

# Disc Plate Method of Microbiological Antibiotic Assay

## I. Factors Influencing Variability and Error<sup>1</sup>

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Several factors are investigated that normally cause variation in zone diameters in conventional disc plate diffusion assay procedures. Of these factors the most serious is the unequal exposure of the individual plates at top or bottom of stacks to temperatures above and below room temperature. This unequal temperature exposure is avoided by novel handling and incubation procedures. A major variable, but one which can be controlled, is the varying time interval between pouring seeded agar and the time of applying the pads with antibiotic to the plates. This influence of time of setting and the effects of several other sequential operations are combined into a composite variable. This variable is then accounted for and normalized by interposing "external" reference plates set with a reference solution in the sequence of approximately 100 plates. No "internal" reference zones are employed. Such factors as volume of agar poured, wedge shape of agar in a dish, volumetric errors in dilutions, and timing considerations are studied and discussed. The results of this study form the basis for a test protocol which is presented in a following paper.

The diffusion assay of antibiotics employing a disc plate method has great virtue of convenience, simplicity, sensitivity, efficiency, and dependability (5, 10). The relation of diameter of inhibitory zones to concentration of antibiotic in a solution applied in cups has been considered theoretically (2, 7). In practice, the diameter is directly responsive to antibiotic concentration, and the limitations on accuracy are the recognition and control of the many subtle variables. The applicability of the system is general, and materials and equipment for the conduct of the procedure are widely available. Paper discs and blow-molded plastic petri dishes are available. An instrument for accurate reading of zone diameters was described by Davis et al. (3) and is manufactured and sold by Fisher Scientific Co., Pittsburgh, Pa.

When studies conducted with antibiotics appear to the experimenter to demand high accuracy of assay, the alternatives of microbiological or physicochemical assay present themselves. Some comparisons between several microbiological procedures are presented by Gavin (5, 6).

A chemical or physicochemical test, if validated as completely specific for the active component, may be more accurate than results generally

achieved with microbiological procedures (1, 6). A chemical or physical test is often more amenable to automation than microbiological procedures (9). For instance, automation has recently made more efficient and accurate the use of turbidimetric and colorimetric assays (8). A chemical or physical test is likely to be very narrow in its application and much more difficult to establish than a microbiological test.

Microbiological assay procedures provide a valid measure of antibiotic activity with little danger, generally, of interference from biologically inactive components or degradation products, and the disc plate method persists in wide use (10).

In our own experience, surprisingly large inaccuracies have been encountered in the results of such testing. Accordingly, we reexamined some of the factors in the disc plate diffusion method and attempted to improve the accuracy of the method without sacrificing its great generality, sensitivity, and convenience.

### MATERIALS AND METHODS

Certain a priori commitments to equipment and procedures were made in initiating the present study. Disposable 3.75 inch (ca. 9.53 cm) blow-molded plastic petri dishes and 0.25 inch (6.3 mm) diameter antibiotic discs were used; application of all solutions were from the same automatic self-filling and -delivering capillary pipette. Six discs were placed on each

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petri dish, all filled with the same solution, consisting of either a known or an unknown. This is in contrast to the more common practice of using "internal standards," i.e., two or three discs with reference solution on each petri dish (10).

The automatic filling and emptying pipette employed here is similar in construction to that described by Davis and McGuire (4) except that it is made with a smaller capacity (17  $\mu$ liters) to deliver an appropriate amount of fluid into the 0.25 inch (ca. 0.64 cm) pads employed. The total capillary capacity of the pads placed on a dry surface is about 27  $\mu$ liters. When a pad is placed on an agar surface, some of this capacity is filled by absorbing fluid from the agar within the few seconds before applying the test solution from the pipette. Enough capillary capacity must remain in the pad when the pipette is applied to pull all of the fluid out of the pipette.

A single antibiotic solution [2  $\mu$ g of Keflin per ml in pH 6.9 (0.2 molar) phosphate buffer] was employed throughout, the same amount of which was applied to each pad of every plate. By this procedure, variables such as the order of pouring the plates, the time and order of "setting" the plates (i.e., placing pads and solution), and placing the plates in stacks for incubation, etc. revealed their separate influences. Even very small variation of zone diameters could be tied to these controlled variables. Zone diameters were measured reproducibly to plus or minus 0.05 mm employing a Fisher-Lilly Zone Reader (3). The use of Keflin solution with *Bacillus subtilis* as the test organism resulted in sharp zone edges.

Usually a total of 102 plates, each bearing six pads, were set and handled in a standard way each day by a single operator. All variations of zone diameters within plates, between plates in each stack, and between stacks were examined for systematic and random relationships, and the variability was interpreted as to cause and as to the possibility of control.

Throughout this study *B. subtilis* was employed as the test organism. A suspension of spores held at 5 C in distilled water was added to hot nutrient agar. The hot seeded agar was generally poured at 50 C. Certain procedures or materials were changed in the course of the study, with only minor accounting for their effect upon accuracy. For instance, we determined that some disposable petri dishes were superior to others, and, after an examination of significant structural

features of the dishes, the practice of using only one specific product was adopted. This was Diamond Plastics petri dishes from mold 3.

The most feasible operation with stacks of plates appeared from the beginning to be to make a unit of handling of about 30 plates with blanks at top and bottom of stacks. The quantity of seeded agar poured per plate was varied, being either 6, 8, or 10 ml, but the 8-ml pour was adopted and considered standard. The composition of nutrient agar was arbitrarily chosen for well-defined zones. [The composition of nutrient agar employed with *B. subtilis* was for 1 liter: 1.5 g of beef extract (Difco), 6.0 g of peptone (Difco), 3.0 g of yeast extract (Difco), and 20.0 g of agar (BBL). This was adjusted to 6.0 with dilute HCl or NaOH and was unbuffered.]

An early decision was made to employ only volumetric displacement burettes in making up the samples of standard or unknown solutions. In this way, inaccuracies due to drainage errors were avoided. Also anticipating the necessity to employ the same diluent for standard solutions as for unknowns (rat plasma, dog plasma, tissue juices, etc.), procedures were chosen so as to be amenable to the employment of microquantities of fluid. Accordingly, standard displacement microburette procedures were established to make all dilutions and to provide accurate and convenient volumetric deliveries in the microliter range. The displacement microburettes were of either 0.2 ml or 2.0 ml total capacity.

## RESULTS

**Application of test solution to pad.** The quantity of solution applied to a pad has a large proportional influence upon the size of resulting zone diameters. A comparison was made of the variability of volumes of solutions applied to pads by a commonly used procedure and the procedure adopted here. Weighed pads were filled with buffer solution by one of the two procedures and then reweighed. The first procedure was that of touching the pad to a supply of the solution in as uniform a manner as feasible saturating the capillary capacity of the pad. The second procedure involved applying solution with the self-filling and

TABLE 1. Statistical comparison of the dipping and pipette methods of delivering test solution onto assay discs

Liquid delivered	Volume delivered ( $\lambda$ )									
	Disc dipping method					Capillary pipetting method				
	N <sup>a</sup>	$\bar{X}$	S <sup>2</sup>	S	S(%)	N	$\bar{X}$	S <sup>2</sup>	S	S(%)
10% NaCl	10	27.38	1.008	1.004	3.65	10	17.99	0.125	0.353	2.00
Water	10	27.21	2.096	1.447	5.35	10	16.86	0.020	0.143	0.83
Water	10	27.09	1.401	1.183	4.36	10	16.88	0.050	0.223	1.32
Average					4.45					1.31

<sup>a</sup> N is the number of pads in each group,  $\bar{X}$  is the mean for the group, S<sup>2</sup> is the variance, S is the standard deviation, and S(%) is the coefficient of variation.

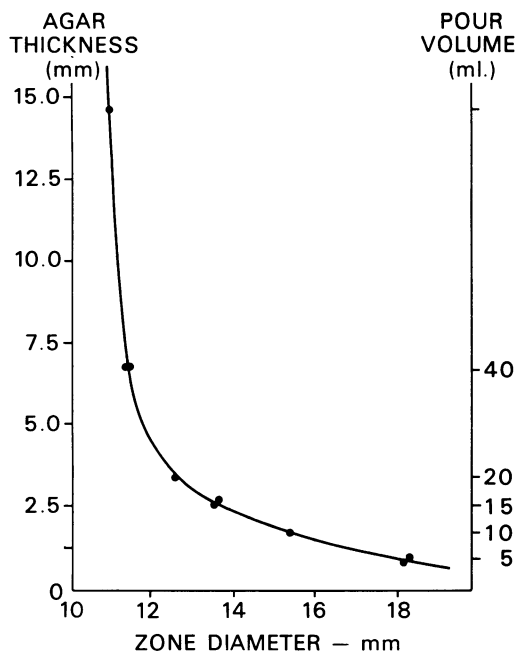


FIG. 1. Dependence of zone diameter upon pour volume and agar thickness. Zone diameters are averages of six zones formed in each of two dishes represented by two data points at each pour volume.

-delivering pipette as described above. Table 1 sets forth the resulting weights of solution and the variability. The variability of weight or volume applied is at least three times as great from the filling of pads by touching solution as from the use of a delivery pipette. The pipette may be expected also to circumvent errors in other procedures due to varying surface tension and viscosity of the sample solution.

**Preparation of test solution.** Large errors generally occur in volumetric dilutions employing delivery pipettes for small volumes, especially where viscous solutions with implicit large drainage errors must be employed. To circumvent these errors, the use of volumetric displacement devices was adopted instead of volumetric delivery devices. Significant errors of delivery from these displacement microburettes occur when delivery of less than 1  $\mu$ -liter is attempted. The protocol presented in the following paper anticipates the delivery of never less than 2.0  $\mu$ liters in the preparation of solutions.

**Agar thickness.** The thickness of the test agar layer greatly affects the resulting zone diameter. Accordingly, the relation of zone diameter versus thickness of test agar layer was examined. The results in Fig. 1 show that the thickness or the pour volume in a petri dish is very critical at pour volumes usually employed. An error in thickness

is a direct and proportional consequence of any error in pour volume. A pouring device allowing delivery of constant volume is therefore essential.

In addition to such error in agar thickness from plate to plate resulting from pour volume error, there is also the possibility of variation in agar thickness *within* a plate. Two sources of such thickness errors exist in disposable petri dishes. The first is the formation of a wedge-shaped layer of agar as a result of tilt of the plate bottom while the melted agar is cooling and setting up. This can be due either to an imperfect plate or to slope of the surface on which the plates are placed for pouring.

The extent of such wedge shape of the agar has been measured by employing a depth gauge to measure the inside contour of the plate bottom. We found that the mold in which the plastic dishes are made generally imposes a sizeable tilt to the dish characteristic of the mold. The amount of tilt characteristic of four mold numbers of Diamond Plastics plates was 0.025, 0.125, 0.15, and 0.175 mm across the 2.5 inch (ca. 6.4 cm) diameter of the circle of zone centers. The products of several other manufacturers were similarly examined and the range of tilt encountered was generally greater than 0.125 mm. The average thickness of agar in an 8-ml pour in a petri dish is approximately 1.25 mm. It is therefore obvious that a few tenths millimeter of wedge shape is a large percentage of variation in thickness. Figure 2 shows for one experiment the interrelationship of tilt (from two sources), wedge shape of agar, position of zones, and zone diameters.

The wedge effect leaves the *average* thickness of agar in a plate unaffected. Whereas the wedge effect causes major variation of zone diameters within a plate, there is of course a tendency of the larger zones resulting on the thin side to compensate for and average out the smaller zone diameters which result from thicker agar on the opposite side. Although this cancelling may not be complete, no regular effect could be observed on the *average* of the six zone diameters of plates when tilt of 0.00, 0.45, 0.72, 0.90, and even 1.20 mm were deliberately imposed. When averaging of all zones in a plate is depended upon to eliminate this source of error, as is done here, the opposite zone should be eliminated also if any zone in a plate is imperfect or unreadable. Such averaging out of the wedge effect would not occur in unsymmetrical arrangement of like zones, as where three unknowns and three internal standard zones are employed (10).

A second systematic variation in the shape of plates which leads to variation of the thickness of agar from zone to zone within a plate is a cup-

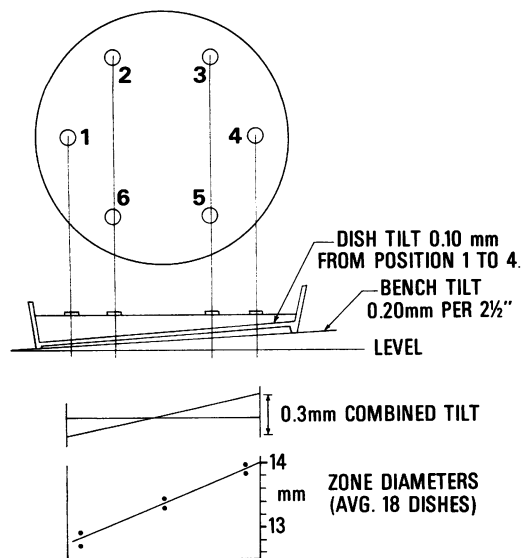


FIG. 2. Relation of zone diameter to pad position and agar thickness in a plate with wedge-shaped agar. The tilt characteristic of the dish is uniformly related to position of the sprue visible on the blow-moulded dish. These plates were oriented and placed on a tilted surface and poured.

shaped contour of the bottom of the plate. The inverse deformity, a center bulge, is also prominent in the products of some manufacturers. This deformity is minimal in the Diamond Plastics plates.

The effect of this second shape factor is not cancelled by averaging. The placement of pads at unequal distances from the center when a cup shape or center bulge exists would cause variation in the zone diameters due to the variation of agar thickness along the line from center to edge. The use of a visual guide for placing the pads is essential. The location of pads, as regards distance from the center, is more critical the greater such deformity of the bottom of the plate and the smaller the amount of agar poured. We have made no measurements on tilt or other deformities in the bottom of glass petri dishes, but they are so conspicuously misshapen that they should not be considered for use.

**Inoculum concentration.** An influence of inoculum concentration on resulting zone size is widely recognized (11). Experiments were performed to determine how critical the density of spore inoculum might be in the present system when other factors are constant. Although wide variation in spore density did produce zone diameter differences (smaller zones for higher inoculum), the most serious consequence was in the quality of resulting zone edges, which were

granular and poorly defined when a too dilute inoculum was used. An appropriate inoculum concentration was found after preparation of each new batch of spores and kept constant by employing a uniform dilution into heated agar.

**Temperature-time exposure.** Major attention was given to several details of the operation which might impose an inequality of temperature exposure of the individual plates handled in a single day. Attention was given to every step of the handling procedure between pouring of plates and grow-out of the test organisms.

In a preliminary experiment, the temperature of seeded agar was measured as it emerged from a pouring tube from the automatic pipetting machine. This was done by having a copper-constantan thermocouple in the outlet tube. The thermocouple EMF was directly recorded on a strip chart recorder throughout this experimental pouring. The seeded agar was held in a constant temperature water bath at 50 C during the pouring. The passage of hot agar through the pipetter and tube gradually heated the pipetter several degrees. Inversely, the agar was initially cooled in passing through the pipetter and the first agar to emerge was not up to the steady state pouring temperature. Accordingly, when the pouring is to be done at 50 C, water at 50 C is pumped through the pipetter until the temperature at the pouring tip is stabilized before pumping the heated seeded agar.

A record was made of the temperature of emerging seeded agar at 50 C after brief (3 min) flushing with water at 50 C and then rejecting five pours (40 ml) of seeded agar at 50 C. The temperature was constant throughout a pour of 100 plates to better than 0.5 C.

An experiment was performed to determine the rate at which 8 ml of hot agar cooled in petri dishes after pouring. A thermocouple was placed in such plates and the temperature was followed with a strip chart recorder. The course of temperature decline to room temperature was asymptotic and rapid. In general, the temperature had fallen to within 1 C of room temperature within 15 min after pouring.

The placing of poured plates in stacks before they reach room temperature slows and prolongs the cooling time. The major consequence of this slower cooling in a stack is that a plate removed from the bench top and placed in a stack at a higher temperature (shorter time after pouring) experiences a much longer exposure to elevated temperature than one placed in the stack after longer cooling on the bench top.

If the relatively slow operation of pouring (10 min), was followed quickly by the relatively fast operation of stacking (2 min), the first plates

TABLE 2. *Dependence of zone diameter on incubation temperature*

Determination	Stack	37 C	30 C	26 C
Zone diameter (mm) <sup>a</sup>	1	12.76	13.49	14.88
	2	12.40	12.42	14.40
Concn with simulated standard curve at 30 C	1	1.70	2.00	2.77
	2	1.77	2.00	2.80

<sup>a</sup> Average of 12 plates.

poured would have cooled much further when stacked than the last plates poured. Experiments in which plates were stacked immediately after pouring has confirmed this expectation, producing significantly smaller zones. Accordingly, a regular practice was adopted of letting the plates cool on the bench top for approximately 20 min after completing the pouring before stacking.

To judge how critical is temperature exposure during holding or incubation on the resulting zone diameter and on the test results, incubation was carried out at several temperatures, and the results are presented as zone diameter versus temperature in Table 2. All the zone diameters for each stack were averaged. Data for only two stacks are presented. A typical conversion of zone diameter to "concentration" shows the high sensitivity of test results, expressed as concentration, which would result from unaccounted variation of incubation temperature.

Upon cooling in a refrigerator or warming in an incubator, heat flows into or out of the stack through some thermal contact of the stack with a heat source or heat sink. For instance, the bottom plates of the stack would deviate prominently in thermal exposure if the stack were placed on a cold refrigerator shelf or on a warm incubator shelf. In all experiments in which both refrigeration and incubation at 37 C were employed, such end-effects were seen. The consequence of refrigeration was always to imprint a depressed temperature exposure (and consequent high zone diameter) upon the plates that had been in closest contact with the refrigerator shelf. Even wrapping the stack and use of an insulating pad did not eliminate the top and bottom effect.

When stacks were placed in an incubator at 37 C for grow-out, similar but more elusive top and bottom anomalies of zone diameters were observed within stacks. Wrapping the stacks in aluminum foil and suspending them by means of masking tape to eliminate solid contact with the incubator failed to eliminate completely the top and bottom anomalies of resulting zone diameters. In persistent effort to eliminate the top and

bottom anomalies in stacks caused by incubation at 37 C, changes were tried to restrict, redirect, or eliminate air currents in the incubator. Such efforts resulted in alteration but not removal of top and bottom anomalies. Since the anomalies were generally most exaggerated in the topmost and bottommost plates, but still detectable several plates from top or bottom, this source of error could be minimized by leaving unset the last four plates on the top and on the bottom. Even this practice did not completely eliminate the anomalies.

Evaporation of even a very small amount of water from a poured plate would cause a large lowering of temperature. Evaporation would occur if the plates in stacks were allowed to be in contact with an unsaturated atmosphere. If the thermal flow into or out of the plate is slow compared to the rate of evaporation, one could anticipate the lowering of the temperature of 8 ml of agar by 5.0 C by evaporation of only 80 mg of water (1% of the 8-ml pour volume). Accordingly, enclosure of stacks was provided to secure and maintain a saturated atmosphere about the plates to avoid the cooling effect of such evaporation.

All efforts to attain uniform or linearly diminishing zone diameters within stacks of plates failed when refrigeration was employed between pouring and setting, and when incubation at 37 C was used. Therefore, a room temperature procedure was devised for storing and incubation to circumvent large temperature swings. There are inherent advantages in room temperature (26 C) handling over conventional procedures that employ refrigeration and incubation at 37 C. These are the absence of temperature swings which cannot be made exactly equivalent for all plates in one or more stacks. Subsequently, work in these laboratories (J. F. Quay, unpublished data) has shown that some test organisms produce better zones when the stacks, enclosed in close-fitting steel cylinders, are incubated at 30 C, or even at 37 C. Relatively little end anomaly results from such incubation if the steel cylinders are employed as described before without intervening refrigerator storage.

**Other factors affecting germination rate.** In addition to temperature, other factors that influence rate of germination include the chemical composition of the medium (which, of course, can be fixed) and pH, which can be adjusted initially and stabilized by inclusion of buffers. Without buffer, pH of agar seeded with *B. subtilis* spores generally drifts upward during grow-out. Such pH changes during grow-out have been observed, but pH variability from plate to plate is not great, and growth is often more

favorable in a very weakly buffered or unbuffered solution.

**Timing considerations.** To find and control the sources of variability in diffusion microbiological testing, attention has been centered upon parameters of the diffusion process and upon parameters of the process of growing out of the test microorganisms. The result of a diffusion-inhibition test is the result of a race between spreading of antibiotic by diffusion and the grow-out of the test organisms. The conditions that influence diffusion rate are largely controllable, and small temperature variation has a relatively small influence upon diffusion.

The moment at which the germinating spores reach some critical stage—perhaps where the resulting vegetative organism first doubles—is the “timing” mechanism for determining the position of the zone edge. Inside this circle the germinating organisms are prevented from passing the critical stage (doubling), whereas outside this circle the concentration of antibiotic is too small to prevent the event. It is generally understood that when the concentration of inoculum increases (here doubles) more antibiotic is required to prevent further growth. Thus, the line within which doubling is prevented by just sufficient antibiotic concentration becomes a fixed zone edge which becomes sharper but does not move significantly with the further divisions and doubling of numbers of test organisms. Outside this circle, a continuing increase of density occurs as the doubling and redoubling of organisms continues.

The initiation of germination for all plates is perhaps that moment when the spore suspension is added to hot agar. Accordingly, the last plate poured should come to the critical timing event referred to above at about the same time, because all plates are poured within a 10-min period. The cooling to room temperature of the first and last plates poured is separated only by this short interval. The cooling is equally rapid for all plates since they all cool on the bench top.

The initiation of diffusion, however, is at the moment when the antibiotic solution is applied to the pad. From the first plate set to the last plate set in a series of 102 plates may be 1 hr. Thus, when “time” is called by the germination event, the last plates set will have had 1 hr less to diffuse, and the zones determined at that moment must be smaller for a given concentration of test solution.

If the setting of the test pads is done on a regular time schedule, this factor can be accounted for in the subsequent treatment of data. The function of a constant standard solution (2  $\mu\text{g}/\text{ml}$ ) set at regular intervals throughout the schedule is to define the influence of the order and time sequence

of this and all other operations carried out in the same numerical sequence. The influence of order of handling within the experiment of 1 day can then be accounted for in the data handling. Representative data show that the diameters decrease approximately linearly with the setting order, or with the time of setting, if a regular schedule of setting is followed. This slope must be greater in proportion to the time required for setting a given number of plates.

An experiment was performed to determine the effect of the time of holding the poured and stacked plates at room temperature before setting them. The time between setting and grow-out (the time allowed for diffusion) must be reduced by longer holding between pouring and setting. A regular decline occurred in the zone diameters produced by 2.0  $\mu\text{g}$  of antibiotic per ml applied to plates held for intervals up to about 5 hr after pouring (see Table 3). When the plates were set at later times, small, unusable, poorly defined zones were formed. From this experiment, we concluded that resulting zones are sharp and usable from plates that have been held up to as much as 3 hr after pouring. This limitation may of course vary greatly with the test organism.

The following paper presents a convenient method of accounting for all the factors of timing;

TABLE 3. Influence of room temperature holding time before setting plates

Holding time (hr)	Zone diameters (mm) <sup>a</sup>	Observations
Expt 1		
1	15.18	Normal zones
2	14.43	Normal zones
3	13.57	Normal zones
4	12.71	Normal zones
5	11.55	Normal zones
6	10.74	Diffuse zone edges
7	9.8	Diffuse zone edges
8	9.9	Diffuse zone edges
10	10.16	Diffuse zone edges
12	10.20	Diffuse zone edges
Expt 2		
1	15.04	Normal zones
2	13.96	Normal zones
4	11.83	Normal zones
5	10.75	Normal zones
6	9.71	Normal zones
7	8.60	Normal zones
8	8.10	Diffuse zone edges
9	8.15	Diffuse zone edges
10	8.25	Diffuse zone edges

<sup>a</sup> Average of 24 zones.

namely, the order of pouring, stacking, and setting. Fortunately, although the effects of these variables cannot be removed, they can be regularized, combined, and accounted for in a manner that removes them as a source of variability or error in assay results.

### DISCUSSION

Of the factors that influence variability and error, some can be controlled by choice of conditions and some by handling procedures. Other factors cannot feasibly be controlled, but, by establishing an order and timing of the operations, the consequence of the combination of factors is a regular decline in zone diameter for a given concentration as the plate sequence number increases. Reference plates inserted at regular intervals permit normalizing this combined variable.

Among the controllable factors, we have shown that special care must be taken to apply a constant volume of solution to the pads. The superiority of using an automatic filling and emptying capillary pipette for applying antibiotic solution over an alternative procedure is demonstrated. The use of the pipette is rapid and feasible. Setting plates was possible at a regular rate of two plates or 12 pads per minute.

Accuracy of diluting test solutions is poor by conventional volumetric delivery glassware if very small volumes are employed. Such difficulties can be circumvented by employing displacement microburettes, which not only provide accurate reading of volumes but also eliminate the hazard of drainage error.

Agar thickness is a critical factor in determining the zone diameter. The factors that contribute to variation in agar thickness are the amount of agar poured (causing a directly proportional error in thickness) and wedge shape. The latter results from sloping petri dishes or from pouring on a surface with tilt.

Another fault of petri dishes which causes uncontrolled variation in thickness of agar is the presence of a nonplanar bottom. Whereas wedge shape produces very large zone diameter variations within a plate, averaging all zone diameters in a plate removes most of the error from this source. In test protocols where "internal" reference zones are employed, both reference and unknown zones should be set only as *opposed pairs*, and, if one zone of agar is unusable, the opposite zone should be dropped.

Different temperature exposure of plates in the different positions within stacks (*viz.*, top and bottom) is unavoidable but can be minimized by enclosure of stacks in close-fitting steel cylinders and avoidance of large temperature shifts, particularly between room and refrigerator temperature. Refrigeration should not be used, and incubation should be at room temperature if the satisfactory growth of test organisms allows. Several factors associated with pouring and stacking have been found that cause inequalities of temperature exposure. Corrective measures were found.

Characteristic growth of test organism and the zone size and sharpness respond to changes in *pH* and buffering. No attempt was made here to optimize these factors for the test. This should be done for each test organism and for each antibiotic, if repetitive testing is to be done.

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