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Variations in MIC value caused by differences in experimental protocol

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ABSTRACT

The minimal inhibitory concentration (MIC) of an antibiotic for a microorganism quantifies the effectiveness in reducing growth or the bactericidal ability of the compound. Measurements of MIC's carried out using different protocols should be comparable. Several of the factors that influence the outcome of the measurement vary between protocols. Variations in the MIC estimate were examined for *E. coli* and amoxicillin as well as tetracycline and for *Pseudomonas putida* and enrofloxacin. Duration of the measurement, density of the starting culture, the use of optical density or cell counts to determine growth and the induction of resistance can cause differences of a factor of up to 8 in the MIC value. While this does not hamper the reporting of trends by researchers adhering to the same protocol, it may affect assessments based on the absolute value of the MIC of a given combination of microorganism and antibiotic.

1. Introduction

The measurement of the Minimal Inhibitory Concentration (MIC) of an antibiotic for a given microorganism is considered to indicate the degree of susceptibility or resistance of this strain. The measured MIC is only valid for the particular "drug/bug" combination for which it has been determined. Even then the MIC gives only a coarse indication of the susceptibility of a bacterial population (Scavizzi et al., 2002). The need for consistent data on antimicrobial resistance has long been recognized (Stelling and O'Brien, 1997; Tenover et al., 2001). Many governments engage researchers to monitor trends in antibiotic resistance. The results from different reports need to be equivalent for comparisons between studies. Methods that assess susceptibility have been standardized by the Clinical and Laboratory Standards Institute (Wikler, 2006). These standards must be purchased and the costs are sometimes considered prohibitive. In addition, there is still some variation in outcome of MIC measurements possible, even if the protocols are followed. Therefore, attempts to further harmonize the methodology are being made (Bronzwaer et al., 2008). In this study we point out a few sources of variation that are known to occur in daily laboratory practice.

Physicians may need to calculate the optimal dose of antibiotics for treatment of severely ill patients (Pea and Viale, 2006; Pea et al., 2005; Roberts and Lipman, 2006; Smith and Gould, 2004). The MIC values used in this type of calculations need to reflect the response of the pathogen in the patient. While this will always be problematic, as many factors may influence the response of a microorganism to a certain antibiotic, a reliable MIC value is essential. Mathematical models describing the dynamics of an infection (Jesse et al., 2008), could be expanded to include antibiotic treatment. In such a model, an exact MIC value is indispensible. The Clinical and Laboratory Standards Institute has many standards for measurements of antimicrobial susceptibility, but even in these some potential sources of variation are not addressed.

In this study we examine factors that can influence the absolute value of MIC measurements, such as duration of the measurement, density of the inoculum, the actual parameter measured and age of the batch of antibiotic. These factors vary between protocols followed by different laboratories and can cause up to 8-fold differences in the outcome.

2. Materials and methods

2.1. Bacterial strains, growth media and growth conditions

For this study *E. coli* MG1655 and *Pseudomonas putida* NCTC10936 were used. *E. coli* was grown in medium described by Evans et al. (1970), containing 55 mM glucose. For shake-flask and MIC readings the Evans medium was buffered using 15.6 g/l $Na_2H_2PO_4$. pH was set to 7.0 using 4 N NaOH. *P. putida* was grown on either Evans medium or, alternatively, M9 medium. M9 medium contents per liter: 1 g NH_4Cl, 6 g $Na_2H_2PO_4$, 3 g KH_2PO_4, 493 mg $MgSO_4 \cdot 7H_2O$, 2.78 mg $FeSO_4 \cdot 7H_2O$, 1.11 mg $CaCl_2$, 3.7 μg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 24.7 μg H_3BO_3, 7.1 μg $CoCl_2 \cdot 6H_2O$, 2.5 μg $CuSO_4 \cdot 5H_2O$, 15.8 μg $MnCl_2 \cdot 4H_2O$,

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1.4 µg $ZnCl_2$, 0.5 mg nicotinic acid, 0.5 mg thiamine•HCl, 1 mg biotin, 0.5 mg choline chloride, 0.5 mg folic acid, 1 mg *myo*-inositol, 0.25 mg Ca-panthothenate and 0.5 mg pyridoxal•HCl. Media were autoclaved for 20 min at 120 °C, with the exception of glucose (10 min, 110 °C) and vitamin solutions (0.2 µm filter-sterilized). Both media were chosen because they are defined, as opposed to the media often prescribed by standardized protocols. Pre-cultures for inoculation of the 96-well plates were grown at 37 °C for *E. coli* and at 30 °C for *P. putida* in a shake-flask and continuously shaken at 200 rpm.

2.2. Minimal inhibitory concentration (MIC) readings

Serial dilutions, ranging from 0 µg/ml to 4096 µg/ml of each antibiotic, were made in 96 well-plates using either Evans or M9 medium, depending on the experiment. Tetracycline and amoxicillin stock solutions of 10 mg/ml were freshly made for each experiment in 1 N HCl. Enrofloxacin (10 mg/ml) was dissolved in 0.1 N HCL and after dissolution brought to pH 10. All stock solution were 0.2 µm filter-sterilized and stored at 4 °C prior to use. Tetracycline stock solutions were wrapped in tin-foil. *E. coli* or *P. putida* were inoculated into each well to an approximate starting OD_{600} of 0.05. For endpoint MIC readings, plates were read at 600 nm for the initial OD ($t=0$) and then incubated in an incubator at 37 °C (for *E. coli*) or 30 °C (for *P. putida*). After 24, 48 and 72 h, the plates were read again at 600 nm. Alternatively, growth was followed in time in the micro-titer plate reader, using 15 min reading and 7.5 min shaking intervals (for the first 8 h) and 1 h reading and 15 min shaking intervals for the remainder of the experiment at the appropriate temperatures. For 96-well measurements a SPECTRAmax 384 Plus with SOFTmax Pro software, manufactured by Molecular Devices (Sunnyvale, California, USA), was used.

The MIC was defined as the lowest concentration of antibiotic that reduced the growth to an OD at the time of the measurement of 0.2 or less. In most experiments, optical density (600 nm) measurements were compared to cell numbers as determined by counting in a heamocytometer under the microscope.

3. Results

The time courses of growth of *E. coli* MG 1655 in the presence of various concentrations of amoxicillin and tetracycline and *P. putida* NCTC10936 in the presence of enrofloxacin are shown in Fig. 1. In fact, the term "growth" may not be appropriate, as OD, rather than cell numbers, was measured during the experiments. In the samples for cell counts, swollen cells were observed in the case of *E. coli* in the presence of amoxicillin and to a lesser degree also for the combination of *E. coli* and tetracycline. The differences in the curves reflect the interaction of the antibiotic with the microorganism. Amoxicillin interferes with cell wall synthesis and this is reflected in the initial OD increase and subsequent decline of *E. coli* exposed to intermediate levels of amoxicillin. At concentrations that allow limited growth, amoxicillin does not reduce the rate of OD increase of *E. coli*, but tetracycline, that inhibits protein synthesis, does. The effect of enrofloxacin, which inhibits DNA synthesis, on growth rate of *P. putida* is similar to that of tetracycline on *E. coli*.

The estimates of the MIC are influenced considerably by the timing of the measurement (Table 1). Though 3 h may be very short for such measurements, the time points of 6, 12 and 24 h could all appear to give valid estimates. The estimates obtained, however can vary by a factor of up to 4 for any combination of antibiotic and microorganism. *E. coli* exposed to amoxicillin seems to have a high MIC after 3 or 6 h, which decreases afterwards. While *E. coli* seems hardly hampered by 2 µg/ml tetracycline after 24 h, after 6 h it seems to be barely growing in comparison to the control. The MIC of *P. putida* in the presence of enrofloxacin appeared to decrease from 6 to 24 h by a factor of 4, though at the very end of the experiment some growth started to occur at 0.5 µg/ml enrofloxacin, indicating the induction of resistance.

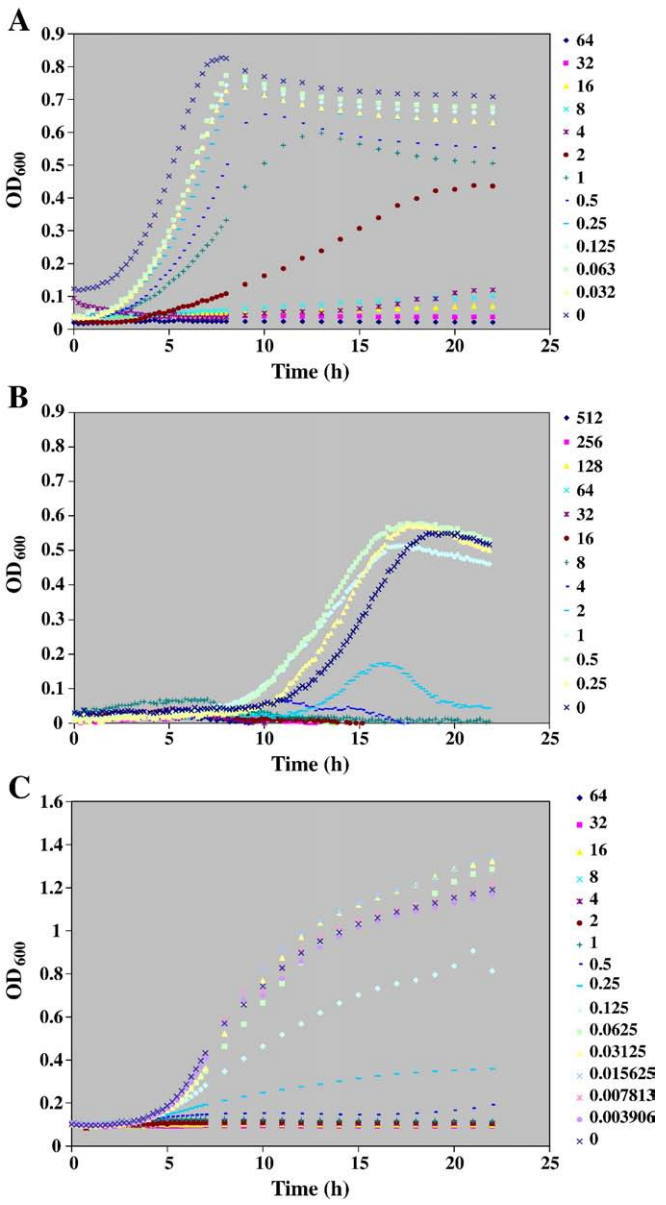

Fig. 1. Experiments measuring the optical density of cultures as a function of time at varying concentrations of antibiotics to determine the MIC. (A) Growth of *E. coli* at various concentrations of tetracycline. (B) Growth of *E. coli* at various concentrations of amoxicillin. (C) Growth of *P. putida* at various concentrations of enrofloxacin.

The density of the culture at the start of the experiment considerably influences the outcome of the MIC measurement (Fig. 2). Low density cultures with an initial OD of 0.01 yield a MIC estimate of between 0.5

Table 1
Estimates of MIC values as a function of the length of the measurement.

Organism	Drug	Time (hours)	Estimated MIC (µg/ml)
E. coli	Tetracycline	3	1.0
		6	2.0
		12	2.0
		24	4.0
E. coli	Amoxicillin	3	32
		6	16
		12	8
		24	8
P. putida	Enrofloxacin	3	ND
		6	0.125
		12	0.25
		24	0.5