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Novel Method for Assessing Postantibiotic Effect by Using the Coulter Counter

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To study postantibiotic effect, viable counts are routinely acquired by the pour plate technique. However, this technique is laborious and time-consuming; the required sample dilutions also cause excessive errors and material wastage. A new total cell counting technique in which a Coulter counter is used to obtain more efficient postantibiotic effect measurements has been developed.

The postantibiotic effect (PAE) is the duration of bacterial growth suppression following the abrupt removal of the test antibiotic from the culture medium (1, 11, 12). This duration is related to the antibiotic concentration and exposure time prior to its removal (11) and is also specific for the antibiotic-bacterium system (1, 12).

Traditionally, assessment of PAE requires that changes in bacterial count be followed for a few hours after antibiotic removal. To obtain bacterial counts, the conventional pour plate technique is widely adopted. Although performance of the technique is simple, it is cumbersome and labor-intensive. In most cases, failed experiments brought about by an incorrect dilution scheme can cause much frustration and the loss of time and materials. There is a clear need for a more practical method of studying PAE. We describe here a total cell counting (TCC) method which allows for simple and efficient assessments of PAE. The method was successfully applied in studies of the PAEs for three different antibiotic-bacterium combinations.

Escherichia coli ATCC 25922, *Streptococcus faecalis* ATCC 29212, and *Staphylococcus aureus* ATCC 25923 were used in the study. Sterilized Mueller-Hinton broth supplemented with 12.5 μg of Mg^{2+} per ml or 25 μg of Ca^{2+} per ml (MHB-S) and nutrient agar were used. Both lyophilized organisms and culture media were acquired from Difco Laboratories, Detroit, Mich. The test organisms were isolated and maintained on agar slants at 4°C. Tetracycline HCl, dicloxacillin, and erythromycin were purchased from Sigma Chemical Co., St. Louis, Mo. The MICs of the antibiotics for the respective microorganisms (tetracycline for *E. coli*, dicloxacillin for *S. aureus*, and erythromycin for *S. faecalis*) were determined by the macrodilution method (9).

Overnight suspensions of the test organisms were prepared. Prior to the experiments, these overnight cultures were allowed to grow in fresh MHB-S at 37°C to attain logarithmic growth. The actively growing culture was then adjusted visually against a 0.5 McFarland standard with MHB-S. To start the PAE studies, 0.1 ml of the adjusted culture was introduced into 0.9 ml of MHB-S containing the test antibiotic to yield a density of approximately 10^6 to 10^7 CFU/ml. The organisms were exposed to four times the MIC of the antibiotic for 1 h (1). The antibiotic was subsequently removed by cell washing, i.e., saline wash and spinning (three times) at an average speed of 4,250 rpm for 5 to 8 min. The bacterial cells were resuspended in 10 ml of fresh MBH-S. The respective total and viable cell counts were quantitated by submitting each sample for TCC with the Coulter counter (Coulter Multisizer II; Coulter Electronics Limited, Luton, United Kingdom) and for colony counting by the pour plate method at time zero, immediately before and after antibiotic removal, and 0.5- to 1-h intervals thereafter. For the pour plate method, the samples (0.1 ml) were serially diluted with sterile saline such that 20 to 200 colonies were obtained on each agar plate following an overnight incubation. For the TCC method, either 0.1 or 0.5 ml of sample (depending on the size of the initial inoculum) was added to an electrolyte solution, which consisted of 2% NaCl and 1% formaldehyde, to yield a final volume of 10 ml before total cell counts (500 to 30,000 cells per 50 μl) were measured in duplicate. PAE is defined as the time required for a 1-log-unit increase in the bacterial count following antibiotic removal relative to that required for the control.

The TCC method requires serial measurements of the total cell count at designated times over the entire experiment and at least one viable count immediately after antibiotic removal. The total cell count (N_T) obtained at any time following antibiotic removal is equal to $N_L + N_D$, where N_L and N_D are the number of live and dead organisms, respectively. Both N_L and N_T were simultaneously measured at the time of antibiotic removal; N_D could therefore be determined. Because dead organisms lose the ability to multiply, N_D should remain unchanged in the samples collected thereafter. Hence, N_L in these samples could be estimated as $N_T - N_D$. Data on estimated N_L were compared directly with the viable colony counts determined simultaneously at each sampling time by the pour plate method.

The following settings of the Coulter counter were used: aperture current of 800 μA, gain of 2, and an orifice diameter of 30 μm. To reduce assay interference, both MHB-S and electrolytes were rendered particle free by filtering them through a 0.22-μm-pore-size filter prior to use. To correct for the possible difference in counting efficiencies between the two methods, a standard curve was constructed by plotting log cell counts (Coulter counter) against log colony counts (pour plate method) for the individual microorganisms during logarithmic growth. Simple nonweighted linear regression was used to test for correlation ($\alpha = 0.05$). The N_T measurements generated by the Coulter counter were subsequently converted to colony counts via these standard curves.

The MIC of dicloxacillin for *S. aureus*, the MIC of tetracy-

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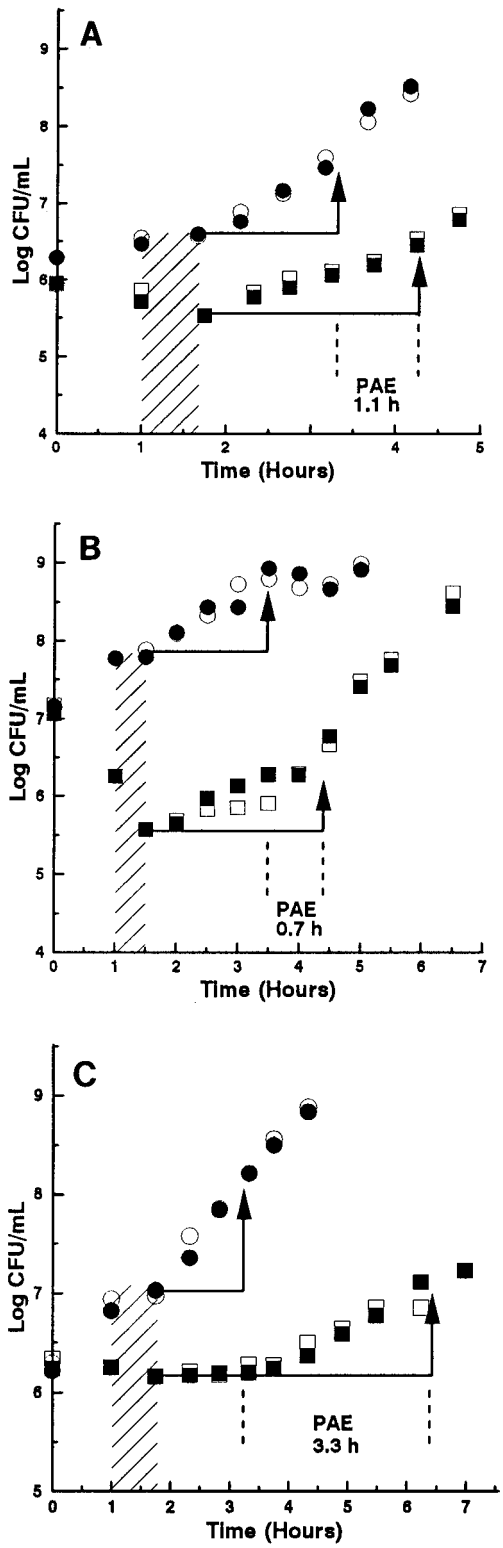

FIG. 1. Colony count-versus-time plots obtained by the TCC method (open symbols) and the pour plate and colony counting method (closed symbols). (A) Dicloxacillin against *S. aureus*; (B) tetracycline against *E. coli*; (C) erythromycin against *S. faecalis*. Antibiotic-treated and control cultures are represented by squares and circles, respectively. The shaded area describes the time required for the cell washing procedures.

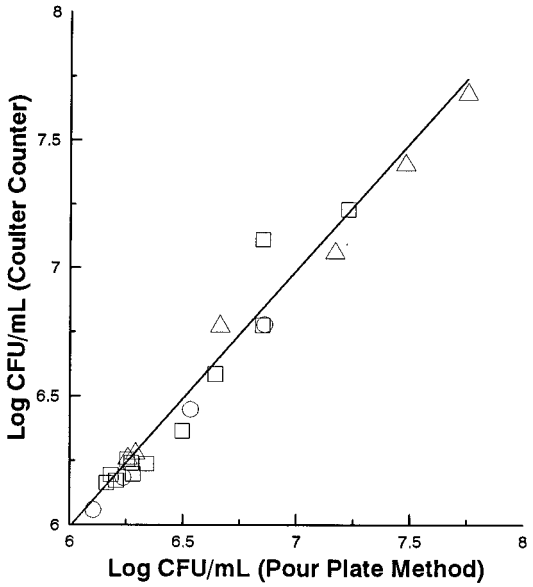

FIG. 2. Correlation of colony counts simultaneously obtained by the two methods for the three antibiotic-treated cultures: *S. aureus* (○), *E. coli* (△), and *S. faecalis* (□). Compared with the line of identity (slope = 1), the slope of the regression line is 0.999 ($r = 0.974$; $P < 0.005$).

cline for *E. coli*, and the MIC of erythromycin for *S. faecalis* were measured to be 0.2, 2, and 2 μg/ml, respectively.

The standard curves showed an excellent correlation ($P < 0.01$) between the two methods for the three microorganisms tested. Prior to the present study, it was not known if the shapes and morphologies of the microorganisms might influence cell counting by the Coulter counter. This was the primary reason for the inclusion of microorganisms with three distinct morphologies. The standard curves showed minimal deviations between the various bacterial species, suggesting that shape and morphology do not play a significant role in TCC. Nevertheless, all three standard curves demonstrated slopes slightly less than unity (0.944 to 0.967) with small positive intercepts (0.421 to 0.430), suggesting that the efficiency of counting is somewhat higher by the TCC method than by the pour plate method.

When compared with the respective controls, all three antibiotic-bacterium combinations demonstrated various degrees of PAEs (Fig. 1). The PAEs and the colony count data collected throughout the experiments by the two methods were essentially superimposable. When regressing all of the colony count data collected, excellent agreement between the two methods was observed (Fig. 2). The utility and practicality of the TCC method were thus verified.

Use of the total cell count data obtained with the Coulter counter has been extensive (2–4, 6). However, the lack of selectivity between live and dead organisms has remained a major concern. Furthermore, the raw cell counts measured with the Coulter counter should not be treated as direct substitutes for colony counts without accounting for the slight difference in counting efficiencies between the two methods.

The rapid generation of usable count data by the TCC method is a major advantage over the pour plate method. This allows continuous assessments of and feedback about an ongoing experiment. Any mistakes or errors can easily be detected during the experiment. The amount of time and effort spent on problematic and unsuccessful experiments can thus be further reduced.

A number of other methods for assessments of PAEs have previously been proposed. These include CO_2 production (5), ATP (7), and optical density (10) measurements. Nevertheless, any additional sample treatment and experimental steps can contribute to added measurement errors, experiment time, and cost. A brief report has previously described the use of a 1-log-unit increase in the total particle count as an endpoint measurement for PAE (8); however, this endpoint is dependent on the numbers of both live and dead organisms in the culture at the time of antibiotic removal. With the correction of the number of dead cells in the culture, operation of the current method is simple, direct, and efficient. Data from the present limited study provide the basis for the TCC method; however, its utility should be established for other antibiotic-bacterium combinations.

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