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Analysis of Certain Variables in the Agar Dilution Susceptibility Test

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This study examined (i) the activity of gentamicin added to agar in ratios of 1:100 and 1:1,000 to produce a final concentration of 1 μg/ml, (ii) the uniformity of distribution of gentamicin in agar in relation to the amount of mixing, and (iii) the effect of agar depth on the activity of gentamicin against *Pseudomonas aeruginosa*. Although the ratio of antibiotic solution to agar had no significant effects on activity of the antibiotic, the amount of mixing did. Agar depth had no significant effect on the activity of gentamicin.

In 1971 the report of the International Collaborative Study (ICS) on antibiotic susceptibility testing, prepared by Ericsson and Sherris (2), recommended the agar dilution method as the reference procedure for determining quantitatively the antimicrobial susceptibility of rapidly growing aerobic and facultatively anaerobic bacteria. This procedure, rather than the broth dilution technique, was selected because of its better reproducibility and greater economy for testing large numbers of tests. If widely adopted, use of the ICS procedure would facilitate comparison of data from different centers.

In the description of the procedure by Ericsson and Sherris (2) and in a subsequent, more detailed description by Washington and Barry (5), it was arbitrarily specified that 1 volume of each dilution of antimicrobial agent be added to 9 volumes of agar and that 25 ml of the resultant agar mixture be poured per 100-mm plate. No data have been published to substantiate the necessity for these particular specifications.

The purpose of our study was (i) to examine the effects of adding 1 volume of antimicrobial agent to agar in ratios of 1:100 and 1:1,000, (ii) to determine the uniformity of distribution of antimicrobial agent in agar in relation to the amount of mixing, and (iii) to evaluate the effect on antimicrobial activity of agar depth.

MATERIALS AND METHODS

Gentamicin was selected as the antimicrobial agent to be used in the study. To minimize variability in its activity due to composition of the medium, only one lot of Mueller-Hinton agar (MHA; BBL, lot 301635) was used.

Ratio of antimicrobial solution to agar. To examine the effects of varying the ratio of antimicrobial solution to agar, gentamicin stock solution was added in a ratio of 1:100 to each of three bottles containing MHA and in a ratio of 1:1,000 to each of three additional bottles containing MHA in order to produce a final concentration in each of 1 μg/ml. Before addition of the gentamicin, the medium had been allowed to equilibrate at 48 C. After the addition of gentamicin, each bottle was inverted at least 10 times to ensure mixing. The contents of each bottle were then poured equally into each of six plates and allowed to solidify on a flat surface. Two circular (7.8 mm) plugs of agar were then removed from different sites on each plate and applied to the surface of pour plates containing *Staphylococcus epidermidis* ATCC 27626. After incubation for 16 h at 37 C, the zone diameters were measured.

Influence of amount of mixing on uniformity of distribution of antimicrobial agent in agar. In this portion of the study, gentamicin stock solution was added to each of 40 bottles containing MHA in a ratio of 1:1,000 to produce a final concentration of 1 μg/ml. The bottles were then divided into four groups of ten each: one group was not mixed, and the other three groups were mixed by inversion one, five, and ten times, respectively. The contents of each bottle were immediately poured equally into each of six plates and allowed to solidify on a flat surface. Gentamicin activity in the agar was determined by the aforementioned bioassay system.

Effect of agar depth. To determine the effect of agar depth on antimicrobial activity, 34 strains of *Pseudomonas aeruginosa* were tested against gentamicin in an arithmetic progression of concentrations ranging from 1 to 10 μg/ml. Four sets of plates per gentamicin concentration were poured so that each of the four plates within a set contained 10, 17, 25, and 35 ml of agar, respectively. After standardization of the inocula, according to ICS specifications (2, 5), they were applied to the solidified agar by means of an inocula replicating device (3). Minimal inhibitory concentrations (MICs) were determined after 16 h of incubation at 35 C.

RESULTS

Ratio of antimicrobial solution to agar. The mean zone diameters obtained from bioassay of the agar plugs removed from each set of plates are shown in Fig. 1. By two-way analysis of variance with repeated measures on one factor (6), the test statistic was an F statistic with 1 and 4 degrees of freedom and a value of 1.33, which was not significant.

We were, therefore, unable to conclude that the ratio of antimicrobial solution to agar or the sequence in which the plates were poured had any effect on the zone diameters of inhibition and, therefore, on the concentration of gentamicin in agar.

Influence of amount of mixing on uniformity of distribution of antimicrobial agent in agar. The mean zone diameters obtained from bioassay of the agar plugs removed from each set of plates are shown in Fig. 2. By two-way analysis of variance with repeated measures on one factor, the F statistic with 3 and 36 degrees of freedom was 16.21 ($P < 0.001$). We were, therefore, able to conclude that the number of inversions performed during mixing had had a significant effect on zone diameters of inhibition and, therefore, the uniformity of distribution of gentamicin in agar. Agar plugs from bottles with five and ten inversions had a higher average zone diameter of inhibition than agar plugs from bottles with zero and one inversions. There was no significant difference between bottles inverted five times and those inverted ten times. Moreover, with the exception of plate set A for the group of bottles with zero inversions, there were no significant differences in zone diameters among plate sets for any group of bottles.

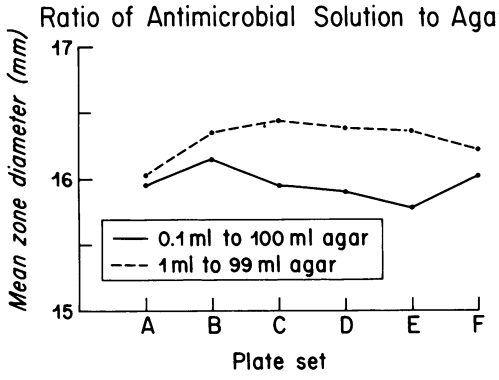

FIG. 1. *Mean zone diameters of inhibition produced by agar plugs removed from each of six sets (A-F) of agar plates prepared after adding gentamicin solution to agar in ratios of 1:100 and 1:1,000 to yield final concentrations of 1.0 μg/ml.*

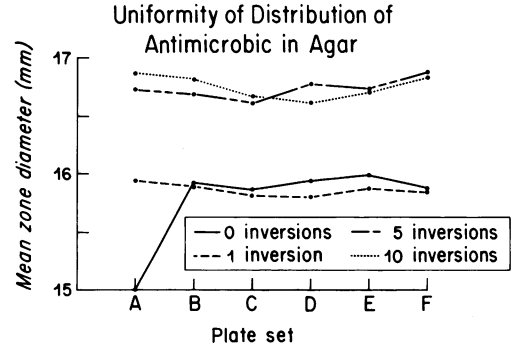

FIG. 2. *Mean zone diameters of inhibition produced by agar plugs removed from each of six sets (A-F) of agar plates prepared after inverting bottles containing antibiotic and agar (1 μg/ml) zero, one, five, and ten times.*

It is apparent, therefore, that a minimum of five inversions of bottles containing agar ensured proper mixing and distribution of the antimicrobial agent.

Effect of agar depth. The MICs of the 34 strains of *P. aeruginosa* were ranked from 1 to 4 (lowest to highest). A one-way analysis of variance for ranked data, Friedman's procedure (6), was then performed on these ranks. The test statistic, a chi-square with 4 degrees of freedom, was 0.2 and was not significant. The volume of medium per plate, i.e., agar depth, therefore had no significant effect on MICs of gentamicin against *P. aeruginosa*.

DISCUSSION

Standardization of quantitative antimicrobial susceptibility tests is of paramount importance to enable different laboratories to compare their data meaningfully; to this end, the ICS agar dilution method has been recommended as the reference procedure. Certainly, for reference work, it is essential to adhere carefully to the details of the procedure as published elsewhere (2, 5). For routine purposes, however, the agar dilution method may be simplified by increasing the size of the dilution steps tested (4). Further convenience and economy may also be achieved by eliminating the preparation of large volumes of diluted stock solutions of antimicrobial agents and by the use of smaller quantities of agar. We, therefore, felt that it was necessary to determine that the ratio of antibiotic solution to agar and the volume of agar per plate, i.e., agar depth, had no significant effects on the MICs.

Desiccation during storage of thin agar plates may affect the MICs. For routine purposes, our plates (15 to 17 ml of agar/plate)

are prepared once or twice weekly and stored unwrapped at 4 C in a laboratory refrigerator that neither defrosts itself nor has circulating air. The MICs of standard organisms used daily in our laboratory for quality control purposes have not varied under these specified conditions. Storage of agar plates at 4 C in Mylar bags is recommended if a refrigerator with circulating air must be used. Finally, for reference work, it is desirable to use the agar plates within 24 h of their preparation (5).

The uniformity of distribution of an antimicrobial agent in agar not only is related to the amount of mixing used but is also, as has been discussed in detail by Cooper (1), a function of the laws of diffusion. With time, therefore, the gradient diminishes between the reservoir of antimicrobial agent in solution and a point located at a specified distance from the reservoir. At infinite time the concentration becomes uniform throughout the medium. Presuming constant temperature and viscosity of the agar, the diffusion coefficient of a particular antimicrobial agent will vary with the radius of the molecule. These theoretical considerations aside, uniform distribution of an antimicrobial agent in agar, before pouring the mixture into plates, is generally ensured by mixing. Obviously, the capacity of the container must permit adequate mixing.

It is apparent from this study that the ratio of antibiotic solution to agar does not significantly affect the uniformity of distribution of antibiotic in agar, providing that there is adequate mixing of the two. It is also apparent that the volume of agar per plate, i.e., the agar depth, has no significant effect on MICs.

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