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NOTES

Agar Shake Tube Technique for Simultaneous Determination of Aerobic and Anaerobic Susceptibility to Antibiotics†

JAMES B. EVANS* AND LIZZIE J. HARRELL

Department of Microbiology, North Carolina State University, Raleigh, North Carolina 27607

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The broth dilution method of determining the minimum inhibitory concentration (MIC) of antibiotics has been adapted to an agar shake tube technique with semisolid media. This permits the simultaneous determination of the aerobic and anaerobic MICs for facultatively anaerobic bacteria such as staphylococci.

There is increasing evidence that anaerobiosis may have a significant effect on the minimum inhibitory concentration (MIC) of antibiotics against facultatively anaerobic bacteria (4, 5, 8, 9). In addition, it has been shown that conditions in abscesses are often anaerobic (3). In such instances, the anaerobic MIC may be a more accurate indicator of the clinical effectiveness of antibiotics against the causative bacteria. The disk diffusion and broth dilution methods currently used for determining the MIC of antibiotics are aerobic and are not easily adaptable to anaerobic determinations (6, 7). An agar shake tube technique has been reported to be convenient for determining aerobic and anaerobic growth and substrate utilization patterns of facultatively anaerobic bacteria (2, 10). We have adapted this technique to provide a convenient method for simultaneously determining the aerobic and anaerobic MICs of antibiotics for staphylococci and other facultatively anaerobic bacteria.

Most of our studies have been with strains of *Staphylococcus aureus*, including the standard reference strain (ATCC 25923) and several recent clinical isolates. Other species of staphylococci and one culture of *Escherichia coli* have also been used. Best results with *S. aureus* have been obtained using a semisynthetic medium containing vitamin-free acid-hydrolyzed casein (1) with 0.3% agar (CH agar). However, any medium that supports good anaerobic growth of the test organism should give satisfactory results, and we have used brain heart infusion and Trypticase soy broth with 0.3% agar added. Mueller-Hinton broth failed to support good anaerobic growth of the staphylococci and could not be used.

The inoculum was prepared by diluting an overnight brain heart infusion broth culture to 2×10^7 colony-forming units/ml, as determined by turbidimetric measurement. The basal medium was prepared in a concentration that provided the desired level of constituents after dilution with the antibiotic solution. The medium was dispensed in 4.0-ml portions in test tubes (13 by 100 mm) with loosely fitting plastic caps, sterilized at 15 lb/in^2 for 15 min, and cooled to 45 to 50°C in a temperature block or water bath. Twofold dilutions of antibiotic were prepared in CH broth at or above room temperature, and 1-ml portions were added to the basal medium and gently mixed. One milliliter of CH broth was added to the control tube. All tubes were inoculated with 0.1 ml of the diluted culture, again gently mixed, promptly chilled in an ice bath, removed, and placed in a 35°C incubator for 18 to 24 h. The lowest concentration of antibiotic that prevented visible growth in the top 1.0 cm of the tube was recorded as the aerobic MIC, and the lowest concentration that prevented growth in the lower portion of the tube was recorded as the anaerobic MIC.

Figure 1 shows the results of a typical experiment in which the anaerobic MIC was fourfold higher than the aerobic MIC. Aerobic MIC values determined by this technique agreed quite well with those obtained by the regular broth dilution method. The anaerobic MIC values obtained with the agar shake technique often were higher than the values obtained by using the broth dilution method in GasPak anaerobic jars. Either we obtained better anaerobiosis with the agar shake technique or we may have avoided problems resulting from the outgrowth of the more resistant cells in the inoculum. Occasionally a few scattered colonies were observed (as in Fig. 2) in the aerobic zone of tubes that had antibiotic concentrations greater than the aerobic MIC. These were not considered in

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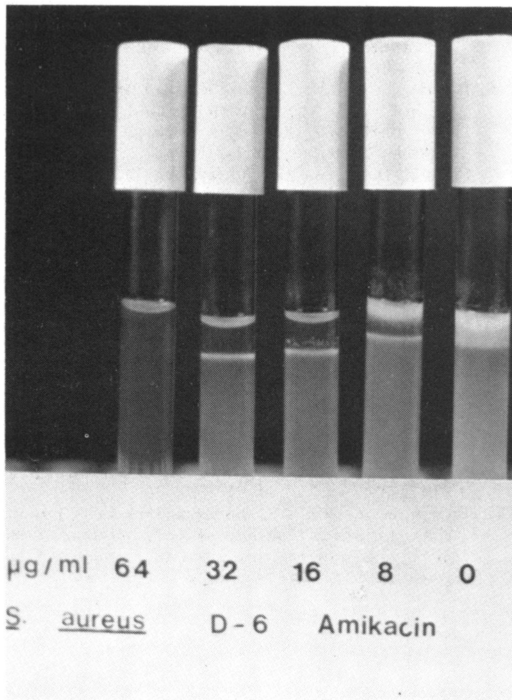

FIG. 1. *Staphylococcus aureus D-6 showing a fourfold increase in resistance to amikacin under anaerobic conditions.*

determining the aerobic MIC, but may serve as a warning of the possible emergence of resistant mutants if marginal levels of antibiotics are used. In some instances (e.g., Fig. 2) we have noted an inverse correlation between the number of "resistant" colonies and the level of antibiotic in the tube. These colonies may have arisen from a subpopulation of resistant cells, as described by Wilson and Sanders (11), or from mutant cells such as the dwarf colony variants studied by Lacey (4).

We made three or more determinations of the aerobic and anaerobic MICs of five antibiotics for 15 bacterial strains with the agar shake technique. The results were highly reproducible. The MICs from the three independent tests were either in complete agreement, or differed by no more than one twofold dilution, over 90% of the time. In agreement with the results of investigators who used other techniques (4, 5), the anaerobic MIC of the aminoglycosides for some strains of *S. aureus* was at least 32-fold greater than the aerobic MIC (Fig. 2). *Escherichia coli* also was substantially more resistant under anaerobic conditions.

We have found this technique to be relatively simple for manual testing of small numbers of cultures. Laboratories with a large volume of testing may be able to modify the procedure to

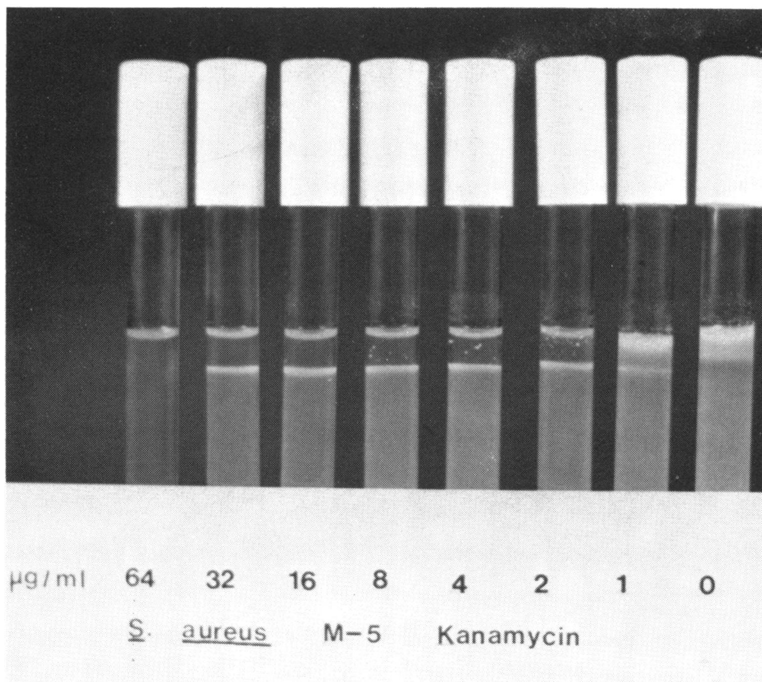

FIG. 2. *Staphylococcus aureus M-5 showing colonies of apparently increased aerobic resistance to kanamycin.*

permit the use of automatic diluters or other automatic equipment.

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LITERATURE CITED

1. **Evans, J. B.** 1975. Uracil and pyruvate requirements for anaerobic growth of staphylococci. J. Clin. Microbiol. 2:14–17.
2. **Evans, J. B., and W. E. Kloos.** 1972. Use of shake cultures in a semisolid thioglycolate medium for differentiating staphylococci from micrococci. Appl. Microbiol. 23:326–331.
3. **Hays, R. C., and G. L. Mandall.** 1974. pO_2, pH, and redox potential of experimental abscesses. Proc. Soc. Exp. Biol. Med. 147:29–30.
4. **Lacey, R. W.** 1969. Dwarf colony variants of *Staphylococcus aureus* resistant to aminoglucoside antibiotics and to a fatty acid. J. Med. Microbiol. 2:187–197.
5. **Reynolds, A. V., J. M. T. Hamilton-Miller, and W. Brumfitt.** 1976. Diminished effect of gentamicin under anaerobic or hypercapnic conditions. Lancet i:447–449.
6. **Rotilie, C. A., R. J. Fass, R. B. Prior, and R. L. Perkins.** 1975. Microdilution technique for antimicrobial testing of anaerobic bacteria. Antimicrob. Agents Chemother. 7:311–315.
7. **Stalons, D. R., and C. Thornsberry.** 1975. Broth-dilution method for determining the antibiotic susceptibility of anaerobic bacteria. Antimicrob. Agents Chemother. 7:15–21.
8. **Traub, W. H., and E. A. Raymond.** 1971. *In vitro* resistance of *Clostridium perfringens* type A to gentamicin sulfate, and reduced activity of the antibiotic under anaerobic atmospheric conditions. Chemotherapy 16:162–165.
9. **Virtanen, S.** 1974. Antibacterial activity of sulphamethoxazole and trimethoprim under diminished oxygen tension. J. Gen. Microbiol. 84:145–158.
10. **Whittenbury, R.** 1963. The use of soft agar in the study of conditions affecting the utilization of fermentable substrates by lactic acid bacteria. J. Gen. Microbiol. 32:375–384.
11. **Wilson, S. G., and C. C. Sanders.** 1976. Selection and characterization of strains of *Staphylococcus aureus* displaying unusual resistance to aminoglycosides. Antimicrob. Agents Chemother. 10:519–525.