfisomadine, sulfadiazone, sulfamethoxazole, and a triple sulfas, MIC determinations were made with moderate success, although the values obtained were higher than those reported in the literature. Preliminary work on sulfapyridine, sulfamerizine, sulfacetamide, and sulfachlorpyridazine indicates that the same results will apply to these sulfas.

**DISCUSSION**

The procedure used for the determination of MIC values was essentially the method described by Harwick, Weiss, and Fekey (3). In the development of the present procedure, Nutrient Broth was first tried as the growth medium, and the MIC was apparent when growth was confirmed by transfer to a TSA plate. This transfer was necessary because of difficulty in determining the growth in the microtiter plates on a turbidity basis alone; however, plating not only involved an extra day, but was not applicable to drugs which were merely bacteriostatic rather than bactericidal. The presence of the indicator in the medium eliminated the problems of reading...
growth strictly on a turbidity basis. The end point was an easy-to-read color change, clearly visible in most cases, and assays could be conducted in biological fluids which are themselves cloudy and tend to obscure results.

In this paper, trimethoprim was chosen to exemplify the reproducibility and accuracy of the method because of the extensive work done on this drug. Except for the sulfonamide derivatives, MIC values were determined successfully with equal reproducibility and accuracy for the other antimicrobial drugs. We suspect that the variability found in sulfonamide determinations, in particular the higher end points, is a result of assaying in biological fluids, which may contain sulfonamide inhibitors, rather than a problem in the method.

The use of broth containing an indicator as the growth medium in microtiter assays, with the color change as an indicator of growth, is a modification having a wide application. Since a variety of bacterial species are able to ferment glucose or other sugars, this facet of the procedure should present no problem in the majority of cases. *Pseudomonas*, although it produces no acid from glucose, was assayed by this method and produced no color change with incubation. The drug end point for this organism was obscure and difficult to determine in comparison with the other bacteria listed. This study primarily involved ATCC bacterial strains. The clinical isolates tested gave results as satisfactory as the ATCC strains; however, extensive tests with recent clinical isolates, which might not readily produce acid, were not included.

All media-indicator combinations listed in this study were used with equal success. Mueller-Hinton Broth was used as a specialized medium in assaying for sulfonamides. Results suggest that a range of growth media as well as other specialized media could be used, depending on the drug or conditions of the assay. When the pH range was critical, the indicator was changed to fit the need; e.g., phenol red was used for a starting pH above 7.0 and bromothymol blue for a starting pH range of 6.5 to 6.8.

In conclusion, this technique is recommended as a rapid, precise, and accurate means of determining the MIC of a number of antibiotics and antimicrobials. This technique appears to be an improvement over usual turbidity methods used for reading end points within the microtiter system, particularly when biological fluids are used.

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