

Susceptibility testing: accurate and reproducible minimum inhibitory concentration (MIC) and non-inhibitory concentration (NIC) values

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R.J.W. LAMBERT AND J. PEARSON. 2000. Measuring the minimum inhibitory concentration (MIC) of a substance by current methods is straightforward, whereas obtaining useful comparative information from the tests can be more difficult. A simple technique and a method of data analysis are reported which give the experimentalist more useful information from susceptibility testing. This method makes use of a 100-well microtitre plate and the analysis uses all the growth information, obtained by turbidometry, from each and every well of the microtitre plate. A modified Gompertz function is used to fit the data, from which a more exact value can be obtained for the MIC. The technique also showed that at certain concentrations of inhibitor, there was no effect on growth relative to a control well (zero inhibitor). Above a threshold value, which has been termed the non-inhibitory concentration or NIC, growth becomes limiting until it reaches the MIC, where no growth relative to the control is observed.

INTRODUCTION

A current definition of the Minimum Inhibitory Concentration, MIC, is “the lowest concentration which resulted in maintenance or reduction of inoculum viability” (Carson *et al.* 1995). The determination of the MIC involves a semi-quantitative test procedure which gives an approximation to the least concentration of an antimicrobial needed to prevent microbial growth. In the recent past, the method used tubes of growth broth containing a test level of preservative, into which an inoculum of microbes was added. The end result of the test was the minimum concentration of antimicrobial which gave a clear solution, i.e., no visual growth (Collins 1964; Davidson and Parish 1989). Currently, the tubes have been replaced by a semi-automated microtitre method. Where the turbidity of the test compound interferes with the test, indicators can be used for the determination of the end-point. These include fluorescein diacetate (Chand *et al.* 1994) and resazurin (Mann and Markham 1998). However, the end-point of all experiments is still the observation of no growth in one of the wells, interpreted as the MIC (Sommers 1980).

The lack of a quantitative standard method has hampered many studies into the action of antimicrobials (Janssen *et al.* 1987; Manou *et al.* 1998). However, the principle problem encountered is that all MIC techniques currently used are

semi-quantitative. Tiina and Sandholm (1989) examined the inhibition of micro-organisms with glucose oxidase/glucose. Using turbidometry, they related the area under the O.D./time curves to the degree of inhibition observed using the ratio of growth of a control to that of the test. A study was carried out to determine whether this basic idea could be used to obtain accurate MIC values, for a given set of conditions, for a range of common antimicrobial substances.

MATERIALS, MICROBES AND METHODS

Preparation of bacterial suspensions for testing

Staphylococcus aureus ATCC 6538, *Escherichia coli* ATCC 11229 and *Pseudomonas aeruginosa* ATCC 2730 were grown overnight in flasks containing 80 ml Tryptone Soya Broth, TSB (Oxoid), with shaking, at 30 °C. The culture was centrifuged at 4000 rev min⁻¹ (510 g, Sigma, Harz, Germany; model 3K-1) for 10 min. The resulting cell pellets were pooled and resuspended in 0.1% peptone water. In general, an inoculum level of approximately 1 × 10⁷ ml⁻¹ was used, which was made reproducible through the use of a calibration standard produced from an investigation of O.D. readings and plate count numbers.

Method of analysis

This method of setting up the experiment was designed to minimize the amount of work required by the operator, and a

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200-well test (2×100 wells) could be prepared in under 2 h, given the availability of the microbes. The tests were carried out on a Bioscreen Microbiological Growth Analyser (Labsystems, Helsinki, Finland), and the experimental set up was based on the use of the non-standard, 100-well plates manufactured for this machine. However, any O.D. reader, or any other machine capable of giving an output signal based on a growth criterion, can be used with suitable modifications to the experimental design.

A stock solution of the antimicrobial under test was prepared in the microbial growth medium being used. From the stock solution, nine 'principle' dilutions were made using the growth medium. The recommended dilutions are from 1 to 0.2 fractional dilutions, e.g. 4 ml stock plus 6 ml growth broth giving the 0.4 dilution fraction. The first column of the plate received 250 μ l of growth broth. To the first wells of each remaining column were added 500 μ l of a principle dilution. All the other wells received 250 μ l of growth broth (assuming a twofold dilution series); 250 μ l of a principle dilution was then added to the next well in the appropriate column and mixed, and the dilution series continued to the end of the column.

Each well, except for one of the controls (well 10), in column one received 50 μ l of the prepared inoculum. The plate was then incubated at the desired temperature and time, with the analyser recording the O.D. of each well at 600 nm every 10 min.

Analysis

The basis of the technique is the comparison of the area under the O.D./time curve of the control with the areas of the tests (the nine controls, wells 1–9, are averaged and the background, well 10, is removed from the data). As the amount of preservative in a well increases, the effect on the growth of the organism also increases. This effect on growth is manifested by a reduction in the area under the O.D./time curve relative to a control well at any specified time. By calculating the area under the O.D./time curves using the trapezoidal rule, the relative amount of growth can be obtained using the ratio of the test area to that of the control, termed the fractional area, FA.

Using an available spreadsheet, the calculations and time required were minimized with the use of the template which was developed. The template received the raw data from the Bioscreen, and outputted the results in the form of concentrations of preservative with the observed fractional areas.

Measurement of NIC and MIC: Fitting of a modified Gompertz function

Data in the form of log concentration *vs* fractional area can be analysed using a modified Gompertz equation (see

Mackey and Derrick 1982 for another example of its use). The altered Gompertz function, in this case, relates the fractional area (y) to the log of antimicrobial concentration (x), Eq. 1.

$$y = A + Ce^{-e^{B(x-M)}} \quad \text{eqn 1}$$

where A is the lower asymptote of y (approximately zero), B is a slope parameter, C is the distance between the upper and lower asymptote (approximately 1) and M is the log concentration of the inflexion point. The values of the NIC and MIC are defined as the intersection of the lines $y = A + C$ and $y = A$, with the equation of the line tangential to the point $(M, (A + Ce^{-1}))$, respectively.

$$\text{MIC} = 10^{(M + \frac{1}{B})} \quad \text{eqn 2}$$

$$\text{NIC} = 10^{(M - \frac{1.718}{B})} \quad \text{eqn 3}$$

The values of A , C , B and M are obtained from a non-linear fitting procedure, commonly found on statistical packages such as JMP (SAS Institute, Cary, NC, USA).

RESULTS

The following experiments have been picked to show the utility of the methodology. Table 1 summarizes the data obtained.

Dodecylpyridinium chloride and *Staphylococcus aureus*

The 100-well plate of the Bioscreen was set up to give a range of dilutions from 83 to 0.03 ppm. Incubation was carried out for just under 18 h. The data obtained were transformed into fractional areas and plotted against concentration, Fig. 1.

In general, plotting the inhibitor concentration on a logarithmic scale gives a characteristic sigmoid-shaped curve. The curve can be split into three principle regions: a region where the presence of the preservative has no effect on the of the organism relative to the control growth (as measured by O.D.), a region where there is increasing inhibition of growth, and a region where there is no measurable growth relative to the control. Terms have been assigned to two specific concentrations, the non-inhibitory concentration, NIC, the concentration above which the inhibitor begins to have a negative effect on growth, and the minimum inhibitory concentration, MIC, which marks the concentration above which no growth is observed relative to the control.

Table 1 MIC and NIC values (mg l⁻¹)

Compound	Organism	NIC	MIC
C8QAC	<i>Staph. aureus</i>	352	594
C8QAC	<i>Ps. aeruginosa</i>	1560	4844
C10QAC	<i>Staph. aureus</i>	47	79.4
C10QAC	<i>Ps. aeruginosa</i>	475	1462
C12QAC	<i>Staph. aureus</i>	4.57	7.9
C12QAC	<i>Ps. aeruginosa</i>	157	346
C14QAC	<i>Staph. aureus</i>	0.61	1.22
C14QAC	<i>Ps. aeruginosa</i>	58.6	83.7
C16QAC	<i>Staph. aureus</i>	0.24	0.51
C16QAC	<i>Ps. aeruginosa</i>	Complex	> 1000
C18QAC	<i>Staph. aureus</i>	0.54	1.02
C18QAC	<i>Ps. aeruginosa</i>	Complex	> 1000
Dodecylcholine Cl	<i>Staph. aureus</i>	5.54	9.5
Dodecylcholine Cl	<i>Ps. aeruginosa</i>	118	152
Dodecylpyridinium Cl	<i>Staph. aureus</i>	1.06	2
Dodecylpyridinium Cl	<i>Ps. aeruginosa</i>	66.3	186
Phenethyl alcohol	<i>E. coli</i>	935	4158
Phenethyl alcohol	<i>Staph. aureus</i>	2468	3733
Phenoxyethanol	<i>Staph. aureus</i>	3619	6080
Triclosan	<i>E. coli</i>	0.0252	0.0825
Silver nitrate	<i>Ps. aeruginosa</i>	2.87	7.9

C_nH_{2n}N(Me)₃ Br; *n* = 8, 10, 12, 14, 16, 18; against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

The straight alkyl chain trimethyl ammonium bromide surfactants (C_nQAC) were examined. Figure 2 displays the

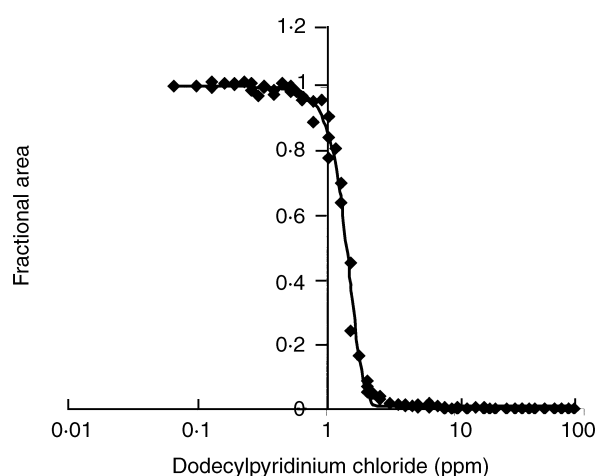


Fig. 1 The inhibition profile of dodecylpyridinium chloride against *Staphylococcus aureus*: (◆), observed FA; (—), fitted Gompertz function

observed and fitted inhibition profiles for a range C_nQAC compounds against *Staph. aureus*. A plot of chain length against the log MIC and log NIC values gave a stepwise drop in susceptibility from *n* = 8 to 14, Fig. 3. A maximum level of inhibition was obtained at *n* = 16; thereafter, a decrease in susceptibility was found. In the case of *Ps. aeruginosa*, a stepwise drop in the log MIC and log NIC values was observed up to *n* = 14. For chain lengths of *n* = 16 and 18, complex inhibition profiles were found e.g. Figure 4.

Phenethyl alcohol and phenoxyethanol against *Staphylococcus aureus*

Phenethyl alcohol, PeA, is described as a poorer inhibitor than phenoxyethanol, PoE (Paulus 1993). An inhibition study was conducted under the same conditions of temperature and inoculum challenge. Under these conditions, it was found that PeA had the lower MIC (Table 1). The slight difference in molecular weight (122.2 vs 138.2 for PeA and PoE, respectively) cannot account for the 2000 ppm difference between the MIC values.

Reproducibility

Dodecylcholine is a quaternary ammonium analogue, but unlike the quaternary ammonium compounds (QACs) used regularly in disinfection, the alkanoylcholines have an

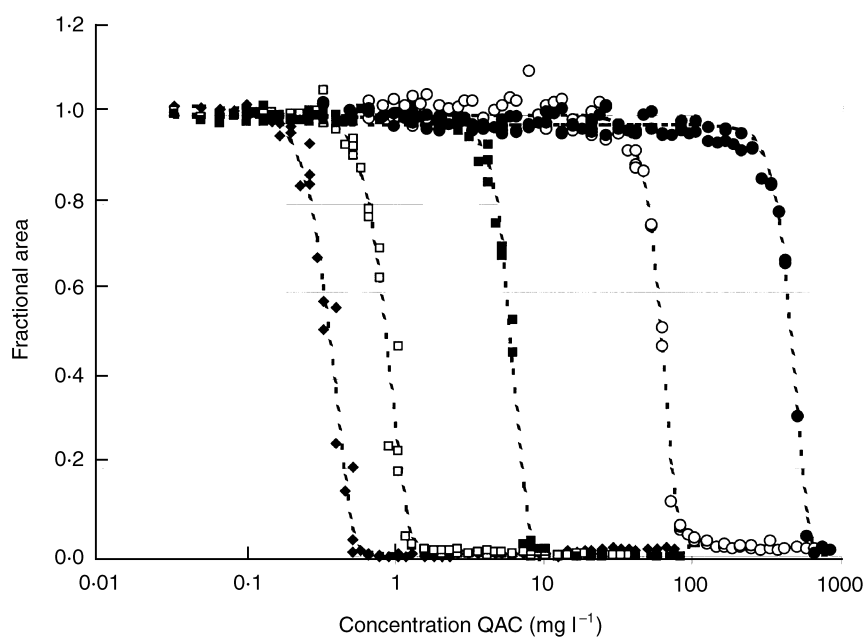


Fig. 2 Observed inhibition profiles and fitted Gompertz models (dashed lines) for CnQAC, $n = 8, 10, 12, 14, 16$, against *Staphylococcus aureus*: (●), $n = 8$; (○), $n = 10$; (■), $n = 12$; (□), $n = 14$; (◆), $n = 16$

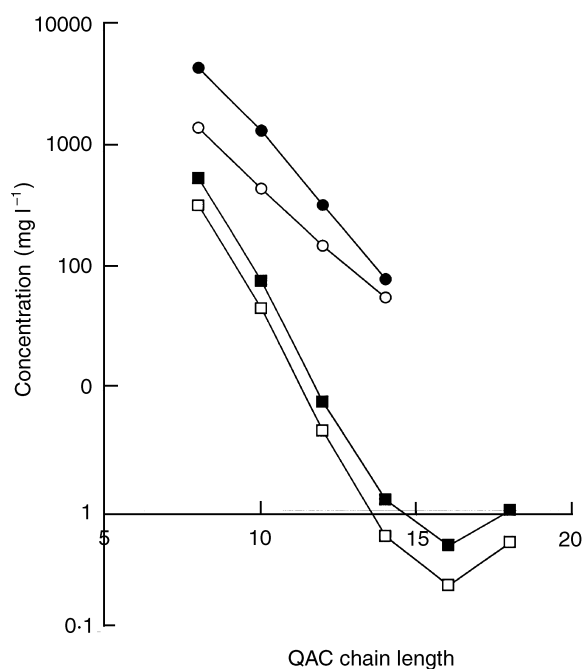


Fig. 3 MIC and NIC dependence on alkyl chain length for CnQAC, $n = 8, 10, 12, 14, 16, 18$, against *Staphylococcus aureus*: (□), MIC, (●), NIC; and $n = 8, 10, 12, 14$ against *Pseudomonas aeruginosa*: (○), MIC, (●), NIC

expected low toxicity due to an ester link which connects the alkyl chain with the choline moiety. The modified Gompertz analysis of five separate inhibition investigations against *Staph. aureus*, done on different days by different operators, gave an $\text{NIC} = 6.16 \pm 0.45$ ppm and an $\text{MIC} = 10.09 \pm 1.04$

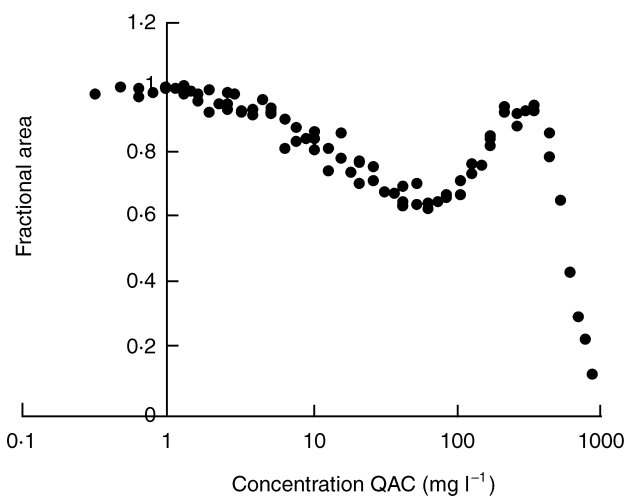


Fig. 4 Inhibition profile of C16QAC against *Pseudomonas aeruginosa*

ppm. The inoculum challenge for these experiments ranged from 0.9×10^7 to 1.59×10^7 organisms ml^{-1} .

Rapidity of the technique

Minimum inhibitory concentration experiments are generally carried out over a specified time, e.g. 24 h. As this technique is based on growth relative to a control, it should allow an estimation of an MIC at any time up to the point of the test termination. In this laboratory, many of the experiments are terminated after 18 h, allowing time for data analysis and the setting up of another experiment(s) based on those results. An analysis of the calculated NIC and MIC values of the inhibition of C14QAC against *Staph. aureus*, with respect to incubation time, was carried out. The NIC and MIC values were calculated every hour, after an initial 4 h incubation, up to the end of the test. The results are shown in Fig. 5. The figure clearly shows a rise in the MIC as incubation time increases, commensurate with an increase in the NIC. Below about 8 h of incubation, the signal to noise ratio of the control wells is low, resulting in large FA values. The time taken for a substantial increase in the signal to noise ratio will be dependent on the inoculum size and the specific growth rate of the organism.

DISCUSSION

Minimum inhibitory concentration is a difficult subject. The traditional methods of analysis do not allow a quantitative measurement; MIC is, in effect, a range of concentrations depending on the dilution series used. Measurement of

the MIC should be a first line technique of discovery for the biocide and preservative scientist, but in many laboratories, it is used as an indicator of activity rather than something of real substantive value. Further, comparison of values obtained from the multitude of different techniques is problematic (Skytta and Mattila-Sandholm 1991). Current turbidometric techniques fail to provide good quantitative data because they do not use all the available data. These techniques are often simply extensions of the tube dilution series method into microtitre wells. The demarcation between growth/no growth is examined, and the concentration of inhibitor in the well with no growth is termed the MIC. All the growth information below the MIC concentration is normally discarded.

By examining all the data from the 'basic' MIC dilution technique, it has been possible to place a value on the data normally rejected. Furthermore, it has been found that these data can lead to the placing of an accurate MIC value on the antimicrobial, and also, a value on the non-inhibitory concentration, the NIC, the concentration below which normal growth is observed.

Definition

Within the context of the work described here there are two working definitions of MIC: one is that the MIC is the minimum concentration required to prevent growth in the broth relative to a control and is in line with the definition of Carson (1995); the other is based on the extrapolation of a tangent from the inflexion point of a fitted Gompertz curve to a lower asymptote (the zero-growth line). The former is a more comfortable definition, whereas the latter is easy to calculate and use and is less prone to experimental or subjective operator error.

The MIC (and the NIC) are dependent on the conditions under which the experiment was run. These conditions should be specified when reporting the results. The most important conditions that have been observed are the incubation temperature, the organism and the inoculum size used, and a fuller examination of these factors will be reported in the near future.

Inhibitor concentration \leq NIC

The NIC is the concentration above which the inhibiting substance begins to have an observable effect on growth. At concentrations below the NIC, growth occurs at a pace equal to the control. The population of microbes is unaffected by the presence of the test substance. In some instances, the FA may exceed one if the test inhibitor leads to better growth conditions than the control. Thus, this methodology could be used for the optimization of growth media as an FA greater than one implies better growth conditions.

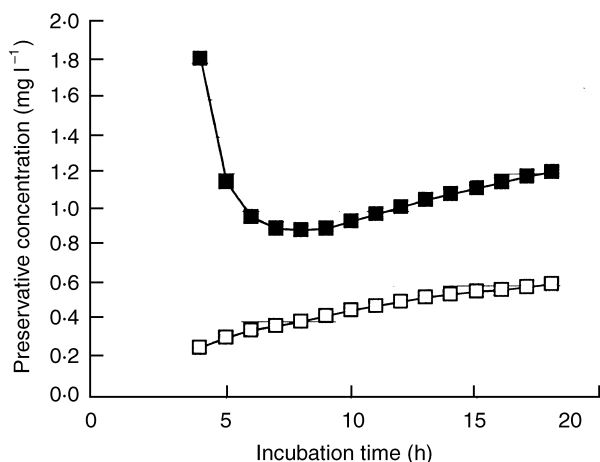


Fig. 5 Examination in the change of NIC and MIC with incubation time for C14QAC against *Staphylococcus aureus*: (■), NIC; (□), MIC

$NIC \leq \text{inhibitor concentration} \leq MIC$

Between the two principle concentrations, growth inhibition occurs. The observed inhibition can have many causes, e.g. a poisoning of the microbial system leading to a slowing down of growth in a dose-dependent manner, or the induction of apparent lags caused by the immediate cessation of growth (see Lambert and Stratford 1999; for a discussion of weak acid-induced lags). In many of the cases examined here, there is a linear relationship between FA and inhibitor concentration between the NIC and the MIC. This is suggestive of a single, encompassing mechanism of inhibition. In others, however, such as the long-chain cationic surfactants, there appears to be a biphasic inhibition suggestive of a complex inhibition mechanism. In the case of *Ps. aeruginosa* and C16QAC, after an initial decrease in FA, increasing amounts of inhibitor lead to an increase in the FA. It is believed that this experiment (which has been repeated many times) is suggesting a link between the MIC and the critical micelle concentration, CMC, of the surfactant.

Devinsky *et al.* (1985, 1991) have shown that there is a relationship between the MIC and CMC of quaternary ammonium compounds. Optimum antimicrobial activity of surfactants is strongly related to the ability to form micelles. Essentially, a micelle represents a microbially-inactive form of the surfactant. The lower the CMC, the less free-bulk surfactant available to interact with the microbial membrane. Our hypothesis to explain the inhibition profile shown in Fig. 4 is that pre-micellization occurs between membrane components of the Gram-negative bacterium and the surfactant biocide. This essentially reduces the effective concentration of the biocide in solution and thus, there is an increase in the FA. As the CMC of the surfactant is approached (416 mg l⁻¹, Mukerjee and Mysels (1971)), this effect reaches a maximum. This effect may be one of the causes of 'blebbing' (Jones *et al.* 1989). At concentrations higher than the CMC, the membrane becomes solubilized by the surfactant and lysis occurs. This phenomenon, in which a lower concentration of inhibitor has a greater inhibitory effect on growth than at a higher concentration, would have been undiscovered if this method of examining the inhibition profile had not been adopted.

$MIC \leq \text{Inhibitor concentration}$

At the MIC, no growth relative to the control is recorded. In some cases, the FA appears to increase past the MIC, but this can be related to an increase in opacity caused by the inhibitor itself at the wavelength used. If the MIC is obscured by the inhibitor in this way, then an adaptation of the methodology of Mann and Markham (1998) may be prudent. The MIC is defined by this method mathematically, based on the Gompertz equation. In some cases, the unbridled use of the

Gompertz will lead to inaccurate MIC values. For example, the use of the modified Gompertz function with the data shown in Fig. 4 would be unwise. In such cases, other approaches may have to be adopted, such as truncating the data and fitting a regression line to those data. The MIC is defined for a particular incubation time. Figure 5 shows that after about 10 h (dependent on initial inoculum size), this method can provide an accurate NIC and MIC. Changes in both values can occur with greater incubation times, and an examination of the rate of change in the NIC and MIC with incubation time may also be prudent.

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