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Combining diagnostic methods for antimicrobial susceptibility testing – A comparative approach

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ABSTRACT

Background: The minimum inhibitory concentration (MIC) is a measure of antimicrobial susceptibility testing (AST) of a given antibiotic but provides insufficient information when bacterial killing is crucial, *e.g.*, when treating immunocompromised patients. In these cases, the minimum bactericidal concentration (MBC) is a more reliable measure of antibiotic activity. Here, we aim to demonstrate and recommend combinations of methods for MIC and MBC measurements. We also aim to emphasize the importance of uniform protocols for these procedures including the time point for reading MIC results, which the authors suggest to be 20 h.

Methods: To address the challenges with obtaining fast and reliable readouts on MIC as well as the kinetic and end-point effects of antibiotics, the broth micro dilution method, a calorimetric method and a microscopy-based screening system (MBSS) were evaluated in this study. For MBC determination, fluorophore staining with SYTO9 and propidium iodide was compared to the broth regrowth method.

Results: Three scenarios for combining the MIC and MBC methods depending on the investigators' primary concern (time, cost or sensitivity) are presented. Further, as the MBSS and the isothermal microcalorimetry method detected delayed bacterial growth up to 18 h after initiation of experiments, the importance of reading MIC testing after a full 20 h is emphasized. A one-fold change in MIC values can be observed when comparing data obtained at 16 h and 20 h of incubation.

Conclusion: The authors suggest that combining MIC and MBC determinations will provide more detailed understanding of the bacteria susceptibility to antibiotic drugs and result in more clinically relevant data and optimized therapies. Furthermore, establishing 20 h as a time point for reading MIC results will provide more uniform data across laboratories.

1. Introduction

In the search for efficient antibiotic therapies, antimicrobial susceptibility testing (AST) is performed *in vitro* in experimental and diagnostic laboratories worldwide. Standardized experimental conditions are thus essential for obtaining reliable and reproducible results. As a measure of AST, the minimum inhibitory concentration (MIC) is used and is defined as the lowest concentration of an antibiotic which inhibits visible growth of a microorganism after 16–20 h of incubation (Andrews, 2001). However, the MIC does not reveal whether the antibiotic is bacteriostatic or bactericidal and is a weak predictor of the antibiotic efficacy *in vivo* (Wiegand et al., 2008).

Especially with regard to antibiotic therapy in patients with inflammatory diseases (Andrews, 2001; Brennan and Durack, 1983) or immunocompromised patients, it is crucial to determine the minimum bactericidal concentration (MBC), since an intact immune system is necessary to eliminate inhibited pathogens (Bär et al., 2009). The MBC is the lowest concentration that kills the bacteria, with a 99.9% or 10^3 reductions in the number of bacterial colonies on subculture (Taylor et al., 1983; Meylan et al., 1986; Lowy et al., 1983; Pankey and Sabath, 2004). Comparing MBC values helps to rationally choose the most efficacious antibiotic for compromised patients (Taylor et al., 1983).

Many studies have used the MBC and the MBC/MIC ratio to assess bactericidal activity of antibiotics, correlate *in vitro* data with possible outcomes of *in vivo* treatments (Brennan and Durack, 1983; Meylan et al., 1986; Lowy et al., 1983) and even predict outcomes of *in vivo* treatments (Lowy et al., 1983; Pankey and Sabath, 2004; Tuomanen et al., 1986). Determination of MBC has received some criticism, as the commonly used test has shown poor reproducibility between laboratories (Taylor et al., 1983; Meylan et al., 1986; Pelletier, 1984). The test is derived from the broth dilution procedure and involves re-growth of bacteria in antibiotic-free media (Taylor et al., 1983). The variability in

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Table 1
MIC and MBC values for the tested antibiotics against *P. aeruginosa*, *S. aureus*, and *E. coli* obtained by using the methods described. MICs and MBCs are expressed as μg/mL. Values represent median value [μg/mL] of up to three independent experiments. A scaling factor of 2 should be considered when performing fluorescence to determine MBC values, due to a consistent underestimation of MBC when this method is used. For all measurements N = 3. Asterisk (*) indicates the sample where N = 2.

Bacteria	Antibiotic	MIC values			MBC values	
		Broth dilution	Microscopy	Calorimetry	Broth dilution	Fluorescence
P. aeruginosa	Gentamicin	0.5	0.5	2	0.5	0.125
	Tobramycin	0.25	0.5	2	0.5	0.125
	Ciprofloxacin	< 0.0625	< 0.0625	0.125	0.125	0.0625
	Colistin	2	2	2	2	2
S. aureus	Gentamicin	32	64	32	64	32
	Tobramycin	32	32	32*	32	16
	Ciprofloxacin	0.125	0.25	0.25	0.25	0.125
	Vancomycin	2	2	2	4	1
	Colistin	16	16	16	16	16
E. coli	Gentamicin	0.25	0.25	2	0.5	1
	Tobramycin	0.5	1	2	1	0.5
	Ciprofloxacin	< 0.0625	< 0.0625	< 0.0625	< 0.0625	< 0.0625
	Colistin	2	2	1	2	2

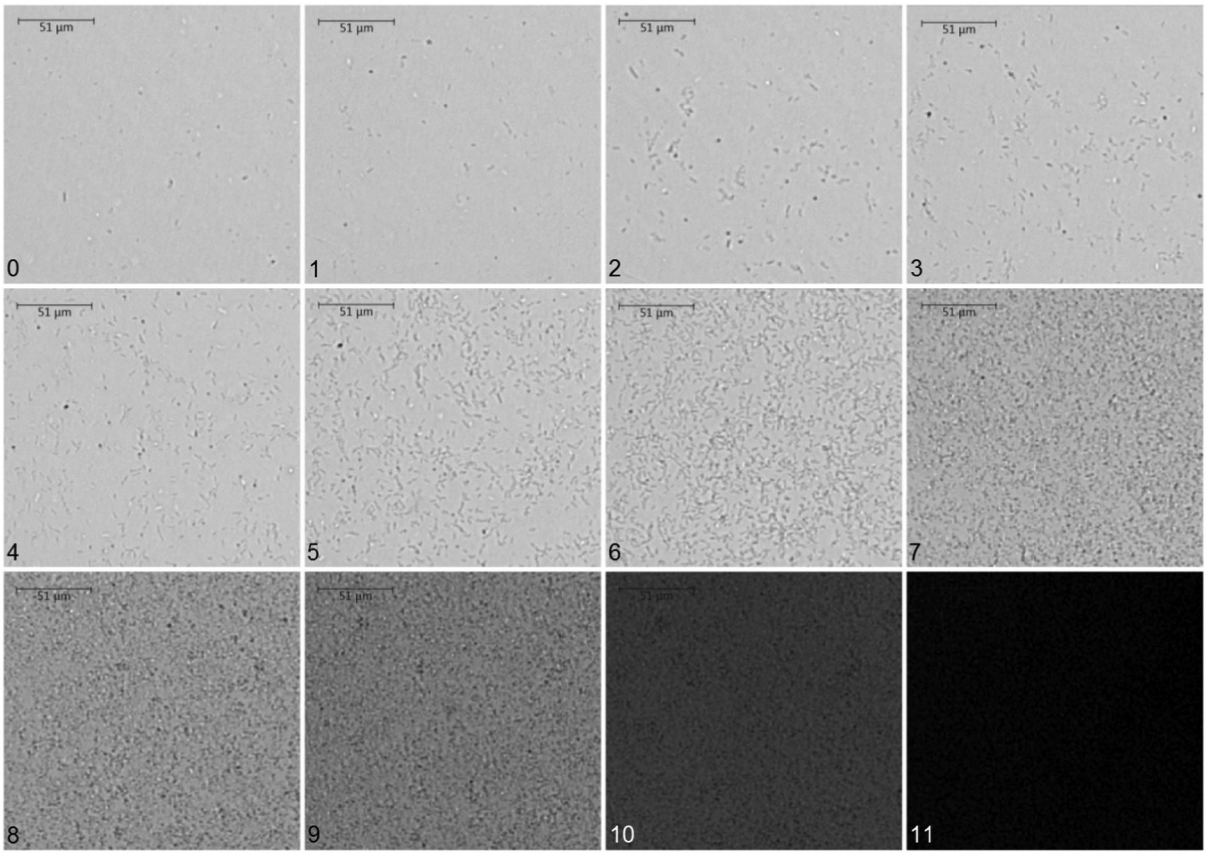

Fig. 1. Representative growth images of *P. aeruginosa* exposed to sub-MIC (0.125 μg/mL) concentration of gentamicin as obtained by using the microscopy-based screening system. The bacteria are evenly distributed in the observed area, with bacterial growth resulting in a gradual increase in light obscuration. The numbers on the image indicate hours after initiation of the measurement (0 denotes the image taken at time 0, 1 indicates image taken after 1 h, *etc.*). Scale bar indicates 51 μm.

reported MBC values is attributed to lack of specific guidelines and differences in technical details of the procedure, such as the size of the inoculum used (Brennan and Durack, 1983), sample mixing (Taylor et al., 1983; Pelletier, 1984), and growth phase of the bacteria (Brennan and Durack, 1983; Meylan et al., 1986). These details do not affect MIC values, yet are critical factors for the outcomes of MBC determinations (Brennan and Durack, 1983; Taylor et al., 1983). Nonetheless, reports suggest that MBC is of higher clinical relevance when predicting the response of bacteria to antibiotic therapy *in vivo*, and thus the determination of both MIC and MBC is advisable (Brennan and Durack, 1983; Bär et al., 2009).

This study reports an objective comparison of methodologies by measuring the effect of a range of antibiotics on bacterial growth inhibition and bacterial killing. The influence of the antibiotics on growth kinetics was also assessed. Thus, the implementation of isothermal microcalorimetry (IMC) and a microscopy-based screening system for real-time determination of MIC values as alternatives to traditional endpoint measurements were pursued. IMC has been used previously to study growth, metabolism, and susceptibility to antibiotics used against *Pseudomonas aeruginosa* (Esarte López et al., 2015; Lago et al., 2011), *Staphylococcus aureus* (Li et al., 2012; Entenza et al., 2014) and *Escherichia coli* (Zaharia et al., 2013; Vazquez et al., 2014; Shi et al.,