Polish Journal of Microbiology
2007, Vol. 56, No 3, 165–168

ORIGINAL PAPER

Reliability of the Etest in Light of the Correlation between an Antibiotic's Critical Concentration (Cc) and MIC Values

MAREK BEDNAR*

Department of Medical Microbiology, Charles University, 3rd Faculty of Medicine, Prague, Czech Republic

Received 24 February 2007, revised 20 July 2007, accepted 24 July 2007

Abstract

The study relates to the theory of diffusion methods for antibiotic sensitivity testing. The aim of the study was to show the relationship between the antibiotic critical concentration (Cc) and its minimum inhibitory concentration (MIC). The results contribute to the explanation of the Etest's reliability and support the scientific basis for MIC determination using agar diffusion methods. Susceptibility among 90 clinical isolates of 12 common aerobic bacterial species to gentamicin, erythromycin, or oxacillin was assessed using the multidisc method (for Cc), by the agar dilution method (for MIC) and by the Etest. The results of all three methods were statistically compared and found to be closely related. The regression equation for Cc values and MIC was $\log_2(MIC) = 0.99 \times \log_2(Cc) - 0.13$; $r = 0.99$; $p < 0.05$; the regression equation for Cc values and Etest-MIC (Et) was $\log_2(Et) = 0.86 \times \log_2(Cc) + 0.34$; $r = 0.96$; $p < 0.05$; the regression equation for Etest-MIC values and MIC was $\log_2(MIC) = 1.12 \times \log_2(Et) - 0.50$; $r = 0.96$; $p < 0.05$.

Key words: Etest, multidisc method, antibiotic critical concentration (Cc), antibiotic sensitivity testing

Introduction

Quantitative antibiotic sensitivity can be estimated by either agar/broth dilution methods, or by agar diffusion methods. The minimum inhibitory concentration (MIC) in dilution methods is not affected by the growth of standard bacterial inoculums because the antibiotic is completely active from the beginning of the incubation period. On the other hand, in diffusion methods (Etest, multidisc method) the concentration of the antibiotic at the edge of inhibition zone at the time of its formation relates to the growing bacterial population. This antibiotic concentration, the actual bacterial density and the time when the edge of inhibition zone is founded are called the critical concentration (Cc), the critical population and the critical time, respectively (Linton, 1961; Cooper, 1963; Barry, 1980; Delignette-Muller and Flandrois, 1994). The critical time generally lasts for a period of several hours after the start of incubation. In slow growing bacteria, the critical time is longer. The critical population is therefore higher than the standard inoculum and, consequently, the critical concentration can theoretically differ from MIC. The question we hope to answer is, does it really differ? If yes, then the Etest wouldn't work properly. However, the Etest has been reported to be reliable by many authors, although it has not been well studied (Kronvall, 2000). The purpose of this paper is to uncover the theoretical explanation for Etest accuracy.

How the antibiotic critical concentration can be measured? If two or more discrete discs with different amounts of the same antibiotic are used, the critical concentration of the antibiotic can be calculated from the content of the discs and from the diameter of their respective inhibition zones. The following rules apply to the theory of inhibition zone formation in the disc method:

- The critical time for the same bacteria strain, antibiotic, and cultivation conditions is independent of the amount of antibiotic in the disc (of the disc content) (Barry, 1980).
- As soon as the edge of a zone is formed, its diameter will not change (Linton, 1961; Barry, 1980).
- The relation between the area of inhibition zone and natural logarithm of antibiotic disc content is linear (Barry, 1980; Kronvall, 1982; Delignette-Muller and Flandrois, 1994).

* Corresponding author: M. Bednar, Department of Medical Microbiology, Charles University, 3rd Faculty of Medicine, Ruska 87, 100 00 Prague, Czech Republic; phone: (42) 26 7162580; fax: (42) 26 7162516; **e-mail: bednarm@cbox.cz**

- The antibiotic diffuses into the agar according to Fick's second law of diffusion (Vesterdal, 1947; Humphrey and Lightbown, 1952; Koch, 1999).

Note on rule 3): the relation between the radius of the inhibition zone and antibiotic disc content is expressed by the equation

$$r_n^2 = a + b \ln(m_n) \qquad \{1\}$$

where r_n is the radius of inhibition zone in n-th disc and m_n is the content of the disc. A graphic image of this dependency is a straight line. Its intercept a and slope b can be calculated from the measured data by common statistical procedures. Measurement of the zone radius from the center of discs provides a better correlation with disc content and size of inhibition zone, in terms of equation {1}, than does measurement of the zone radius starting from the edge of the disc (Kronvall, 2000).

Note on rule 4): a derived radial law for two-dimensional cylindrical diffusion applies to the disc method (Humphrey and Lightbown, 1952; Koch, 1999).

$$Cc = \frac{m_n}{4\pi D_t d} e^{-\frac{r_n^2}{4Dt_c}} \qquad \{2\}$$

where Cc (µg/ml) is the critical concentration of antibiotic, m_n (µg) is the antibiotic disc content at the beginning of diffusion, r_n (cm) is the radius of the respective inhibition zone, measured from the disc center D (cm^2/s) is the diffusion constant of the antibiotic, t_c (s) is the critical time, d (cm) is the agar depth, and e is the base of natural logarithms. The denominator $4\pi Dt_c d$ reflects the drop in antibiotic concentration in the disc during the critical time and the agar depth. Dimensional analysis of the formula yields µg/ml, which confirms the mathematical validity of the formula.

It can be proven that $b = 4Dt_c$. This is in agreement with the research done by others (Cooper, 1963; Drugeon *et al*., 1987; Delignette-Muller and Flandrois, 1994; Koch, 1999). In formula {2}, you can substitute slope b for $4Dt_c$ and we can substitute $a + b\ln(m_n)$ for r_n^2. By consequent reduction, we arrive at resulting formula combining equations {1} and {2}

$$Cc = \frac{1}{\pi b d} e^{-\frac{a}{b}} \qquad *\{3\}$$

where a is the intercept, b is the slope of regression line according to equation {1}, and d is the agar depth. Formula {3} calculates the critical concentration of an antibiotic without knowing its diffusion constant or critical time (*i.e.*, without calibration). If we use at least three discs, the reliability of the result can be ascertained from the correlation of the logarithms of the discs' content and the sizes of the respective inhibition zones.

* Equation {3} used in this work was briefly described in the Medical Science Monitor (2000) 6: 168–170.

Experimental

Materials and Methods

Susceptibility among ninety clinical isolates of common aerobic bacterial species to gentamicin, erythromycin, or oxacillin was assessed. We performed 90 concurrent sensitivity measurements using the multidisc diffusion method, standard agar dilution method and Etest. Sensitivity was measured 33 times for oxacillin, 27 times for gentamicin, and 30 times for erythromycin. These antibiotics were chosen due to their diverse mode of action. They also provide sharp, clear zones of inhibition in the diffusion method.

Bacterial strains and culture media. Forty-five strains of *Staphylococcus aureus*, seventeen strains of *Staphylococcus epidermidis*, six strains of *Pseudomonas aeruginosa*, six strains of *Proteus mirabilis*, six strains of *Escherichia coli*, four strains of *Klebsiella* spp., three strains of *Acinetobacter baumanii*, two strains of *Enterobacter* spp. and one strain of *Enterococcus faecalis* were used. All the strains were isolated during routine investigations of various clinical specimens in a hospital laboratory. The strains were chosen according to the qualitative sensitivity testing (NCCLS, 1993a) so as to obtain three sets of strains for measurements of the sensitivity to given antibiotic over a wide range of MIC values. Erythromycin or oxacillin sensitivity was checked in Gram-positives whereas gentamicin sensitivity in Gram-negatives. The actual experiment involved a quantitative assessment of the sensitivity to a particular antibiotic in selected strains using the multidisc method, agar dilution method, and Etest. The tests were run concurrently for each inoculum. We used Mueller-Hinton agar (Oxoid, Unipath Ltd., Basingstoke, Hampshire, England) poured to a depth of 4 mm in Petri dishes (diameter 90 mm) for all three methods. The inocula for all three methods came from colonies that were suspended in physiological saline solution to a density of 0.5 on the McFarland scale. Plates used for the multidisc method and the Etest were inoculated by swabbing.

Etest. Etest strips (Gentamicin low range; Erythromycin; Oxacillin) were used according to the manufacturer's instructions (AB Biodisk, Solna Sweden). Agar plates were incubated at 35°C. Results were read after 24 hours according to the manufacturer's reading guide.

Antibiotics used. Antibiotic solutions were prepared by dilution of injectable preparations: Gentamicin LEK Pharmaceuticals and Chemical Co., Slovenia (gentamicin), Erythrocin Abbott Laboratories, USA (erythromycin), and Prostaphilin Bristol-Myers Squibb S.p.A., Italy (oxacillin).

Critical concentration (Cc) determinations (multidisc diffusion method). Four 6 mm – diameter blank paper discs (Oxoid, Unipath Ltd., Basingstoke,

Table I
Antibiotic disc content in individual discs A, B, C, and D in multidisc method rounded to three significant digits

Antibiotic	Disc content (μg)							
	Resistants				Sensitives			
	A	B	C	D	A	B	C	D
Erythromycin	1000	200	40.0	8.00	200	40.0	8.00	1.60
Gentamicin	800	256	64.0	16.0	256	64.0	4.00	1.00
Oxacillin	5000	714	102	14.6	102	14.6	2.08	0.298

In order that the number of discs in the multidisc method can be reduced to four, strains were initially subdivided into groups that were either sensitive or resistant using a routine qualitative disc method. Because of practical reasons, the ratio between the neighboring discs' contents is usually (but not obligatory) regular.

Hampshire, England) were placed onto the inoculated agar. An appropriate amount of antibiotic, dissolved in 20 μl distilled water, was dropped onto discs using a pipette. The amount of antibiotic on the discs was estimated on the basis of previous qualitative sensitivity testing of all strains (NCCLS, 1993a) so as to achieve a minimum of two measurable yet distinct inhibition zones when using four discs (Table I). Agar plates were incubated at 35°C for 24 hours. The diameters of inhibition zones were measured using an electronic calliper. Non-rounded zone diameters and data on antibiotic disc content were used to calculate the slope *b* and intercept *a* according to equation {1}. These constants were subsequently employed for the calculation of the critical concentration of the antibiotics using formula {3}. Where at least three inhibition zones formed, the percentage of variation in the zones explained by the disc content logarithms (coefficient of determination R^2 written as a percentage) was also calculated to show the reliability of the result.

MIC determination. The assessment of MIC using the agar dilution method was performed in accordance with NCCLS guidelines (NCCLS, 1993b). The antibiotics were the same as those used in the multidisc diffusion method. Final concentrations of antibiotics in the agar ranged from 0.012 to 512 mg/l. The dilutions were based on a geometrical order (factor 2) and were related to concentrations of 1 mg/l and 1.5 mg/l (0.012, 0.016, 0.023, 0.031, 0.047, 0.063, 0.094, 0.125, 0.19, 0.25, 0.38, 0.5, 0.75, 1.0, 1.5, 2, 3, 4, 6, 8, 12, 16, 24, 32, 48, 64, 96, 128, 192, 256, 384, 512 mg/l). The inoculum was applied to the agar surface by means of a pin replicator. Agar plates were incubated at 35°C. Results were read after 24 hours.

Statistical evaluation of results. The results were classified by the applied method only, not by bacterial strain or the antibiotic used. The correlations of Etest results with MIC, multidisc diffusion method results with MIC and multidisc diffusion method results with Etest results, were expressed using the Spearman rank correlation coefficient. After transforming the results into base 2 logarithms, we expressed them by means of the Pearson correlation coefficient. Statistical values were calculated by Statistica for Windows (StatSoft Inc.). Differences no greater than a twofold dilution factor between the MIC and the Etest or between the MIC and the multidisc method, were used to calculate agreement (Pfaller *et al*., 2000).

Results

Agreement of Cc with MIC was observed in 89 out of 90 concurrent critical concentration measurements. Regression straight line is shown in Figure 1. We observed two disagreements between the MIC and

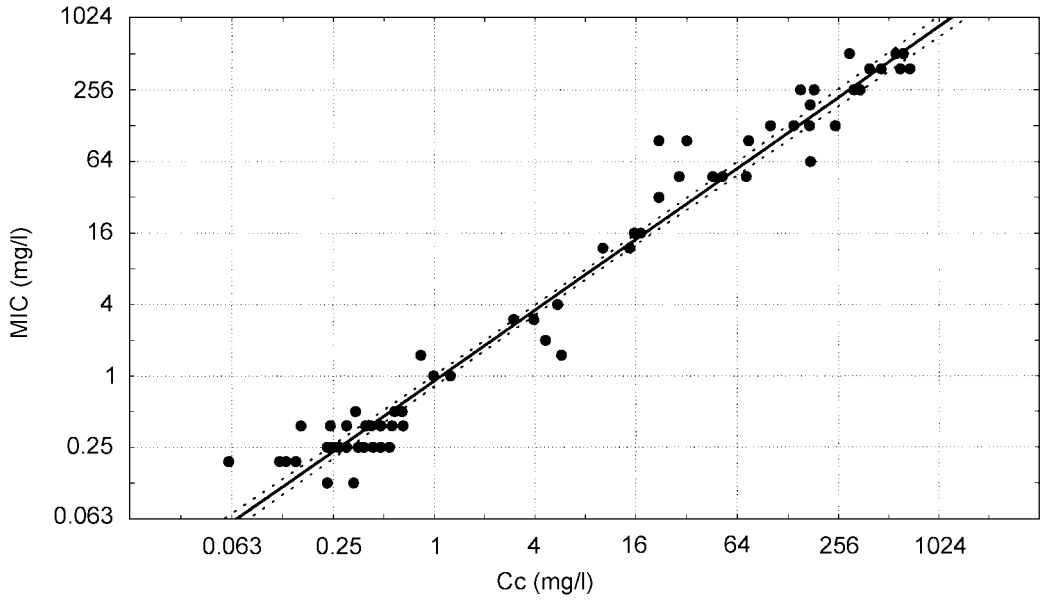

Fig. 1. Correlation of results of sensitivity measurements by multidisc method (Cc – critical concentration) and agar dilution method (MIC – minimum inhibitory concentration). Broken lines indicate the 95% confidence band; some points overlap

the Etest. All disagreements occurred among resistant strains. Both the multidisc method and the Etest correlated well with the dilution agar method: both the Spearman and Pearson coefficients reached at least 0.9. The relation between the results of the multidisc method (Cc) and MIC was $\log_2(MIC) = 0.99 \times \times \log_2(Cc)0.13$; $r = 0.99$; $p < 0.05$. The relation between the Etest results (Et) and MIC was $\log_2(MIC) = 1.12 \times \log_2(Et)0.50$; $r = 0.96$; $p < 0.05$. The relation between Cc and Et was $\log_2(Et) = 0.86 \times \log_2(Cc) + 0.34$; $r = 0.96$; $p < 0.05$. The reliability of the multidisc method expressed as the average percentage of variation in the zones explained by the disc content logarithms was 98.85% (92.36–100.00%). The *b* value in formula {1} was not related to the sensitivity of the strains. Thus, the critical time was independent from strain sensitivity. Neither species-dependent nor antibiotic type-dependent irregularities in Cc-MIC relationship were found.

Discussion

According to the classical theories (Cooper, 1963; Barry, 1980; Hedges, 1999), the bacterial growth rate impacts the inhibitory zone diameter. In the Kirby-Bauer qualitative disc diffusion method, this phenomenon is solved by the interpretative standards (NCCLS, 1993a). The crucial question is whether such "inaccuracy" substantially influences the Etest result. The results of our work show that it does not, because the antibiotic (critical) concentration underneath the edge of the forming zone practically equals MIC. So in Etest the bacterial growth rate may impact the inhibitory zone shape but not its point of intersection with the scale on the strip.

In the multidisc method, the concentration of antibiotic on each additional discs is, optimally, four to seven times lower than on the preceding disc (depending on the type of antibiotic). Because the experimental design initially divided the strains into either sensitive or resistant, we were able to reduce the number of discs used to four and still obtain at least two measurably distinct zones of inhibition for an accurate calculation of the critical concentration. Without this initial categorization, five or six discs would be required to test over the full scale of an Etest strip. Such increasing disc number brings the multidisc method closer to the Etest, which can be imagined as a chain of antibiotic discs with exponentially growing antibiotic content. The primary data in both methods are the critical concentrations of antibiotics. The Etest and multidisc method do have similarities – the zones (including the zero zones) are always formed after the critical time passes. In the case of the Etest, the critical concentration if $r = 0$ estimates MIC using a printed scale.

The correlation between the critical concentration and MIC is not a new finding. Nevertheless, the extent of the correlation is surprising. It implies that in diffusion quantitative methods, bacterial growth up until the critical time does not influence the result. This observation contributes to an understanding of the accuracy of the Etest on a wide variety of organisms, and indicates that the results obtained with quantitative diffusion methods (E-test, multidisc method) can be expressed as MICs without any conversion.

Acknowledgement

The author would like to thank Marie Duskova and Zorka Haasova for their technical laboratory assistance and Jiri Horacek for the software production. The program for the critical concentration calculation is downloadable on http://www.lf3.cuni.cz/ustavy/mikrobiologie/download/atb_cc.zip; last accessed 7/07/07.

Literature

Barry A.L. 1980. Procedure for testing antibiotics in agar media: Theoretical consideration, pp: 1–23. In: V. Lorian (ed.) *Antibiotics in Laboratory Medicine*. The Williams and Wilkins Co., Baltimore/London.

Cooper K.E. 1963. The theory of antibiotic inhibition zones, pp: 1–85. In F. Kavanagh (ed.) *Analytical Microbiology*. Academic Press, New York and London.

Delignette-Muller M.L. and J.P. Flandrois. 1994. An accurate diffusion method for determining bacterial sensitivity to antibiotics. *J. Antimicrob. Chemother.* 34: 73–81.

Drugeon H.B., M.E. Juvin, J.Caillon and A.L. Courtieu. 1987. Assessment of formulas for calculating critical concentration by the agar diffusion method. *Antimicrob. Agents Chemother.* 31: 870–875.

Hedges A.J. 1999. The influence of factors affecting the 'critical population' density of inocula on the determination of bacterial susceptibility to antibiotics by disc diffusion methods. *J. Antimicrob. Chemother.* 43: 313.

Humphrey J.H. and J.W.Lightbown. 1952. A general theory for plate assay of antibiotics with some practical applications. *J. Gen. Microbiol.* 7: 129–143.

Koch A.L. 1999. Diffusion through agar blocks of finite dimensions: a theoretical analysis of three systems of practical significance in microbiology. *Microbiology* 145: 643–654.

Kronvall G. 1982. Analysis of a single reference strain for determination of gentamicin regression line constants and inhibition zone diameter breakpoints in quality control of disk diffusion antibiotic susceptibility testing. *J. Clin. Microbiol.* 16: 784–793.

Kronvall G. 2000. MIC determination of fusidic acid and of ciprofloxacin using multidisk diffusion tests. *Clin. Microbiol. Infect.* 6: 483–489.

Linton A.H. 1961. Interpreting antibiotic sensitivity tests. *J. Med. Lab. Technol.* 18: 1–20.

NCCLS. 1993a. National Committee for Clinical laboratory Standards. Performance Standards for Antimicrobial Disk Susceptibility Tests – Fifth Edition: Approved Standard M2-A5. NCCLS, Villanova, PA, USA.

NCCLS. 1993b. National Committee for Clinical laboratory Standards. Methods for Dilution Antimicrobial Susceptibility Tests for bacteria that Grow Aerobically – Third Edition: Approved Standard M7-A3. NCCLS, Villanova, PA, USA.

Pfaller M.A., S.A. Messer, A. Houston, K. Mills, A. Bolmstrom and R.N. Jones. 2000. Evaluation of the Etest method for determining voriconazole susceptibilities of 312 clinical isolates of Candida species by using three different agar media. *J. Clin. Microbiol.* 38: 3715–3717.

Vesterdal J. 1947. Studies on the incubation zones observed in the agar cup method for penicillin assay. *Acta Pathol. Microbiol. Scand.* 24: 272–282.