Spiral Plate Method for Bacterial Determination

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A method is described for determining the number of bacteria in a solution by the use of a machine which deposits a known volume of sample on a rotating agar plate in an ever decreasing amount in the form of an Archimedes spiral. After the sample is incubated, different colony densities are apparent on the surface of the plate. A modified counting grid is described which relates area of the plate to volume of sample. By counting an appropriate area of the plate, the number of bacteria in the sample is estimated. This method was compared to the pour plate procedure with the use of pure and mixed cultures in water and milk. The results did not demonstrate a significant difference in variance between duplicates at the α = 0.01 level when concentrations of 600 to 12 x 10^4 bacteria per ml were used, but the spiral plate method gave counts that were higher than counts obtained by the pour plate method. The time and materials required for this method are substantially less than those required for the conventional aerobic pour plate procedure.

Agar pour plate procedures are used routinely for the quantitative determination of aerobic bacteria. Koch introduced the technique in 1880 with the development of agar media, and by 1895 it was a recognized procedure. Analysis of the procedure by Breed and Dotterer (2) in 1916 resulted in the procedures used today. In the aerobic pour plate procedure, an unknown sample is diluted many-fold and a known sample of each dilution is mixed with liquid agar in a petri dish. After incubation, that plate which has between 30 and 300 colonies is counted, and the resultant count is multiplied by the appropriate dilution to obtain the bacterial concentration in the sample. All other plates that did not have between 30 and 300 colonies are discarded. This procedure is simple, can cover a large concentration range, and at present is probably the most precise method for determining those bacteria that will grow in the agar media.

Much research has been done to find a better method than the pour plate, primarily because it is time-consuming and costly. The drop plate and the surface plate are two methods in use today for mesophilic aerobes in which liquids are deposited on the surface of an agar plate. The surface plate method is widely used in Europe but is not officially recommended in the United States. Both methods offer cost savings over the pour plate method. Several of the advantages and disadvantages as listed by Thatcher and Clark (12) appear to be applicable to the method presented in this paper. Some new methods have found applications in specific areas of bacterial enumeration or identification, such as particle counting (6), metabolic production of radioactive 14CO2 (5), measurements of various enzyme concentrations, bacterial growth in capillary tubes (1), microscopic scanning of stained organisms (8), and gas chromatography of bacteria. Some of these methods have been discussed by Mitz (7). Generally, the results are obtained more quickly by these methods than by the pour plate technique, but in each instance, disadvantages have prevented them from being routinely used in most bacteriological laboratories.

There are numerous aids for preparing agar pour plates, such as automatic dispensers of agar, automatic diluters, and various pipetting aids. There are also aids for inoculating the surfaces of a prepared agar plate. There are motors (Lab Line Instruments, Inc., Melrose Park, Ill.) that rotate a prepared agar plate while a hand-held loop is used to inoculate the surface. Trotman (13) has developed an automatic plate streaker with which the inoculum is placed on the surface of the agar with a pipette and a mechanically moved loop streaks the inoculum over the surface of the plate.

The basic concept of our plating machine is to deposit continuously a small amount of
undiluted sample on the surface of a rotating agar plate. The resultant track on the agar surface is in the form of an Archimedes spiral. The amount of sample is controlled and decreased while the dispensing stylus is moved from the center to the edge of the rotating agar plate. On incubation, the colonies develop along the lines where the liquid was originally deposited. The number of colonies per unit length of line or per unit area on the agar surface is dependent upon the bacterial concentration in the deposited liquid. Because of the different densities from the center to the edge of the plate, some area of each plate can be counted with ease.

**MATERIALS AND METHODS**

The parts of the plating machine are numbered in Fig. 1, and these are the numbers which appear in brackets.

**Construction of the machine.** To maintain structural rigidity, 0.5-inch (1.3-cm) aluminum plate was used as the base, and 0.25-inch (0.64-cm) aluminum plate was used for the sides. The upper carriage is mounted on three linear ball bearings that ride on two case-hardened 0.5-inch rods. A half nut connects the carriage to a lead screw (13 threads/inch, NC). As the lead screw is rotated, the carriage is moved over the rotating platform. The lead screw is connected to the shaft of the motor by a beaded chain, which goes through reducing sprockets to achieve the correct speed ratio. A variable-speed laboratory stirring motor rotates the platform holding the agar plate at approximately 200 rev/min. The rotation ratio of the plate and the lead screw is about 3:1, which results in a 0.65-mm separation between adjacent lines of the Archimedes spiral. A rotation counter was added to record...
the position of the rotating agar plate, and to facilitate finding the beginning and ending points of the Archimedes spiral. The rack and pinion [5] is mounted on the carriage and attached to an arm [2] that follows the shape of the cam [1]. The movable portion of the rack and pinion is attached to the hollow plunger of the backflow syringe [4]. The barrel of the syringe is fixed to the carriage and connected to the thin-walled Teflon spaghetti tubing [7] which extends through the stylus [6] to the surface of the agar plate. The stylus is mounted on pin pivots that give it free vertical movement but minimal lateral movement. The vertical movement of the stylus allows the tip of the Teflon tubing to maintain contact with the surface of the agar while the plate is rotating.

Operation. A liquid sample is introduced into the tip of the Teflon tubing by applying a vacuum to the two-way valve [3] and pulling the liquid up through the tubing and syringe. This flushes any residual sample from the Teflon tubing as the liquid moves in the reverse manner into the vacuum trap [13]. A bleed valve [14] controls the vacuum applied to the system. When the tubing and syringe are filled with liquid, the valve attached to the syringe [3] is closed. An agar plate is placed on the platform [8], the Teflon tip is placed on the agar surface, and the motor is started. As the platform moves, gravity causes the arm to follow the contour of the cam. Since the arm is connected to the rack and pinion which controls the syringe, the fall of the arm is proportional to the movement of the plunger in the syringe. The arm drops rapidly at first and then more slowly as the carriage moves over the agar plate. This results in most of the sample being deposited close to the center of the plate, with a decreasing volume toward the edge of the plate. When the operation reaches the 80th revolution as shown by the rotation counter [19], the operator lifts the stylus from the agar surface, elevates the arm from the surface of the cam, and opens the two-way Teflon valve to remove residual sample from the system. The inoculated agar plate is removed from the rotating platform. The direction of the motor is reversed, causing the carriage to move back to the starting position. There the system is flushed with a 5% solution of sodium hypochlorite and rinsed with sterile water, after which the next sample is drawn into the Teflon tubing and the operation is repeated.

Counting grid. The counting grid shown in Fig. 2 is used to relate the colonies on a spiral plate to the volume in which they were contained. The grid covers the area of an agar plate and was prepared by dividing the area into four concentric circles and into eight wedges or octants. Thus, each octant is subdivided into four arcs. The relation of liquid volume to area was determined for different portions of an octant and is shown as the shaded areas in Fig. 2. On each plate, only one area with well-separated colonies is counted, and the area which is counted is dependent upon the number of colonies. Colonies are counted in one octant from the outer edge towards the center until at least 25 colonies are observed. The remainder of that arc where the 25th count occurred must be counted. A like area of an opposite octant is counted, and that count is added to the first count. If there are not 25 colonies on one octant, then the colonies on the total plate are counted. The colony count is divided by the liquid volume corresponding to the area over which the colony count was obtained, resulting in the bacterial concentration of the sample.

Three methods were used to calibrate the volume of each portion of an octant. (i) The volume of water dispensed was weighed on an analytical balance and related to the number of revolutions that the plate had turned. (ii) The volume of an octant was obtained by the use of a radioactive solution of 125I; again, by starting and stopping at known positions and determining the radioactivity of the resultant deposited liquid, the volume was determined. (iii) Different bacterial solutions were plated with the machine, and the resultant colony counts, divided by the concentration, gave the sample volume in that area where the colonies were counted.

Cultures. The cultures used in this study, representative of certain bacterial genera found in milk and other foods, were *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Lactobacillus casei*.

*B. subtilis* spores were preserved in 95% ethyl alcohol held at 4 C, and were supplied to us in distilled water concentrations of approximately 2 × 10⁷ spores/ml.

Cultures of *E. coli*, *P. aeruginosa*, and *S. aureus* were maintained at room temperature in 10 ml of nutrient broth (NB, Difco) contained in Pyrex glass tubes (18 by 150 mm) and on nutrient agar (NA, Difco) slants; *L. casei* was maintained in Trypticase soy broth (TSB, BBL). Before the cultures were used in comparative plating studies, they were inoculated into tubes of broth and incubated at 35 (± 1) C for 18 to 24 hr. The bacterial concentrations of the broth

![COUNTING GRID](http://aem.asm.org/)
cultures (or, in certain instances, growth from an NA slant of E. coli suspended in NB) were adjusted by diluting the culture with phosphate-buffered dilution water to a specific optical density known by the desired concentration. This concentration was diluted to give 10 serial dilutions of each culture at equal intervals ranging in count from approximately $10^0$ to $10^7$ cells/ml. These 10 dilutions were plated in duplicate by directly inoculating the surface of prepared agar plates (Standard Methods [SMA, BBL] contained in plastic petri dishes (150 by 25 mm)) with the spiral plating method. When examined by the agar pour plate method, further dilutions were made when necessary and plated in duplicate (SMA, BBL) in plastic petri dishes (100 by 15 mm). Two or more comparative plating studies were made with each culture, and in two studies the cultures (E. coli and S. aureus) were mixed and then diluted and plated as described for single cultures.

All plates were incubated at 35 ($\pm$ 1) °C. The spiral plates were counted after 16 to 24 hr of incubation, with the exception of L. casei cultures, which were incubated for 40 to 44 hr. The agar pour plates were counted after 48 ($\pm$ 3) hr of incubation (with the exception of E. coli plates, which were counted after 18 to 24 hr).

In addition to the comparative studies with pure cultures, 14 split milk samples (4) consisting of inoculated raw milk, cream, and pasteurized milk products were compared for bacteria by the agar plate and the spiral plate methods.

**RESULTS**

Sixty-five replicate plateings were made of a spore suspension of B. subtilis to test the precision of the spiral plating method on replicate counts, and to determine whether the counts were normally distributed under the logarithmic transform. Pearson and Hartley (10) have presented the test and tables used to examine the assumption of normality. A value of $\alpha = 0.072$ was computed for the 65 replicate observations obtained in this experiment, and the assumption of normality under logarithmic transform could not be rejected at the $\alpha = 0.01$ level. The variance as given by Ostle (9) was 0.00196 in log10 units, and the percent coefficient of variation was 9.97 as computed from the raw counts per milliliter of B. subtilis culture.

Table 1 compares the bacterial concentrations obtained by the spiral and pour plate methods for different concentrations of P. aeruginosa. In this comparison, as in all comparisons, blanks of sterile water were plated with the spiral plating machine immediately after the most concentrated samples to check for contamination between samples; none was found. The variance between duplicate plates at all concentration levels is given for each method at the bottom of each column, and shows both methods to be comparable. A least squares fit of the values from Table 1 is graphically presented in Fig. 3, where the linear relation between the two methods can be seen. Figure 3 is the result of the regression of the log10 pour count on the log10 spiral count. The proportion of sum of squares due to this regression is $r^2 = 0.997$.

Table 2 summarizes the variances and the percent differences obtained from all 17 studies in which the spiral and the pour plate methods were compared. Two of the 17 comparisons are presented in Table 1. The last column of Table 2 represents the percent difference between geometric means of the two methods.

**Table 1. Comparison of spiral and pour plate methods with Pseudomonas aeruginosa**

| Organisms/ml | Expt 11 | Expt 12 |
|--------------|---------|---------|
| Spiral       | Pour    | Spiral  | Pour    |
| 14,800,000   | 13,300,000 | 10,300,000 | 12,500,000 |
| 14,100,000   | 15,000,000 | 10,000,000 | 9,500,000  |
| 8,500,000    | 8,100,000  | 5,810,000 | 6,300,000  |
| 8,400,000    | 7,200,000  | 5,800,000 | 6,500,000  |
| 0            | 0        | 0       | 0        |
| 2,160,000    | 1,810,000 | 1,610,000 | 1,320,000 |
| 2,250,000    | 1,580,000 | 1,420,000 | 1,030,000 |
| 1,230,000    | 760,000   | 900,000  | 820,000  |
| 1,110,000    | 850,000   | 720,000  | 810,000  |
| 187,000      | 164,000   | 145,000  | 127,000  |
| 234,000      | 162,000   | 142,000  | 139,000  |
| 117,000      | 86,000    | 81,000   | 71,000   |
| 111,000      | 94,000    | 76,100   | 62,000   |
| 18,600       | 18,400    | 12,700   | 14,500   |
| 19,800       | 17,300    | 16,200   | 13,800   |
| 9,400        | 8,700     | 10,100   | 5,600    |
| 10,000       | 8,100     | 8,050    | 7,500    |
| 2,070        | 1,440     | 1,840    | 1,200    |
| 2,040        | 1,520     | 1,880    | 1,140    |
| 920          | 870       | 820      | 650      |
| 820          | 610       | 690      | 640      |
| Variance     | 0.000207  | 0.000863 | 0.00079  | 0.00264  |
compared to the pour plating method. The counts of the spiral plating method averaged 17% higher than those of the pour plate method. The replicate variances obtained by both methods were about the same, indicating that both methods produce duplicate plates with less than 0.003 variance (about 13% coefficient of variation).

A comparison of the spiral and pour plating methods, performed with split milk samples, shows (Table 3) that the counts obtained by the spiral plating method were 14% higher than those obtained by the pour plating method. The variances between duplicate plates for the two methods were again comparable, as they were with the counts obtained from the pure cultures in buffer solutions. The plates of the split milk samples were counted after 18 and 44 hr of incubation at 35 C. A few colonies (presumably E. coli) were counted after 18 hr, but the pinpoint colonies of micrococcus appeared only after the longer incubation time.

Many microbiological tests are affected by the technique of the analyst. Therefore, an experiment was designed to determine whether the variation among analysts would affect the results obtained by the spiral plating method (Table 4). Five analysts were chosen for the study, three of whom were totally unfamiliar with the operation of the spiral machine. These three were given 15 min of instruction in operational procedure, and then each of the five analysts examined three samples in duplicate. An analysis of variance was used to test the null hypothesis that results for analysts were equal, and no significant difference among analysts was found.

Typical plates as prepared by the spiral plate-making method are presented in Fig. 4 and 5. The E. coli count of the dilution used to make the plate in Fig. 4 was about 10^6 bacteria/ml. The colonies towards the center of the plate were too numerous to count, whereas towards the outer edge individual colonies were countable. When this plate was placed on the grid, the countable portion of the plate was related to volume, as described previously.

### DISCUSSION

Several variables can influence the amount of fluid deposited on the surface of an agar plate. Some examples are the syringe size, the amount the plunger is depressed, and the vertical drop of the cam. The arc through which the rack and pinion arm moves determines the depression of the syringe barrel, and the shape of the cam controls the arm's rate of descent. The cam on this machine allows the arm to drop according to the equation: $Y = \left(\frac{1}{3}\right)X^2$. Y is the vertical position of the arm on the cam, and X is the horizontal movement;
thus, when \( x = 0, y = 10 \text{ cm}, \) and when \( x = 1, y = 3.3 \text{ cm}, \) etc. Since the counting grid is fixed, the area of the plates over which bacteria can be deposited must conform to the limits set by the area of the counting grid. The stylus must begin at the same place on the surface of each plate and cover the same horizontal distance while moving across the plate. Each of these parameters has been set in the machine as it is described; if other conditions are desired, however, each parameter could be altered accordingly. For example, a cam could be designed to allow deposition of the same volume of liquid per unit area on the entire agar surface instead of the density gradient that is now used.

**Table 3. Analysis of split milk samples by spiral and pour plating methods**

| Sample | Milk type | Culture added | Organisms/ml* |
|--------|-----------|---------------|---------------|
| 1      | Homogenized + vitamin D | \( L. \) casei | 14,500 | 14,000 |
| 8      | Chocolate | A | 14,200 | 15,000 |
| 2      | Homogenized, 18% cream* | Micrococcus | 10,000 | 8,150 |
| 4      |          | B | 9,800 | 9,200 |
| 3      | Homogenized, 15% cream* | Micrococcus | 17,400 | 17,000 |
| 7      |          | B, \( L. \) casei | 20,000 | 16,100 |
| 5      | Homogenized, 2% cream | Micrococcus | 24,000 | 19,800 |
| 6      |          | A | 13,500 | 15,900 |
| 9      | Raw, 6% cream | \( L. \) casei | 125,000 | 115,000 |
| 11     |          |                   | 170,000 | 108,000 |
| 10     | Raw, 6% cream | Micrococcus | 105,000 | 101,000 |
| 13     |          | B | 106,000 | 79,000 |
| 12     | Raw, 6% cream | Micrococcus | 155,000 | 154,000 |
| 14     |          | A | 155,000 | 142,000 |

Variance: 0.00342
Average difference: 0.00281

**Analysis of variance**

| Source | Sum of squares | Degrees of freedom | Mean square | F-ratio |
|--------|----------------|-------------------|-------------|---------|
| A, Methods | 0.04067 | 1 | 0.04067 | 13.04* |
| B, samples | 12.79446 | 13 | 0.98419 | 315.45* |
| AB | 0.08812 | 13 | 0.00678 | 2.17* |
| Error | 0.08722 | 28 | 0.00312 | — |
| Total | 13.01047 | 55 | — | — |

* Significant at \( \alpha = 0.01 \) level.
* Significant at \( \alpha = 0.001 \).
* Significant at \( \alpha = 0.01 \).

**Table 4. Analysis of three \( B. \) subtilis suspensions by different operators of the spiral plating machine**

| Suspension | Organisms/ml by |
|------------|-----------------|
| Laboratory technician | Secretary | Stock clerk | Laboratory director | Chemist |
| A | 2.770 | 2.900 | 3.170 | 3.400 | 3.170 |
| B | 24.000 | 22.700 | 25.600 | 31.200 | 30.000 |
| C | 276,000 | 424,000 | 301,000 | 242,000 | 272,000 |

**Analysis of variance**

| Source | Sum of squares | Degrees of freedom | Mean square | F-ratio |
|--------|----------------|-------------------|-------------|---------|
| Operator | 0.01081 | 4 | 0.00270 | 0.85 |
| Sample | 19.39731 | 2 | 9.59666 | 3.069.20* |
| Operator-sample | 0.02071 | 8 | 0.00259 | 0.82 |
| Error | 0.04736 | 15 | 0.00316 | — |
| Total | 19.47619 | 29 | — | — |

* Significant at \( \alpha = 0.01 \) level.

**Fig. 4. Spiral plate inoculated with \( 10^6 \) organisms of \( E. \) coli/ml.**
Various syringes (0.10 to 2.5 ml) have been used to dispense different volumes of liquid onto the agar surface. Also, different sizes of Teflon spaghetti tubing in the stylus have been used to deposit the liquid on the agar surface. Tubing size used in the described machine is 28 American Wire Gauge, but 26- and 30-gauge tubing have also been used.

It is important that the liquid being dispensed from the Teflon tube onto the agar surface remain in continuous contact with the agar. For this reason, the tip of the Teflon tube should ride flat on the agar surface, and the agar surface should be reasonably flat. Irregular patterns may be obtained if these conditions are not met, and recutting the Teflon tube may be necessary to obtain an even pattern. An even cut of the Teflon tube has been obtained by using a thin razor blade and a jig that holds the tube at a fixed position while being cut. To determine quickly that the liquid is distributed evenly on the agar surface, a saturated solution of crystal violet dye from the spiral plating machine should be deposited onto the agar surface and the pattern of the liquid should be observed visually. If an uneven pattern is produced, the Teflon tip should be recut until a satisfactory pattern is obtained.

Sample to sample contamination has not been a problem. The nonwetting characteristic of Teflon, the size of the tubing, and the reverse flow by which a sample is applied to the plate all contribute to preventing contamination between samples. With a new sample, only a few tenths of a milliliter is used to rinse the system of the old sample and load the syringe for the deposition of the new sample. A sample of approximately 30 μl is deposited on the surface of the agar, and that volume is contained in the lower portion of the Teflon tubing. Contamination was observed when spores of *B. subtilis* were used, and excessive rinsing with the sample was necessary to assure no cross-contamination; to eliminate this excessive rinsing, a 1-sec rinse with sodium hypochlorite (NaClO, bleach) was used to eliminate cross-contamination of the bacteria tested, and this operation was incorporated into the loading procedure.

The variances obtained in the 17 experiments in Table 2 demonstrate that the spiral and pour plating methods can produce comparable results in duplicate plates over the concentration levels tested. A variance of 0.0030 is equal to about 13% coefficient of variation, which is a generally accepted variation between duplicate samples for a given analyst using the pour plating method. The recoveries in the last column in Table 2 are all positive, except one, ranging from −6 to 34%, with an average of 17%. Three factors that might cause these differences are (i) the variation in lots of agar (11) and in methods of preparation that can produce media with different efficiencies.
of growing bacterial colonies, (ii) error in the amount of liquid dispensed from the spiral plating machine, and (iii) a greater breaking up of clumps of bacteria as a result of spreading a small volume of liquid on a surface (14) compared to mixing a solution with melted agar, as in the pour plating method. Clark (3) found 70 to 80% higher colony counts from chicken processing samples when surface plating was compared to pour plating. The difference was related to both type and strain of bacteria.

The split milk samples (4) were part of a group of replicate samples that were distributed nationally to various laboratories as part of a laboratory certification program. The analyzed samples included raw milk, homogenized milk, cream, and chocolate milk, and were of unknown cultures and concentrations. The samples containing 15 and 18% cream were more viscous than the other samples and took longer to be pulled into the Teflon tube, but no other difficulties were encountered.

In contrast to pour plates, the spiral plating-making procedure uses all plates; no plates are thrown away. Each plate is put onto the grid and counted. In most cases, between 50 and 100 colonies will be counted, but the position of the area on the grid over which the 50 to 100 colonies are distributed will vary with the sample concentration. A lower limit of 20 colonies per plate (600 bacteria per ml) was imposed, because the average variance obtained with fewer than 20 colonies per plate in the 17 experiments was 0.00053 compared to 0.00261 when plates with fewer than 20 colonies were excluded. An arbitrary upper limit of 100 colonies per one-fourth of the outer arc (about 10 million bacteria per ml) was set for convenience in counting; however, the variance was not altered when more than 100 colonies were counted.

The operator’s time for the spiral plating machine is about 2 min per plate, which is five times as fast as for the pour plating procedure. Much of the saving in time is achieved because no dilution of the sample is made in the spiral plating procedure, thus making the use of pipette, dilution bottle, and sterile water unnecessary. The machine shown in Fig. 1 is being modified to reduce operator time by automating some of the functions now performed by the operator. Counting spiral plates is somewhat faster and easier than counting pour plates, primarily because of the equal size of the colonies.

The technique of depositing bacteria on an agar surface along the fixed lines of an Archimedes spiral has advantages not found in the pour plate procedure. The growth rate of the same species is the same, resulting in colonies of about the same size. In many cases, colonies of different species may be visually differentiated and counted while using the counting grid. Another advantage of spiral plating is that the Archimedes spiral stops when the meniscus of the agar is reached. Thus, colonies are counted only on the clear, level portion of the agar. These factors are important not only in visual counting but also in developing an electronic counter. We are currently investigating, with apparent success, an electronic laser system for counting spiral plates.

The short preparation time, the speed with which the sample is applied to the plates, the broad concentration range, and the probability of automating the spiral plating machine and having an electronic counter, all make the future of the spiral plating method appear very promising.

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