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Standard Guide for Determination of a Survival Curve for Antimicrobial Agents Against Selected Microorganisms and Calculation of a D-Value and Concentration Coefficient[1]

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INTRODUCTION

A variety of testing procedures have been devised almost from the beginning of disinfection and antisepsis as disciplines. From the first, there was recognition of the importance of time and rates of kill. After many decades and numerous test procedures involving carriers, the approach of establishing a death rate curve (often described as a survivor curve) is reclaiming its importance in establishing the basic kinetics of the killing process after exposure to antimicrobial chemicals.

D-values (historically, log death time or decimal reduction time), kill or survivor curves, processing calculations and rates of kill are discussed in many texts. There is extensive theoretical discussion but little applied material on how to perform testing to establish kill curves and D-values and associated calculations.

The guideline form has been selected to permit the inclusion of background information and a model procedure for determining D-values and their calculation. A related function, the concentration coefficient (η) can be calculated from a series of D-values calculated for different concentrations of the test antimicrobial and defines the loss of activity as the material is diluted. This information has value for application in disinfectants because many are sold to be diluted in use.

Specific procedural details are presented in descriptions of methods routinely used to establish a kill curve. The user should establish a protocol for the process that best fits their needs.

An experimental kill curve provides data for a calculated D-value derived from test data used to construct the kill curve.

BACKGROUND

Scientists concerned about antimicrobial testing have debated the value of suspension tests in contrast to tests using simulant carriers with dried microorganisms. U.S. regulation has been committed to carrier tests, while Europeans have emphasized suspension tests combined with practical applied tests using materials as carriers on which the disinfectant actually will be used.

The examination of the kinetics of kill for various disinfectants provides basic information on the activity of antimicrobials. The early history of microbiology reveals a strong momentum directed toward clarification of these reactions. From the earliest years of microbiology, the ideas of rate-of-kill and killing reactions as first order reactions (from chemical kinetics) have been involved in the estimation of antimicrobial activity.

Kronig and Paul (1897) were the early pioneers who developed the concept of bacterial destruction as a process. They used anthrax spores dried on garnet crystals and assessed the survivors by plating washings from the garments after treatment with disinfectants. Chick (1908) found that the number of survivors after disinfectant exposure, when plotted against time of treatment, produced a straight line that showed similarity to chemical, equimolecular reactions. Distortions in the expected straight-line reactions were noted by Chick as well as in subsequent investigations. Over the years, the most common type of deviation from the expected, straight-line survivor curve is a sigmodial one displaying a shoulder, a lag or delay in logarithmic kill, and ending in distinct tailing, sometimes indicating a resistant population.

There has been a variety of procedures advanced for accumulating data that can be used to calculate D-values and construct survivor curves.

Esty and Meyer (1922) introduced the terminology we currently use in relation to bacterial kill

whether for spores or vegetative bacterial cells in devising thermal processing to eliminate *Clostidium botulinium* in the canning industry. They also devised end-point analysis for interpretation of the results of heat exposure and for processing calculations. Their procedure involved sampling multiple tubes or other containers of product and analysis of the number remaining positive to determine the number of survivors by Most Probable Number (MPN) analysis using the pattern of positive and negative tubes. (**1**)[2] This analysis is done after an exposure period when there are fewer bacterial cells or spores in the container and positive and negative tubes can be expected on recovery.

Single-sample subculturing of aliquot samples from a reaction vessel containing the test organism and the test antimicrobial has been the basic means for establishing survival curves. Usually a suspension of target microorganisms is exposed to a disinfectant\sterilant and aliquots are withdrawn at specific time intervals and assessed for survivors, usually with plate counts. Because of tailing problems and difficulty in enumerating small numbers, when only a few survivors are left, MPN methods of enumeration are recommended and often used (**1, 2, 3**). A common method derived from thermal processing in the canning industry is the end-point method, described above, in which the number of positive and negative tubes from replicate sampling (such as tubes or cans) is used alone or in the combination with single sampling to construct a survivor curve and plotted to determine D-values. (**4**)

Many antimicrobial formulations available for test are diluted in use. When D-values are determined and calculated at more than one concentration (dilution) of an antimicrobial, the concentration coefficient, designated as the Greek letter eta or η, denotes the effect of dilution on the activity of a chemical or formulation.

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[2] The boldface numbers given in parentheses refer to a list of references at the end of the text.

1. Scope

1.1 This guide covers the methods for determining the death rate kinetics expressed as D-values. These values can be derived from the construction of a kill curve (or survivor curve) or by using other procedures for determining the number of survivors after exposure to antimicrobial chemicals or formulations. Options for calculations will be presented as well as the method for calculation of a concentration coefficient.

1.1.1 The test methods are designed to evaluate antimicrobial agents in formulations to define a survivor curve and to subsequently calculate a D-value. The tests are designed to produce data and calculate values that provide basic information of the rate-of-kill of antimicrobial formulations tested against single, selected microorganisms. In addition, calculated D-values from survivor curves from exposure at different dilutions of antimicrobial can be used to show the effect of dilution by calculation of the concentration exponent, η (**2**). D-value determination assumes the ideal of first-order killing reactions that are reflected in a straight-line reduction in count where a count-versus-time plot is done. The goal here is not to determine the time at which no survivors are found, but to determine a standard value that can be used in processing and exposure determinations or used to estimate dilutions.

1.1.2 As an example of potential use of kill curve data, the published FDA, OTC Tentative Final Monograph for Health-Care Antiseptic Drug Products, Proposed Rule, June 17, 1994 has suggested the testing of topically applied antimicrobial products using survival curve (or kill curve) calculations. The methods described in this guide are applicable to these products, but adjustments such as the use of antifoaming agents when the reaction mixture is stirred may be necessary to counteract the presence of detergents in many formulations. Frequently the sampling for these tests is done after very short intervals of exposure to the formulation, such as 30 and 60 s. This methodology also has been applied to preservative testing of antimicrobial ingredients in more complex cosmetic formulations (**5**).

1.2 The test methods discussed should be performed only by those trained in microbiological techniques.

1.3 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Terminology

2.1 *Definitions:*

2.1.1 *D-value or decimal reduction time*—(often referred to as log death time) relates reaction kinetics and inactivation rate. It is defined as the time (usually in minutes) to reduce the microbiologic population one $\log_{10}$ or to reduce it to 90 % or reduce it to 10 % of the initial population.

2.1.2 *Fn = fraction negative (FN) data*—(quantal data) are experimental results in the form of a dichotomous response: the unit tested is either positive (showing growth) or negative (showing no growth).

2.1.3 *concentration exponent, η: (dilution coefficient)*—measures the effect of changes in concentration (or dilution) on cell death rate. To measure η, the time necessary to produce a comparable degree of death in a bacterial suspension for at least two different concentrations is measured (D-value) (**6**).

2.1.4 *most probable number (MPN)*—data in which a fraction of the replicate units are negative and can be analyzed statistically using the MPN technique to yield the probable number of survivors at the respective exposure time.

3. Summary of a Basic Test Method

3.1 This test method is conducted on selected microbial species cultured to produce high-count suspensions that are exposed to the test antimicrobial agent or formulation(s) under standardized conditions of temperature and agitation. Samples from this reaction mixture are withdrawn at pre-set times, neutralized and cultured to determine survivors, using standard procedures. A D-value is calculated from the post exposure survivor data utilizing published and accepted methods.

3.2 This test method involves testing a high count suspension of a microorganism as the initial challenge inoculum; at least 10^7 to 10^8 cfu/mL, to achieve a 10^6 cfu/mL when added to the reaction chamber and exposed to disinfectant and to sporicidal chemicals.

3.3 A growth medium for the inoculum must produce a high numbers of vegetative cells or spores within a reasonable time period with consistent resistance to chemical disinfectants.

3.4 Where possible agitation of the reaction chamber is recommended.

3.5 Currently a test temperature of 20 ± 1°C is recommended. This temperature is lower than most environmental temperatures in practice (room temperature). A more typical temperature range is suggested at 22 ± 1°C. The activity of many antimicrobials is increased with increasing temperature. An alternative temperature may be selected for testing, but must be controlled and constant.

3.6 An alternative testing technique to single sequential timed samples may be included in execution of this method because a major problem has occurred with many reported studies. Many kill or survival curves have shown a rapid kill of several logs after an exposure period expected to eliminate survivors, yet leaving a few survivors, usually ten or fewer ranging to 1000. This number fluctuates for an extended time with repeated sampling and has been termed, tailing. A change from single sampling to replicate - unit sampling is recommended as a means to alleviate this problem.

3.7 Repetition of the estimation of a survival curve is recommended. Recommendations for three to five replications with sampling at five time points have been made.

4. Significance and Use

4.1 The different procedures and methods are designed to be used to produce survival data after microorganisms are exposed to antimicrobial agents in order to calculate values that can be used to analyze and rationalize the effectiveness of antimicrobial agents when tested using other, often applied test methods.

4.2 The data from these test procedures may be used in the selection and design of other tests of effectiveness of antimicrobial agents, some of which may be required by regulatory agencies to establish specific claims. Basic kinetic information about killing rate often serves as the initial information on which a testing program can be built.

5. Basic Materials and Reagents

5.1 Some basic materials will be required regardless of the specific method selected. This list may need to be supplemented depending on the techniques selected.

5.1.1 *Colony Counter,* any of several types may be used.

5.1.2 *Membrane Filter Holders and Microbially Retentive Membranes,* (0.22 µm) with vacuum equipment for filtration.

5.1.3 *Incubator*—Any incubator capable of maintaining a temperature within a ±2°C of the recommended optimal temperature for the growth of a specific microorganism under test.

5.1.4 *A Glass Reaction Vessel,* of appropriate size and design to permit required sampling.

5.1.5 *A Realistic Means of Agitation,* such as a hot-plate with a magnetic stirring feature.

5.1.6 *Temperature Controlled Water Bath,* with agitation, when available.

5.1.7 *Sterilizer.*

5.1.8 *Spectrophotometer.*

5.1.9 *Timers*—An interval timer, such as a stop watch for determining elapsed time to remove test samples from the reaction chamber.

6. Additional Materials and Reagents

6.1 Depending on the specific method used, additions may have to be made to the materials and reagents tested.

6.1.1 *Petri Dishes,* 100 by 15 mm required for performing standard plate count.

NOTE 1—Presterilized\disposable plastic dishes are available from most local laboratory supply houses.

6.1.2 *Bacteriologic Pipets,* 10.0 and 2.2 or 1.1. mL capacity. Micropipet types may also be used.

NOTE 2—Presterilized\disposable bacteriological pipets are available from most local laboratory supply houses.

6.1.3 *Liquid Media,* appropriate for the test microorganism. A soybean casein digest agar[3] or equivalent may be used for culturing the test microorganism.

6.1.4 *Agar Plates,* (spread- or pour-plates) of appropriate, optimal media for the test microorganism for culture of test and control samples.

6.1.5 *Neutralizer Solution,* specific for each antimicrobial tested incorporated into diluent and optionally into recovery medium.

6.1.6 *Test Tubes,* with closures of appropriate size for samples and ten-fold dilution of samples.

6.1.7 *A Selection of Flasks and Tubes,* required for culturing of the test microorganisms.

[3] *United States Pharmacoepia XX;* United States Pharmacopeial Convention, Inc. Rockville, MD. Chapter: Microbial Limits Test.

6.1.8 *Diluent Tubes,* for dilution of the test and control samples. Diluent may have phosphate-buffered normal saline or other appropriate diluent for specific microorganisms and neutralizers specific for the test disinfectant should be added to the diluent.

6.1.9 *An Automatic Mixer,* such as a Vortex mixer.

7. Procedure

7.1 The procedures detailed in this guide represent an example of a method that can be used. Alterations will be required in the method if MPN replicate sample techniques are selected for the longer-time samples where low counts are expected. Membrane filtration may also be selected for recovery and counting of surviving organisms.

7.1.1 Prior to a test with a selected microorganism, adjust the culture (after growth for approximately 24 to 48 h at optional temperature) spectrophotometrically to contain between 10^8 and 10^9 cfr for 1 mL. This high count is required so that a small volume, 0.1 to 0.01, can be added to the test disinfectant solution to achieve count of 10^6 to 10^7 in the reaction chamber. Adjusting the inoculum to produce the count in 0.1 mL is recommended.

7.2 Agitate the suspension to produce a uniform suspension by shaking or use of a Vortex mixer.

7.3 *Preparation of Test Chemical or Formulation*—Dilute, or dissolve, or prepare the test material as directed. Attention to the diluent (sterile DI water, tap water, or hard water) is important. Normally for this test, sterile deionized, distilled or RO water is selected, but the test may be performed with other diluents.

7.3.1 Aliquote a specific volume of test material sufficient to permit the number of test samples specified for withdrawals, while still maintaining the necessary testing volume.

7.3.2 Add a required volume of standardized inoculum to produce an initial challenge count of 10^6 to 10^7 to the test material and the timer started.

7.3.3 *Estimation of Initial Challenge Count*—At the same time as the standardized inoculum is added to the test material in the reaction chamber, add to a dilution tube a volume of inoculum to achieve the same ratio used in the test. Count this same dilution of inoculum by dilution, plating, incubation, and enumeration.

7.3.4 At each pre-designed exposure sample time, a sample, usually 1 mL is removed from the reaction chamber, diluted, plated, incubated and enumerated. Withdraw a minimum of duplicate samples for enumeration. If MPN enumeration is selected, change the sampling procedure to the replicate sample method. The estimated number of survivors is expected to reach approximately 1000 cfu/mL or fewer.

7.3.5 The replicate sample procedure (MPN) requires at least ten, individual 1 mL samples added to ten individual tubes of appropriate neutralizer-recovery medium (10 to 20 tubes may be used). After incubation, record the number of positive and negative tubes. Analyze these results using the Most Probable Number Method (7) of analysis of data to product microbial counts when the number of survivors is expected to be small.

7.4 *Enumeration of Bacteria in Test and Control Samples:*

7.4.1 Enumerate samples by a standard plate count procedure using soybean - casein digest agar or another appropriate recovery agar for the specific test microorganism. Membrane filtration may be used to enumerate survivors, but it is not appropriate for all microorganisms. Incubate plates or membrane filters at the optimal temperature for the specific organism tested.

7.4.2 When ten inoculated tubes are used to estimate the microbial population at the tail-end of the curve (MPN), calculation procedures for this method are described in the Standard Methods of the Examination of Water and Waste Water (7).

8. Construction of a Survival Curve

8.1 The average cfu/mL of a sample, beginning with the initial inoculum count and ending with the last timed sample are plotted on the ordinate or vertical axis of semi - log graph paper (or the $\log_{10}$ of the average) and the time in minutes of the corresponding time sample on the abscissa or horizontal axis.

8.2 To calculate a D-value from the survivor information, select two values: the initial inoculum count, and the count of the timed sample where the survivor curve produces a straight line. Calculate the D-value using the following formula. The average counts must be converted to $\log_{10}$ prior to calculation to yield log reduction. The D-value is normally expressed in minutes, but seconds or even hours may be necessary (3).

9. D-Value Formula

9.1 Different calculations can be performed with the survivor curve information and with the replicate sample information as follows:

9.1.1 *Calculation:*

$$D = t/N_0 - N_u \quad (1)$$

where:

N_0 = $\log_{10}$ of initial microbial population,
N_u = $\log_{10}$ of microbial population surviving at time t, and
t = time of exposure.

t is the exposure time at a constant temperature.

9.2 The D-Value calculation can be made with survivor counts determined from plate counts, membrane filters or MPN calculations.

9.2.1 If the multiple replicate approach is used exclusively, the fraction negative method (FM) can be used to estimate survivor curves. In a fraction negative procedure, the fraction of the replicate units that are negative and positive are recorded and can be analyzed using the MPN technique to find the surviving microbial population (8).

9.3 When MPN techniques are used to yield the probable number of survivors at the respective exposure time, the MPN equation of Halvorsan and Ziegler is used (9):

$$N_u = ln(n/r) = 2.303 \log_{10}(n/r) \quad (2)$$

where:

n = number of units exposed,

r = number of units sterile, and
N_u = is the population per multiple replicate unit after exposure for time, u.

10. Concentration Coefficient

10.1 Calculation of the concentration exponent can be made if the D - value from several dilutions of the same chemical or formula is available (**2**). This determination measures the effect of dilution on the antimicrobial agent and is calculated from the formula as described:

$$\frac{(log\ death\ time\ at\ Conc\ C_2) = (log\ death\ time\ at\ Conc\ C_1)}{log\ C_1 - log\ C_2} = \eta\ (\text{concentration exponent}) \quad (3)$$

log death time = D-value

10.2 If the log of a death time (D-value) is plotted against the $\log_{10}$ of the concentration of the antimicrobial, a straight line is usually produced. The slope of the line is η or the concentration exponent.

11. Precision and Bias

11.1 A precision and bias statement can not be made for this test method at this time.

12. Statistical Treatment of Data

12.1 Five replications of the survival curve are recommended. The standard error of the mean for each time period should be calculated and plotted for each point. A regression analysis using least squares can be used to fit the line of the survival curve if it is a straight line plot. The D - values can be calculated as described.

13. Keywords

13.1 concentration coefficient; concentration exponent; D-value; death rate curve; decimal reduction time; fraction-negative (FN); log death time; most probable number (MPN); survivor curve

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