Tube Dilution Antimicrobial Susceptibility Testing: Efficacy of a Microtechnique Applicable to Diagnostic Laboratories

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A microtechnique for determining antibiotic susceptibilities by the serial dilution method was evaluated in a clinical diagnostic microbiology laboratory. As compared with the standard tube method, an agreement of 94% was achieved for determining minimal inhibitory concentration with ± one tube dilution as the criterion of comparison. The experience with this system suggests that it could easily be incorporated into diagnostic laboratories as a routine procedure.

In recent years, the increasing availability and types of antimicrobial agents have added appreciably to the problems confronting the diagnostic laboratory. In many clinical situations, the tube dilution method of determining antimicrobial susceptibilities is generally recognized as providing more accurate and useful information as compared with results obtained by the disc method. However, from a practical point of view, cost, time, and personnel factors frequently require that the tube dilution method be used only with isolates from the problem patient.

A number of papers (1, 4, 9) have appeared which question disc antibiotic susceptibility testing procedures. Discrepancies frequently arise when one compares the results obtained with the disc and tube dilution methods.

These studies were undertaken to evaluate the feasibility of an in vitro susceptibility testing technique in a clinical laboratory which is reproducible, accurate, and reliable, but lacks the disadvantages of being time-consuming, expensive, and tedious.

For this purpose, the Microtiter (Cooke Engineering Co.) system, as used routinely in many serological laboratories (10), was investigated as a possibility to resolve the inherent difficulties in the standard accepted method.

MATERIALS AND METHODS

Stock standards of antibiotics generously provided by pharmaceutical companies, including cephalothin, ampicillin, colistin, neomycin, tetracycline, kanamycin, polymyxin B, streptomycin, chloramphenicol, gentamicin, cephaloridine, lincomycin, erythromycin, penicillin G, carbenicillin, and cloxacillin, were weighed on an analytical balance, diluted in glass-distilled water to a concentration of 1,000 μg/ml, sterile filtered, and stored as 1 ml of frozen stock.

Macromethod. For each antibiotic examined, 0.2 ml of 1,000 μg/ml was added to 1.6 ml of Brain Heart Infusion broth (BHI), and nine serial twofold dilutions were made in 0.9 ml of broth. Subsequently, 0.1 ml of a 10^-4 dilution of an 18-hr broth culture of the organism was added to each tube. One tube without antibiotic served as the organism control. After 18 hr of aerobic incubation at 37 C, tubes were examined for evidence of turbidity or sediment. The tube exhibiting no visible growth and containing the least amount of antibiotic was considered the minimal inhibitory concentration (MIC).

Micromethod. Disposable transparent V plates, (Cooke Engineering Co. or Linbro Chemical Co.), containing 96 cups arranged in 8 rows of 12 cups, were sterilized by being rinsed in ethyl alcohol, drained in an inverted position overnight, and placed under a germicidal ultraviolet source for 1 hr. Working solutions of antibiotics were prepared weekly by diluting the frozen stock to 200 μg per ml of BHI. Each week a disposable plate containing 12 different antibiotics was prepared. Each cup, in a vertical row of 12 cups, was filled with a different antibiotic and stored at 4 C. At the end of a week, the working stock standards were discarded and fresh solutions were prepared. For the microtitration procedure, 0.025 ml of BHI was added to each well with a calibrated pipette dropper. A heat-sterilized, 0.025-ml multi-microdilutor was utilized for diluting 12 antibiotics simultaneously (Fig. 1), by placing the microdiluter in the plate containing the working stock of antibiotics and transferring to the first row of the titration plate, mixing, and transferring to subsequent rows until the titration was complete.

The complete twofold serial titration of 8 dilutions of 12 different antibiotics usually took less than 1 min. An inoculum of 0.025 ml of an 18-hr BHI
broth culture diluted $10^{-4}$ was added to each cup. After inoculation, the plate was covered with transparent sealing tape, a small pin-hole was made at the center of each cup, and the plate was incubated at 37°C for 18 hr. The end point of the titration was determined by examining the plate, with transmitted light against a dark background, for growth in the first cup (50 µg/ml) and each succeeding cup (Fig. 2).

**RESULTS**

Isolates of various gram-negative rods, including *Pseudomonas*, *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter*, and *Escherichia*, gram-positive organisms such as coagulase-negative and coagulase-positive *Staphylococcus*, and other rapidly growing organisms were examined against a variety of antibiotics. As noted in Table 1, if one accepts ± one tube dilution as the criterion for comparison between methods, an excellent correlation was achieved. When the methods were compared, a correlation of 78% was achieved initially (series 1). However, after technical familiarization with the technique was established (series 2), a correlation of 89% was noted. Subsequently, after the technique was firmly established (5 months), a correlation of 94% was achieved between the two methods.

**DISCUSSION**

Variabilities (4, 7, 9, 11) encountered in antibiotic disc susceptibility testing have been attributed to diffusibility of the antibiotics, size and distribution of the inoculum, moisture content of the agar, type of medium used, interpretation of narrow zones, failure to set the disc properly, and other variables. The multidisc technique, two discs, or the use of a single high- or low-concentration disc are additional variations of the technique which tend to cause confusion in the interpretation of results from a clinical laboratory.

The serial tube method of susceptibility testing is considered the common method of reference, but the tedious nature of the macromethod has prevented universal usage on routine isolates.

The obvious advantage of determining discrete end point susceptibilities which can be correlated with therapeutic levels obtainable in the patient is that the physician is afforded a more sophisticated approach in the management of bacterial infections. Although this approach is generally recognized as ideal, tube dilution susceptibility testing usually is relegated to the research oriented laboratory. An initial modification of the macro-technique utilizing spot depression plates (3) was reported from this laboratory in 1965. Although the spot plate compared favorably with the tube technique, the time required and the glassware used remained significant problems to be resolved. Subsequently, commercially available microtitration equipment and disposable plates became available and provided an alternate methodology which could overcome inherent problems in manual pipetting procedures.

These data and other reports (8; L. A. Chitwood, S. Med. J. 60: 1358; J. D. MacLowry and H. H.
Larsh, Intersci. Conf. Antimicrob. Agents Chemother., 8th, 1968, New York) suggest that an excellent correlation exists between the microand macrotechniques and that the microtechnique can be incorporated into a routine diagnostic laboratory.

The most time-consuming procedure of the technique is the addition of the diluent to each cup. However, with the use of an automatic dispensing instrument (Cooke Engineering Co.), automation of the technique becomes a possibility.

Decreased activity of antibiotics, usually by two tubes, was noted when the 10^{-3} dilution of the test organism was inadvertently allowed to remain at room temperature for several hours before being added to the antibiotics. Obviously, the size of the inoculum requires standardization. Although a 10^{-3} dilution was empirically established for most rapidly growing organisms, a 10^{-3} dilution was used for more fastidious organisms such as beta- and alpha-hemolytic streptococci and pneumococci. In addition, with these and similar organisms, it was found advantageous to add 5% fresh sheep blood as an indicator of growth. Triphenyl tetrazolium chloride in the media has been used as a visible indicator of growth in the microtechnique (6).

Occasionally, spurious results do occur which have been attributed invariably to a mixed culture for the inoculum; however, by maintaining a log of tube dilution susceptibilities by organism, such errors are detected easily.

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