New Turbidimetric Assay for Quantitation of Viable Bacterial Densities

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A turbidimetric assay was developed and validated against Escherichia coli for the quantitation of viable bacterial densities. The Abbott MS-2 research system was employed for continuous 5-min measurements of optical density. A linear standard curve was obtained by regressing the initial bacterial density (log CFU per milliliter) against the time required for bacterial growth causing a 5% decrease in optical transmittance. Slope and intercept values obtained from eight standard curves showed excellent assay reproducibility. Results obtained by the turbidimetric assay compared favorably to those obtained by the conventional pour plate assay. Prior to the application of the new assay, possible interferences of postantibiotic effect induced by the test antibiotics were excluded. The turbidimetric assay, which is presumably more efficient and less expensive, was implemented for the time-kill studies of three different β-lactam antibiotics against E. coli.

The MIC is the most commonly used measurement to quantitate the antimicrobial effect of antibiotics. However, this endpoint provides little information on the time-dependent changes of a bacterial population during incubation with the antibiotic. Time-kill studies, which rely solely on the pour plate and colony-counting assay, remain the only means to examine these time-related changes. Automated systems such as the spiral plater (6) have been available to facilitate dilutions; however, this system is expensive and uses a concept similar to that of the pour plate assay. Considering the need for a more efficient and less expensive assay, we sought to develop a turbidimetric assay for the quantitation of viable bacterial densities. The new assay was applied to the time-kill studies of three different β-lactam antibiotics against Escherichia coli.

E. coli ATCC 25922 (Difco Laboratories, Detroit, Mich.) was maintained on nutrient agar slants at 4°C before use. Mueller-Hinton broth (BBL, Cockeysville, Md.) supplemented with cations (50 mg of Ca2+ per liter and 25 mg of Mg2+ per liter) (MHB-S) and nutrient agar (BBL) were used. Amoxicillin and penicillin G were purchased from Sigma Chemical Co., St. Louis, Mo. Amdinocillin (mecillinam) was a gift from Hoffmann-La Roche Inc., Nutley, N.J. The MICs of the three antibiotics against E. coli were determined by the macrodilution method (9).

The Abbott MS-2 research system (MS-2) (Abbott Diagnostics Division, Irving, Tex.) was used for continuous optical density (OD) measurements at 5-min intervals. This system can independently process 16 cartridges (each with 11 growth chambers) simultaneously. The growth chambers are maintained at 35°C with constant agitation between OD measurements.

A suspension of E. coli was prepared from an overnight plate. The culture was allowed to grow at 35°C to attain a logarithmic growth phase and was adjusted visually to the density equal to a 0.5 MacFarland standard by diluting with MHB-S. A volume of 2.5 ml of the adjusted bacterial culture was introduced into the first chamber of the MS-2 cartridge. The initial inoculum size of the adjusted culture was determined by the pour plate assay. Chambers 2 through 11 were each filled with 2.0 ml of sterile MHB-S. To achieve a series of 1 to 5 dilutions for chambers 2 through 10, 0.5 ml of the bacterial culture from the preceding chamber was transferred and mixed with the MHB-S in the next chamber. When this procedure was completed, the final volume in each chamber was 2.0 ml. Chamber 11 served as a negative growth control. When the fully loaded cartridge was inserted into the MS-2, incubation at 35°C and 5-min OD measurements were started. The time required for a 5% decrease in transmittance (T5%) was recorded for each chamber. To obtain a linear standard curve, the initial log10 CFU per milliliter values for each chamber determined from the initial inoculum of the adjusted culture and the dilution factor were plotted against the corresponding T5% values (see Appendix). Orthogonal linear least-squares regression was used to determine the best fit line through the points. Bacterial density of any test sample was obtained by interpolating its T5% value from the regression equation. From the regression equation, the instantaneous growth rate constant (K) of the test organism was calculated as the absolute value of the regression slope multiplied by 2.303. The x-intercept theoretically represents the time necessary for a single organism to multiply and produce an inoculum causing a 5% decrease in transmittance. The y-intercept approximates the assay threshold, i.e., the bacterial density required for the MS-2 to detect a 5% decrease in transmittance.

Prior to the use of the turbidimetric assay for time-kill studies with the three β-lactam antibiotics, possible interferences of postantibiotic effect (PAE) were evaluated by the procedures described by Craig and Gudmundsson (2) at 4× MIC.

The turbidimetric assay was compared with the pour plate assay (reference assay) to determine its accuracy. Thirteen non-antibiotic-treated bacterial cultures of unknown initial colony counts were assayed by both methods. In addition, to validate the use of the turbidimetric assay in antibiotic-treated culture, time-kill data at 0.04 and 0.08 μg of ampicillin.
nocillin per ml obtained over a 6- or 10-h period by both turbidimetric assay and pour plate assays were compared. In both cases, orthogonal least-squares regression was applied to analyze the colony count data for correlation. The slope of the regression line was compared with the line of identity (slope = 1) by using a t test (α = 0.05).

The pour plate assay involved the dilution of 0.1 ml of bacterial sample with 9.9 ml of sterile saline. Additional dilutions were performed depending on the anticipated bacterial density. One milliliter of the diluted sample was mixed with 10 ml of molten agar at 45°C and was poured onto a culture plate. After an overnight incubation at 37°C, the number of CFU on each plate was counted. Data were recorded for plates containing 20 to 200 CFU.

To demonstrate the applicability of the turbidimetric assay, time-kill studies were performed at 0.5× MIC of the three β-lactam antibiotics with an initial inoculum of ~10⁶ CFU/ml and a total volume of 10 ml. At 1-h intervals, 0.1 ml of sample was withdrawn and immediately subjected to a 10-fold dilution in MHB-S. Then 0.1 ml of the diluted sample was promptly added to 1.9 ml of MHB-S in the growth chamber and was submitted to 5-min OD measurements. With the two dilution steps, the antibiotic activity was effectively negated by the resulting 200-fold dilution. By adopting other dilution schemes, higher orders of dilution could also be attained. The T₅₀% value recorded for each sample was subsequently converted into colony count measurements by using the standard curve with correction for the dilution factor.

The MICs were measured to be 0.16, 8, and 50 μg/ml for amoxicillin, amoxillin, and penicillin G, respectively.

A representative standard curve of log₁₀ CFU per milliliter versus T₅₀% is shown in Fig. 1; T₅₀% values decreased linearly with increasing log₁₀ CFU per milliliter (r = 0.999). The K values from eight standard curves performed on different days ranged from 1.84 to 1.98 h with a mean (± standard deviation) of 1.91 ± 0.04 h⁻¹, i.e., an average doubling time of 21.75 min. This value compares favorably to the value for E. coli in the literature (6, 7). The theoretical threshold to detect a 5% decrease in transmittance (y-intercept) for E. coli was estimated to be 10(6.76 ± 0.03) CFU/ml. The average time for a single growing E. coli bacterium to multiply and to reach the required density causing a 5% decrease in transmittance was 8.13 ± 0.16 h (x-intercept). These three parameters were highly reproducible with coefficients of variation of ≤2.1%.

The excellent agreement in the bacterial counts determined by the reference and the new turbidimetric assay in either non-antibiotic- or antibiotic-treated cultures verifies the accuracy of the turbidimetric assay. In both cases, a significant correlation (P < 0.01) was obtained between the two assays. The slope of the regression line obtained for the non-antibiotic-treated cultures was not different from the slope of 1 when evaluated statistically (P > 0.05). Similarly, the slope of the regression line for the amoxicillin-treated cultures was not statistically different from 1 (P > 0.05) (Fig. 2).

The apparent lack of PAE for the three β-lactams tested against E. coli at 4× MIC was confirmed by the results of the present study (Fig. 3), in good agreement with previous study results that penicillins and cephalosporins, in general, do not or only minimally elicit a PAE against gram-negative bacteria (1, 2, 5, 8). The turbidimetric assay performs optimally for antimicrobial agents which do not exhibit a PAE. However, the assay can tolerate the existence of a short PAE without compromising the accuracy of the assay. In time-kill studies, the usual bacterial density after sample dilution ranges between 10⁶ and 10⁷ CFU/ml. Over this range
of density and using a hypothetical 15-min PAE, i.e., a 15-min delay in $T_{90x}$, as an example, the regression analysis shown in Fig. 1 produces only a 4 to 10% error in the estimated bacterial count. The magnitude of this error, if it exists, compares favorably to that caused by the conventional pour plate technique.

When PAE following antibiotic exposure is assessed, dilution has been extensively used as a means to negate antibiotic activity (1, 3, 5, 8). In these studies and among other procedures, a 1:100 to 1:1,000 dilution scheme has been adopted as a standard procedure to negate antibiotic activity at 5× to 10× MIC, including that of β-lactams. For example, if a dilution factor of 1,000 was acceptable in these PAE studies for antibiotic removal at 10× MIC (final concentration after dilution was 0.01× MIC), the 200-fold dilution scheme employed in the present time-kill studies at 0.5× MIC (final concentration was 0.0025× MIC) should provide more than adequate dilution. However, as the dilution requirement gets large at higher antibiotic concentrations, the sensitivity of the assay is reduced. For a 200-fold dilution scheme, the initial colony count must be $\geq 10^3$ CFU/ml in order to obtain accurate counts. In this respect, the sensitivity is similar to that obtained by the pour plate assay. Nevertheless, for β-lactams with the ring readily hydrolyzed by β-lactamase, a 1:10 dilution along with the addition of the stable enzyme may be useful to enhance sensitivity of either assay by reducing the extent of sample dilution required.

Time-kill curves for individual antibiotics at 0.5× MIC are shown in Fig. 4. A lag phase, a killing phase, and a regrowth phase were typical for time-kill curves of amoxicillin, penicillin G, and amdinocillin. Several investigators had attempted to use continuous OD measurements to study the time-dependent antimicrobial activity by mixing the test organisms with the antibiotic (4, 10, 11). By doing so, optical interference by the continuous formation of dead cells and morphological changes of viable cells obscures any relationship between viable cell count and OD measurement. Such a problem is circumvented by the sample dilution prior to OD measurements. This dilution step significantly removes any optical interference and permits normal bacterial growth of the remaining viable cells. In conclusion, two requirements must be met prior to utilization of the turbidimetric assay. First, the sample dilution must be sufficient to effectively eliminate antimicrobial activity or the antibiotic activity must be removed by some other methods. Second, there must be no or only a minimal PAE. When the two requirements are met, the turbidimetric assay described in this paper can substitute for the laborious conventional pour plate assay.

As suggested by the present data on β-lactams against E. coli, the turbidimetric assay appears to be a more efficient and less laborious alternative for the pour plate assay when performing time-kill studies. The conversion of OD measurements to colony count data requires the assumption that visible light absorption by the bacterial culture can be described by Beer's law (see Appendix); therefore, the application of the new turbidimetric assay to other antibiotic-bacterium combinations will require further testing. This is particularly relevant for bacterial species that grow in clusters or chains.
APPENDIX

Assuming that Beer's law applies to the absorption of visible light (605 nm) by the bacterial culture, the following equation can be used to describe the relationship between light absorption and bacterial density:

\[ \text{Abs} = a \cdot b \cdot N \]  

(1)

where Abs is absorbance, \( a \) is the absorption coefficient of bacteria in media, \( b \) is the length of the path of light, and \( N \) is the bacterial density in CFU per milliliter.

In addition, absorbance can be defined in terms of transmittance as

\[ \text{Abs} = -\log_{10} \text{transmittance} \]  

(2)

When transmittance decreases by 5% (\( tr_{5\%} \)) from its original value because of the exponentially increasing bacterial density from its initial value \( (N_0) \) at a growth rate \( (K) \) over a period of \( T_{5\%} \), equations 1 and 2 can be combined to yield

\[ -\log_{10} tr_{5\%} = a \cdot b \cdot (N_0 \cdot e^{K \cdot T_{5\%}}) \]  

(3)

After dividing both sides of equation 3 by \( a \cdot b \), the equation can be rewritten as

\[ \frac{-\log_{10} tr_{5\%}}{a \cdot b} = N_0 \cdot e^{K \cdot T_{5\%}} \]  

(4)

By taking logarithms on both sides of equation 4, we can show that

\[ \log_{10} \left( \frac{-\log_{10} tr_{5\%}}{a \cdot b} \right) = \log_{10} N_0 + \left( \frac{K}{2.303} \cdot T_{5\%} \right) \]  

(5)

After rearrangement of equation 5, the final equation (equation 6) takes the form of \( y = -mx + b \) (equation for a straight line, where \( m \) is the slope, \( x \) is the independent variable, and \( b \) is the intercept)

\[ \log_{10} N_0 = \left( \frac{K}{2.303} \cdot T_{5\%} \right) + \log_{10} \left[ \frac{-\log_{10} tr_{5\%}}{a \cdot b} \right] \]  

(6)

REFERENCES