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Mathematical Model for Comparison of Time-Killing Curves

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The relevance of mathematical modeling to investigations of the bactericidal effects of antimicrobial agents has been emphasized in many studies of killing kinetics. We propose here a descriptive model of general use, with four parameters which account for the lag phase, the initial number of bacteria, and the limit of effectiveness and bactericidal rate of antimicrobial agents. The model has been applied to several kinetic datum sets with amoxicillin, cephalothin, nalidixic acid, pefloxacin, and ofloxacin against two *Escherichia coli* strains. It is a useful tool to compare killing curves by taking into account model parameter confidence limits. This can be illustrated by studying drug effects, strain effects, and concentration effects. For the antibiotics used here, concentration effects had an influence mainly on the length of the lag phase and the minimum number of living cells observed. It is therefore clear that differences in the killing curves with changes in one or more parameters could occur.

The time-killing-curve method has been used in many studies, since bacterial death is evaluated with more information by this method than by end-point methods. When several antibiotics with several antibiotic concentrations are studied by this method, the number of curves to compare increases, leading to difficulty in adequately managing the results. As a means of data analysis, mathematical modeling is anticipated to be a useful tool to describe and compare kinetics, leading to a quantitative appraisal of bactericidal effects. The early stage of bacterial death induced by several means is said to be exponential in many cases (6, 7). However, in many cases, there are deviations from the exponential function (5). For instance, when bacteria have been exposed to quinolones, the decreasing phase of the killing curve corresponds to a biexponential function (4). In this case, the absence of mortality observed at the beginning of the killing kinetics is not taken into account. More-complex functions have been used to describe the bactericidal activities of β-lactams, but they are too antibiotic specific (9, 10). Until the present, there has been no standard mathematical model encompassing the initial growth plateau and the bactericidal phase including the so-called tail of the killing curves. In this paper, we propose a general descriptive mathematical model that fits every bacterial kinetics curve with a tail, whatever the antimicrobial agent used. A quantitative comparison of the phenomena has thus become possible, as has objective analysis utilizing the parameter confidence limits determination. The advantages of this new model are illustrated by comparing the bactericidal effects of two β-lactams and three quinolones of major medical interest, at various concentrations, against two *Escherichia coli* strains.

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MATERIALS AND METHODS

Bacterial strains. *E. coli* KL16 (4) and *E. coli* ATCC 25922, the reference strain for the quality control of antibiogram susceptibility, were used throughout the study.

Antimicrobial agents. The following antibiotics were used: amoxicillin (Beecham Pharmaceuticals, Paris, France), cephalothin (Glaxo Laboratories, Paris, France), nalidixic acid (Winthrop Laboratories, Clichy, France), pefloxacin (Roger Bellon Laboratories, Neuilly-sur-Seine, France), and ofloxacin (Roussel Uclaf Laboratories, Paris, France). These antimicrobial agents are prescribed in many cases of *E. coli* urinary tract infections.

Determination of MICs. MICs were determined by a standard macrodilution test (1) in Mueller-Hinton (Ca^{2+} at 25 mg/liter and Mg^{2+} at 12.5 mg/liter) broth (Diagnostics Pasteur, Paris, France). The inoculum was 10^6 bacteria per ml. The same batch of broth was used throughout the study.

Killing curves. Each strain was grown overnight in Mueller-Hinton broth. The initial number of cells was adjusted to 10^5 bacteria per ml with a nephelometer (ATB 1510; bioMerieux, Marcy l'Etoile, France). Preheated Mueller-Hinton broth (35°C) was used for all dilutions. This suspension was used to perform five separate assays in 50-ml borosilicate glass flasks. Each assay mixture contained 19.9 ml of bacterial suspension and 0.1 ml of working antibiotic solution. The final concentrations of each antibiotic were selected on the basis of the particular MIC for each test strain and were multiples of the MIC (1× MIC, 2× MIC, 3× MIC, and 5× MIC). A flask without antibiotic was used as a control. The flasks were incubated aerobically at 35°C with constant agitation (incubator shaker; New Brunswick Scientific [100 rpm]). Sampling for colony counts was performed every 30 min between 0 and 6 h. The flasks were immediately reincubated following each sampling procedure.

The killing curves were obtained by plotting the decimal logarithm of CFU per milliliter against time.

Carryover of antimicrobial agents. The absence of carryover in killing curves was verified with amoxicillin (5× MIC), cephalothin (5× MIC), nalidixic acid (5× MIC),

pefloxacin (5× MIC), and ofloxacin (5× MIC) against both *E. coli* strains as follows. A 1-ml volume of the antibiotic solution was passed through a 47-mm-diameter (0.45-μm-pore-size) filter membrane (Sartorius, Palaiseau, France), and then an inoculum of about 100 bacteria per ml was passed through. A control without antimicrobial agents was prepared in the same manner. The membranes were then immediately washed twice with 100 ml of sterile aqueous Tween 80 (0.02% [vol/vol]) and soya peptone (0.1%) (bio-Merieux), aseptically transferred onto Iso-Sensitest agar, and incubated at 35°C in an air incubator. The numbers of CFU were determined after 24 h. Carryover was studied with 12 replicates of each antibiotic. Growth in the antibiotic-free controls was compared with that in assay mixtures containing antibiotics by means of a nonparametric statistical procedure (Mann-Whitney U test; level of significance, α was fixed to 5%). When no significant difference between controls and tests was observed, the absence of significant antimicrobial-agent carryover was established.

Bacterial counts. Colony counts were determined by removing 10 and 100 μl and 1 ml of the broth at specified times. Samples were passed through 47-mm-diameter (0.45-μm-pore-size) filter membranes (Sartorius), or a 0.4-ml volume of broth was first diluted in borosilicate glass test tubes containing 3.6 ml of sterile aqueous Tween 80 (0.02% [vol/vol]), after which 10 μl of test dilution was filtered. The membranes were washed twice with 100 ml of sterile aqueous Tween 80 (0.02% [vol/vol]) and soya peptone (1%), transferred onto Iso-Sensitest agar, and incubated at 35°C for 24 h. They were examined with a magnifying lens to count the colonies which had been colored by spraying with triphenyltetrazolium (0.5%). Results were calculated from filters with counts between 20 and 300 CFU when the original sample was less than 1 ml, or with counts between 0 and 300 CFU when the original sample was 1 ml.

Mathematical analysis. Mathematical modeling was carried out with log-transformed data (when the log transformation was possible, i.e., with killing curves for which CFU per milliliter was not null). Since CFU tended to decline over several decimal logarithms, use of the log-transformed data served to stabilize the variance of the errors on bacterial counts.

The parameters of the mathematical function were estimated by nonlinear regression. The ordinary least-squares criterion was used to fit the model to the data. The minimum of the sum of the squared residuals between the observed and theoretical points was computed with a program written in Mathematica language using the subroutine FindMinimum (Mathematica; Wolfram Research, Inc., Champaign, Ill.).

The confidence limits of parameter values ($1 - \alpha$; α is the level of significance and is fixed to 5%) were defined according to the method described by Beale (3) and were determined with a previously described program (8).

To study the variations of antimicrobial effects observed among antibiotics, antibiotic concentrations, and bacterial strains, parameter values were statistically analyzed by the following tests: (i) the equality between two estimated parameter values was rejected (i.e., two estimated parameter values were regarded as significantly different) when their confidence intervals ($1 - \alpha$) did not overlap or (ii) the equality between an estimated parameter value and a theoretical and fixed value was rejected (i.e., an estimated parameter value was significantly different from a theoretical value) when the theoretical value did not belong to the confidence interval ($1 - \alpha$) of the estimated parameter value.

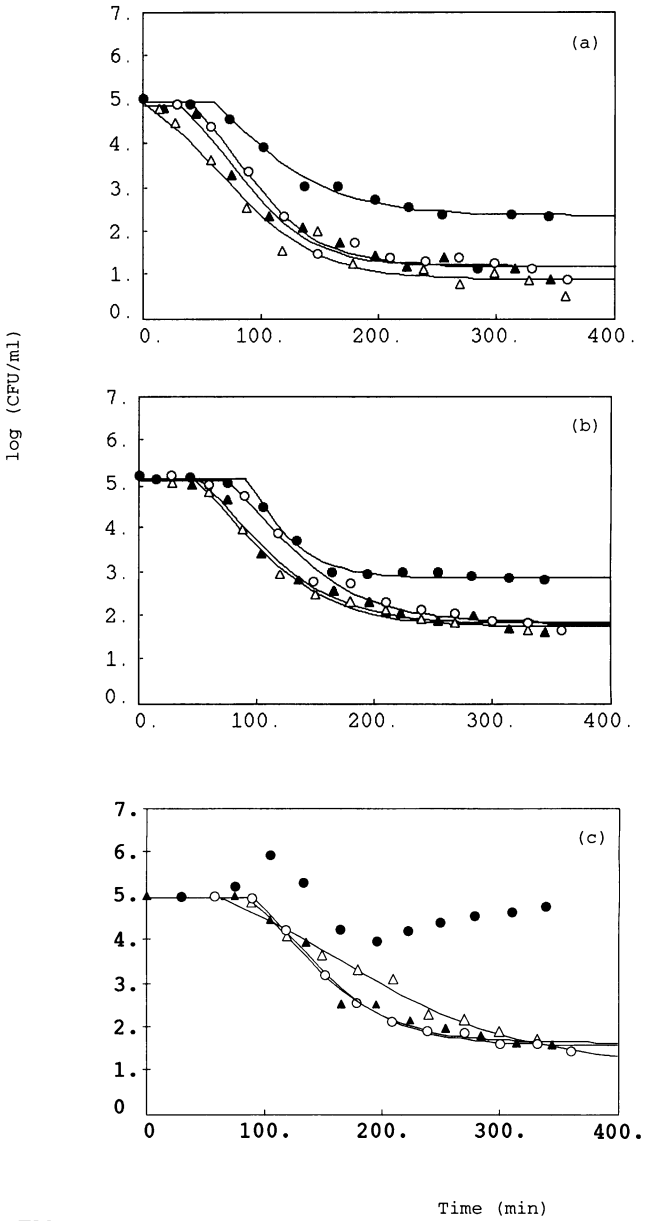

FIG. 1. Survival of *E. coli* KL16 in broth containing antibiotics. Bacterial concentrations are plotted against time, together with the theoretical fitted curves (solid lines). ●, 1× MIC; ○, 2× MIC; ▲, 3× MIC; △, 5× MIC. (a) Ofloxacin; (b) amoxicillin; (c) nalidixic acid.

RESULTS

MICs. The MICs for *E. coli* ATCC 25922 exposed to nalidixic acid, pefloxacin, ofloxacin, amoxicillin, and cephalothin were 2, 0.06, 0.06, 4, and 16 μg/ml, respectively. The MICs were 4, 0.06, 0.06, 4, and 8 μg/ml, respectively, in the case of *E. coli* KL16.

Killing curves. A decrease in the bacterial population was always observed at all antibiotic concentrations. Occasionally, regrowth was observed after 150 min for the lowest concentrations of nalidixic acid and pefloxacin. The shapes of the killing curves when bacterial strains were exposed to β-lactams or to quinolones were very similar: a plateau (or

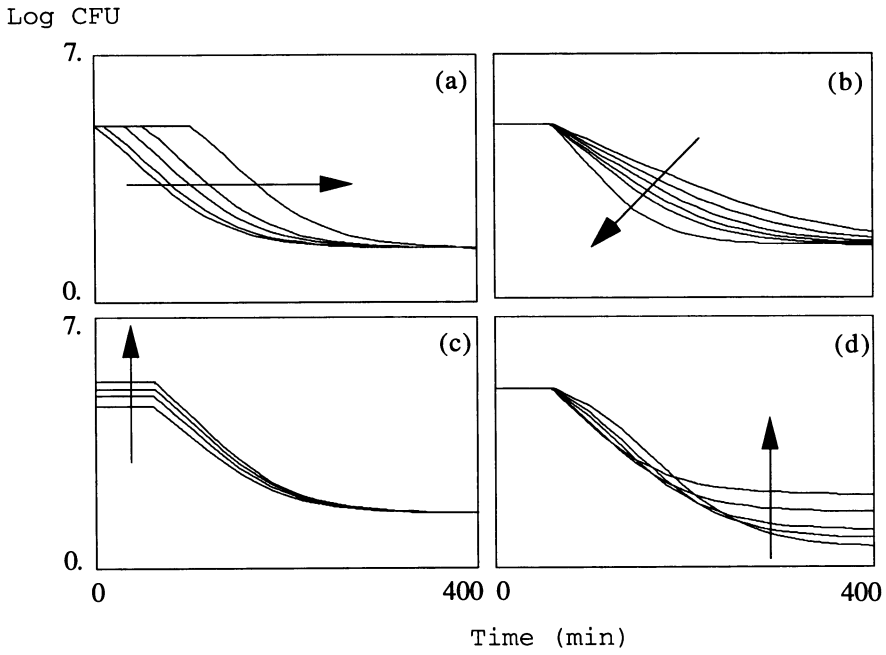

FIG. 2. Simulations of the mathematical model. The decimal logarithms of bacterial counts (log [CFU per milliliter]) are plotted against time (minutes). Parameters have been varied individually to illustrate their effect on the shape of the curve. Default parameter values were as follows: Δt, 60 min; α, 0.02/min; Y_O, 5.0; Y_m, 1.5. Arrows show the increasing values of the parameters. Values for Δt were 0, 10, 30, 50, and 100 min; values for α were 0.01, 0.012, 0.016, and 0.025/min; values for Y_O were 4.5, 4.8, 5.0, and 5.2; values for Y_m were 0.5, 0.75, 1.0, 1.5, and 2.0. (a) Δt; (b) α; (c) Y_O; (d) Y_m.

lag) sometimes preceded the decrease in the bacterial population, and a tail that indicated a marked deceleration in the rate of bacterial death was always observed. Figure 1 shows the killing curves of *E. coli* KL16 exposed to two of the antibiotics.

Mathematical modeling. The general form of the mathematical function used was obtained by letting $Y(t) = \log_{10} N(t)$,

$$\begin{cases} Y(t) = Y_O & \text{if } t \leq \Delta t \\ Y(t) = Y_O + Y_m - \left(\dfrac{Y_O\, Y_m\, e^{\alpha(t - \Delta t)}}{Y_O - Y_m + Y_m\, e^{\alpha(t - \Delta t)}} \right) \end{cases} \quad (1)$$

where t (minutes) is the time, $N(t)$ is the bacterial-population density (CFU per milliliter) at time t, and Δt (minutes), Y_O (dimensionless), Y_m (dimensionless), and α (1/min) are parameters of the mathematical model which have to be estimated by nonlinear regression. Δt is the time from which the decrease of the bacterial population was observed; Y_O is the decimal logarithm of the initial number of cells; Y_m is the decimal logarithm of the minimum observed number of living cells; α accounts for the intensity of the bactericidal rate. Figure 2 illustrates the influence of each parameter on the shape of the curve.

When the parameter Δt was 0, the model could be reduced to the following function:

$$Y(t) = Y_O + Y_m - \left(\frac{Y_O\, Y_m\, e^{\alpha t}}{Y_O - Y_m + Y_m\, e^{\alpha t}} \right) \quad (2)$$

The number of parameters of the partial model (equation 2) was then reduced to three. These two mathematical models (equations 1 and 2) are referred to as nested models (2). To decide which was the simplest nested model to fit a datum set adequately, we used a likelihood ratio test (2), described below. We let S denote the sum of squares and v denote the degrees of freedom, with subscripts f and p denoting the full model with four parameters (equation 1) and the partial model with three parameters (equation 2), respectively. On the other hand, we let $F(v_e, v_f; \alpha)$ denote the table value of the Fisher-Snedecor distribution, with v_e (the subscript e is for extra [$v_e = v_f - v_p = 1$]) and v_f ($v_f = 4$) denoting degrees of freedom, at the level of significance α (5%). If the ratio $(S_f - S_p)/(S_f/v_f)$ is lower than $F(v_e, v_f; \alpha)$, the partial model is sufficient (2) and can be used. The Δt parameter value is then fixed to 0.

The full or partial model fitted to killing curves for which no regrowth during the early phase of bacterial mortality was observed. The values of the parameters and their confidence intervals ($1 - \alpha$) are listed in Table 1. Figure 1 shows examples of the theoretical curves fitted to the log-transformed data.

Parameter analysis. The concentration-dependent bactericidal effect was defined by the statistically significant variation of one or more parameters of the model fitted to the bactericidal kinetics at various concentrations. A significant concentration effect on the parameter Δt (duration of the lag phase) occurred for *E. coli* ATCC 25922 exposed to amoxicillin and ofloxacin, whereas significant concentration effects on the parameter Y_m (the decimal logarithm of the minimum observed number of living cells) occurred for *E. coli* KL16 (Table 1). Only *E. coli* ATCC 25922 was susceptible to the variations of nalidixic acid concentrations which acted on Y_m. No relation between any parameter and pefloxacin and cephalothin concentration within the concentration range employed was observed.

The statistically significant variation of a parameter of the model fitted to the killing kinetics of various drugs for the

TABLE 1. Values of the parameters and their respective confidence intervals (95%) of the LDL model fitted to experimental data

Antimicrobial agent and MIC multiplication[a]	Value for *E. coli* ATCC 25922[b]				Value for *E. coli* KL16[b]			
	Δt	α	Y_O	Y_m	Δt	α	Y_O	Y_m
Ofloxacin								
1	74 (16, 100)	0.0172 (0.0093, 0.0366)	5.099 (4.755, 5.500)	2.212 (1.349, 2.673)	61 (0, 99)	0.0218 (0.0102, 0.0601)	4.937 (4.349, 5.566)	2.3214 (1.774, 2.710)
2	9 (0, 51)	0.0191 (0.0117, 0.0302)	5.121 (4.579, 6.469)	1.441 (0.996, 1.753)	40 (0, 70)	0.0291 (0.0184, 0.0527)	4.932 (4.456, 5.552)	1.199 (0.088, 1.477)
3	0 –[c]	0.0205 (0.0148, 0.0292)	5.029 (4.428, 5.658)	1.297 (0.863, 1.675)	30 (0, 57)	0.0283 (0.0156, 0.0558)	4.881 (4.343, 5.957)	1.178 (0.742, 1.520)
5	0 –[c]	0.0330 –[d]	4.970 –[d]	0.900 –[d]	0 –[c]	0.0238 (0.0174, 0.0325)	4.930 (4.179, 5.721)	0.879 (0.489, 1.263)
Pefloxacin								
1	ND	–[e]	–[e]	–[e]	–[e]	–[e]	–[e]	–[e]
2	64 (44, 80)	0.0272 (0.0198, 0.0395)	5.014 (4.796, 5.285)	1.807 (1.606, 1.984)	37 (0, 76)	0.0160 (0.0106, 0.0260)	5.135 (4.717, 5.689)	1.712 (1.185, 2.073)
3	43 (7, 71)	0.0186 (0.0132, 0.0330)	5.032 (4.723, 5.390)	1.620 (1.209, 1.949)	0 –[c]	0.0146 (0.0064, 0.0257)	5.016 (3.882, 6.801)	0.353 (0.025, 2.664)
5	23 (0, 47)	0.0163 (0.0113, 0.0219)	5.058 (4.697, 6.056)	1.687 (1.324, 1.950)	0 –[c]	0.0116 (0.0095, 0.0127)	5.023 (4.668, 5.397)	1.457 (0.668, 1.614)
Nalidixic acid								
1	–[e]	–[e]	–[e]	–[e]	–[e]	–[e]	–[e]	
2	83 (0, 120)	0.0200 (0.0064, 0.1076)	5.088 (4.645, 6.183)	2.2961 (1.979, 3.391)	91 (73, 110)	0.0236 (0.0185, 0.0333)	4.982 (4.768, 5.240)	1.600 (1.387, 1.833)
3	56 (0, 100)	0.0124 (0.0071, 0.0260)	5.160 (4.772, 5.885)	2.188 (0.990, 2.762)	87 (18, 130)	0.0233 (0.0126, 0.0826)	4.947 (4.342, 5.710)	1.621 (0.904, 2.093)
5	38 (0, 100)	0.0094 (0.0068, 0.0132)	5.093 (4.722, 5.950)	0.846 (0.206, 1.885)	63 (0, 110)	0.0127 (0.0087, 0.0187)	4.934 (4.384, 5.801)	1.098 (0.304, 1.765)
Amoxicillin								
1	59 (22, 94)	0.0174 (0.0120, 0.0300)	5.096 (4.814, 5.408)	1.804 (1.339, 2.124)	91 (66, 100)	0.0349 (0.0200, 0.0589)	5.118 (4.921, 5.314)	2.845 (2.652, 3.016)
2	37 (0, 58)	0.0197 (0.0138, 0.0275)	5.024 (4.713, 5.533)	1.573 (1.266, 1.819)	74 (29, 100)	0.0228 (0.0133, 0.0425)	5.093 (4.739, 5.510)	1.825 (1.337, 2.139)
3	19 (0, 41)	0.0198 (0.0114, 0.0276)	5.110 (4.791, 5.811)	1.544 (1.259, 1.790)	51 (12, 73)	0.0232 (0.0148, 0.0399)	5.118 (4.792, 5.539)	1.794 (1.409, 2.093)
5	0 –[c]	0.0199 (0.0182, 0.0217)	5.077 (4.920, 5.239)	1.129 (1.394, 1.614)	47 (10, 74)	0.0233 (0.0153, 0.0397)	5.102 (4.728, 5.558)	1.734 (1.427, 1.984)
Cephalothin								
1	61 (19, 90)	0.0173 (0.0103, 0.0277)	4.863 (4.570, 5.205)	1.860 (1.249, 2.196)	53 (27, 75)	0.0278 (0.0157, 0.0370)	5.132 (4.819, 5.458)	2.102 (1.840, 2.320)
2	26 (0, 57)	0.0166 (0.0117, 0.0251)	4.929 (4.544, 5.669)	1.538 (1.144, 1.842)	54 (30, 80)	0.0236 (0.0161, 0.0446)	5.179 (4.863, 5.506)	2.188 (1.945, 2.415)
3	31 (0, 56)	0.0210 (0.0141, 0.0310)	4.941 (4.612, 5.447)	1.521 (1.247, 1.776)	49 (26, 67)	0.0197 (0.0143, 0.0287)	5.153 (4.941, 5.413)	2.196 (1.929, 2.407)
5	18 (0, 55)	0.0223 (0.0108, 0.0484)	4.962 (4.246, 7.683)	1.425 (0.839, 1.816)	34 (0, 66)	0.0192 (0.0113, 0.0388)	5.183 (4.761, 5.734)	2.062 (1.567, 2.395)

[a] Numbers refer to the MIC (e.g., 5 = 5× MIC).
[b] Numbers in parentheses are 95% confidence intervals. ND, no data.
[c] When no lag phase, the model was reduced to the partial model without the parameter Δt.
[d] Confidence intervals could not be estimated according to Lobry's program procedure (8).
[e] The model parameters were not determined because of regrowth in the bacterial populations.

same concentration expressed in terms of the MIC defined the drug-dependent effect of that parameter. The parameter Y_m was drug dependent in the case of *E. coli* KL16 and was significantly higher for cephalothin than for ofloxacin at 2× MIC, 3× MIC, and 5× MIC; it was the same for ofloxacin and amoxicillin at 5× MIC.

DISCUSSION

The kinetics of antimicrobial activity are generally used to evaluate and compare new drugs and study differences and changes in the antimicrobial susceptibilities of clinically important bacterial isolates (1). The new model was empirically built as a tool to describe data without the aim of providing a mechanistic explanation; it is an exclusively descriptive model. We have chosen to include as mathematical parameters indices accounting for the experimentally observed effects of antimicrobial agents on the shapes of killing curves. In order to take into account the lag phase and the tail of each killing curve, two parameters in addition to the standard exponential model have been included. Δt, Y_O, and Y_m can be read directly when log CFU is plotted versus time.

This descriptive model fits the killing kinetics curve with a tail and with a possible lag phase, obtained with different antimicrobial agents so that killing curves are described in the same way, thus allowing them to be compared by using the estimated parameter values. Determination of the confidence intervals of the model enables statistical tests to emphasize significant differences among the parameters so that comparisons are objective.

In this model, significant variations of parameters demonstrate drug-dependent and concentration-dependent effects. It is clear that the antimicrobial effects observed at various drug concentrations or with different drugs may occur with parameters, especially the lag phase or the minimum observed number of living cells, other than the bactericidal rate. The modeling of only the decreasing phase by the exponential model leads to a loss of information, because the lag phase and the tail of the killing curves are not used. However, these last two parameters are important for an accurate evaluation of in vitro antibiotic action to differentiate antibiotic action adequately and to know the killing potencies of the antibiotics tested.

This new mathematical model could be a useful tool to study the bactericidal effects of new drugs, since it describes quantitatively the early antimicrobial activities of drugs for which a tail is characteristic. It adequately takes into account all phases of the killing process. Furthermore, it enables objective comparisons among drugs on a statistical basis. It would also be useful in antibiotic-combination studies to point out significant differences between the effects of the combinations and those of a single drug.

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