Journal of Microbiological Methods xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Journal of Microbiological Methods

journal homepage: www.elsevier.com/locate/jmicmeth

The *Integral Method*, a new approach to quantify bactericidal activity☆

Waldemar Gottardi [a], Jörg Pfleiderer [b], Markus Nagl [a,*]

[a] Department of Hygiene, Microbiology and Social Medicine, Division of Hygiene and Medical Microbiology, Medical University of Innsbruck, Schöpfstr. 41, A-6020 Innsbruck, Austria
[b] Institute of Astro- and Particle Physics, Leopold-Franzens University of Innsbruck, Technikerstr. 20, A-6020 Innsbruck, Austria

ARTICLE INFO

Article history:
Received 10 March 2015
Received in revised form 4 May 2015
Accepted 4 May 2015
Available online xxxx

Keywords:
Quantitative killing assay
Killing curve
Specific bactericidal activity
Antimicrobial agents
Antiseptic

ABSTRACT

The bactericidal activity (BA) of antimicrobial agents is generally derived from the results of killing assays. A reliable quantitative characterization and particularly a comparison of these substances, however, are impossible with this information. We here propose a new method that takes into account the course of the complete killing curve for assaying BA and that allows a clear-cut quantitative comparison of antimicrobial agents with only one number.

The new Integral Method, based on the reciprocal area below the killing curve, reliably calculates an average BA [$\log_{10}$ CFU/min] and, by implementation of the agent's concentration C, the average specific bactericidal activity SBA = BA / C [$\log_{10}$ CFU/min/mM].

Based on experimental killing data, the pertaining BA and SBA values of exemplary active halogen compounds were established, allowing quantitative assertions. *N*-chlorotaurine (NCT), chloramine T (CAT), monochloramine (NH_2Cl), and iodine (I_2) showed extremely diverging SBA values of 0.0020 ± 0.0005, 1.11 ± 0.15, 3.49 ± 0.22, and 291 ± 137 $\log_{10}$ CFU/min/mM, respectively, against *Staphylococcus aureus*. This immediately demonstrates an approximately 550-fold stronger activity of CAT, 1730-fold of NH_2Cl, and 150,000-fold of I_2 compared to NCT. The inferred quantitative assertions and conclusions prove the new method suitable for characterizing bactericidal activity. Its application comprises the effect of defined agents on various bacteria, the consequence of temperature shifts, the influence of varying drug structure, dose–effect relationships, ranking of isosteric agents, comparison of competing commercial antimicrobial formulations, and the effect of additives.

1. Introduction

The performance of killing of pathogens by antimicrobial chemicals is of considerable importance in medicine, because it indicates the usability of a given agent under conditions of practice. The results of such tests are presented by killing curves that demonstrate the surviving colony forming units (CFU) per ml in a suspension of test bacteria in the presence of a test agent after defined incubation times. The forms of killing curves substantially depend on the nature of the bacteria and their initial number as well as on the nature of the agent and its concentration. This implies that under standardized conditions, i.e., the same bacterial strain and initial log CFU, the specific activity of an agent can be determined. By *visual* examination of killing curves, therefore, a comparison of individual agents is possible, yielding *qualitative* information concerning their relative bactericidal activity (BA).

For the evaluation of disinfectants and antiseptics in human medicine, the European Union has issued several norms based on the quantitative suspension test (http://www.en-standard.eu/, e.g., EN 13727 and EN 1040 for bactericidal activity in the medical area European Norm (EN) 1040, 2005; European Norm (EN) 13727, 2012, EN 12791 for surgical hand disinfection European Norm (EN) 12791, 2005, EN 1276 and EN 13697 for bactericidal activity in food, industrial, domestic and institutional areas European Norm (EN) 1276, 2009; European Norm (EN) 13697, 2002). The results of such protocols demonstrate whether a product has passed the respective EN test requirements, mostly ≥5 log reduction within 5 min. However, these standards give only a punctual account without any information about the kinetics of the bactericidal process. As a general standard for approval of disinfectants, it appears to be sufficient.

However, for scientific characterization of microbicidal agents, particularly those that come directly in contact with human tissue under different conditions (antiseptics, antibiotics), a *quantitative* measure that would allow a more exact judgment of the microbicidal activity is of interest. Such a measure should take into account the course of killing curves, and it should be easily accessible from the curves.

Abbreviations: BA, bactericidal activity; CAT, chloramine T; CFU, colony forming units; DL, detection limit; DM-NCT, *N*-chloro-dimethyltaurine; NCT, *N*-chlorotaurine; SBA, specific bactericidal activity; t_{DL}, killing time; tg, tangent.

☆ This article is dedicated to Professor Manfred Rotter (Medical University of Vienna), the recognized expert in the assessment of disinfecting procedures, on the occasion of his 75th birthday.

* Corresponding author at: Department of Hygiene, Microbiology and Social Medicine, Division of Hygiene and Medical Microbiology, Medical University of Innsbruck, Schöpfstr. 41, 1st floor, A-6020 Innsbruck, Austria.
E-mail address: m.nagl@i-med.ac.at (M. Nagl).

http://dx.doi.org/10.1016/j.mimet.2015.05.002
0167-7012/

A method to gain such information could consist in determining the killing time t_{DL} necessary to reach the detection limit (DL) of CFU in quantitative killing assays. A DL of 1 $\log_{10}$ (2 $\log_{10}$), for instance, indicates that a count below 10 CFU/ml (100 CFU/ml) is not detectable. Generally, such DLs originate from small volumes plated (usually ≤ 0.1 ml) and from dilutions in solutions that inactivate the test agent. However, determination of t_{DL} is inaccurate and even impossible if DL is not reached at all, as it is the case in incomplete killing curves (see also Section 2.1).

Early investigations of the kinetics of disinfection revealed a first order reaction for the killing of living bacteria caused by heat or toxic agents (Chick, 1910). This approach indicates that within the same time intervals the same percentage of CFU will be destroyed. A semi-logarithmic graph, i.e., log of surviving CFU (ordinate) vs time (abscissa), yields a straight line that intersects the abscissa with an angle α, the tangent of which suggests itself as a possible *quantitative* measure for the average BA.

$$\text{tg } \alpha = -\text{d}(\log_{10} \text{ CFU})/dt = \text{BA} \left[\text{min}^{-1}\right]. \quad (1)$$

Instead of straight lines, such graphs in practice often show curved ones, with a high killing rate at the beginning, which gradually decreases towards the detection limit. This curvature preferably occurs with highly active agents (HOCl, active bromine compounds) (Gottardi et al., 2014), and can be explained by the depletion of the agent at the beginning of the killing process, and also by the enhanced tolerability of clumped bacteria or bacteria in a special state (e.g., small colony variants, persisters) (Glaser et al., 2014; Heras et al., 2014; Wood et al., 2013), which might be responsible for an overlong tail of the curve. As a consequence, BA (tg α) are not constant quantities, but can be specified by one characteristic number only as averaged values. The challenge was, therefore, to find a straight line that best approximates the bactericidal activity presented in the log CFU/time curve and whose slope (tg α) is a measure for BA of the tested sample. It is needless to say that the results achieved should confirm the ones of the visual comparison of killing curves.

In a study dealing with isosteric chlorine and bromine compounds (Gottardi et al., 2014), extremely differing killing times were observed, which suggested quantifying them numerically. For approximating the killing curve with a straight line, whose slope equals BA, the authors used the averaged log reductions and exposure times (see method #3, Appendix A). Because the concentrations used differed by a factor of up to 1000, presentation on the same graph and visual check of the killing curves were not always possible. By including the concentration C (in mM), the *specific bactericidal activity* (SBA) of the agent was obtained, which enabled a reliable comparison throughout.

$$\text{SBA} = \text{BA}/\text{C } [\log_{10} \text{ CFU}/\text{min}/\text{mM}]. \quad (2)$$

Using this parameter, results based on differing agent concentrations could be compared, where SBA values differing by a factor up to 40,000 (range 0.003 to 120 $\log_{10}$ CFU/min/mM) allowed a relative ranking of the investigated chlorine and bromine agents (Gottardi et al., 2014). In spite of this acceptable performance, the method exposed the shortcoming that in case of small differences in BA or SBA, a comparison of the calculated BA occasionally tended to disagree with the trend suggested by the curves. By removing the longer exposure times, slopes were obtained that finally concurred with the visual examination.

This is a serious flaw. Omitting regular measuring points to obtain, or even support, a certain result is a strong indication of an inapt data interpretation. It was, therefore, of interest to find a method that approximates the average slope of killing curves without these inadequacies. For this purpose, conceivable ways for finding a suitable approximation were investigated (see Appendix A: "Theoretic considerations") which finally led to the "Integral Method" as the best solution for assessing tg α.

2. Methods

2.1. One-number interpretation of killing curves (Integral Method)

Contrary to earlier methods to quantify bactericidal activity (BA) by one number we make use of not only the measurements (data points) of the killing curves but also of the detection limit (DL). It gives, by definition, the same result for any log CFU < DL, namely no detection at all. This approach allows to differentiate between relevant data (log CFU > DL) and irrelevant ones (log CFU ≤ DL), as well as to establish a relevance order by using a "killing relevance variable" (K) as the difference of the data (log CFU) to the irrelevance level (DL),

$$K = \log \text{CFU} - \text{DL}. \quad (3)$$

Under these auspices, a look on any killing curve suggests that the area between the killing curve and the detection limit, which is the integral over the killing curve with DL as abscissa level, or the integral over the relevance coordinate K, is a reciprocal measure of the average BA. It is directly apparent that the smaller the area, the faster killing occurs.

The straight line that represents the average BA by its corresponding tangent is the one, which, starting from the same first data point log CFU (t = 0), provides the same integral. Mathematical details are given in Appendix A, a calculation program (Excel file) in Appendix B.

It should be mentioned that the choice of the detection limit is not very critical. That is, our method works rather well even if DL is, for some reason, not securely established. Also, killing curves with not too large differences in log CFU (t = 0) can be directly compared. Nevertheless, a good experimental methodology is recommended.

2.1.1. Explanation of the Integral Method based on Fig. 1

By addition of the trapezoids 1–5 the area A of the killing curve down to the detection limit (DL = 2 $\log_{10}$ CFU) is found. A rectangle of equal area has the ordinate y = $\log_{10}$ CFU (t = 0) − DL, while the abscissa comes to x = A / y. The rectangle x × y is transformed into an orthogonal triangle with the same area. Its hypotenuse forms with the abscissa the angle α, whose tangent, tg α = y / 2x, represents the sought average BA.

A calculation program (excel file) is presented in Appendix B.

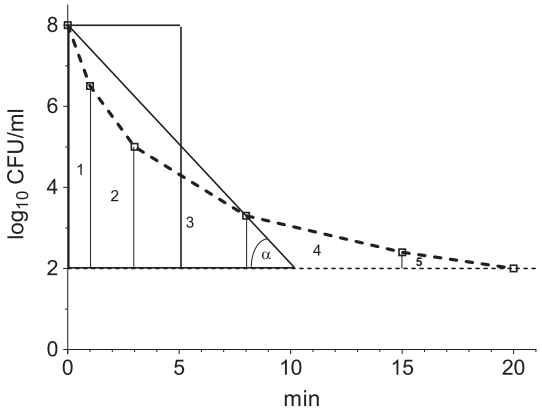

Fig. 1. Schematic explanation of the Integral Method. Derivation of the averaged bactericidal activity from the area below the killing curve. Killing curve (open squares and thick dotted line), detection limit (thin dotted line). A = area below the curve = sum of trapezoids 1 to 5 = 5.25 + 7.50 + 10.75 + 5.95 + 1.0 = 30.45 $\log_{10}$ CFU × min. Abscissa of rectangle = A / ($\log_{10}$ $\text{CFU}_{t=0}$ − 2) = 30.45 / 6 = 5.075 min. Abscissa of triangle = 2 × 5.075 = 10.15 min. Area below the curve = area rectangle = area triangle. tg α = 6 / 10.15 = 0.591 $\log_{10}$ CFU/min. tg α = ($K_0^2 - K_n^2$) / 2A = (36 − 0) / 60.9 = 0.591 $\log_{10}$ CFU/min (Eq. (7)).

Please cite this article as: Gottardi, W., et al., The *Integral Method*, a new approach to quantify bactericidal activity, J. Microbiol. Methods (2015), http://dx.doi.org/10.1016/j.mimet.2015.05.002