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Original Scientific Paper

Optimisation of the microdilution method for detection of minimum inhibitory concentration values in selected bacteria

Stoimir Kolarević[1*], Dragana Milovanović[1], Merisa Avdović[1], Mariana Oalđe[2], Jovana Kostić[1,3], Karolina Sunjog[1,3], Biljana Nikolić[1], Jelena Knežević-Vukčević[1] and Branka Vuković-Gačić[1]

1 Chair of Microbiology, Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia

2 Chair of Plant Morphology and Systematics, Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade, Takovska 43, 11000 Belgrade, Serbia

3 Institute for Multidisciplinary Research, University of Belgrade, Kneza Višeslava 1, 11000 Belgrade, Serbia

ABSTRACT: In this study we investigated the influence of preparation of the bacterial inoculum for a microdilution susceptibility test, e.g., the effect of its optical density, on assessment of the minimum inhibitory concentrations (MIC). The approach employed in the majority of microdilution susceptibility studies is use of the same optical density for preparation of inoculums for different bacterial strains. In the present work, this approach was questioned by determining the ratio between the optical density and the number of bacteria in cultures. We also investigated whether the number of bacteria in inoculums can affect assessment of the MIC value for two antibiotics of broad spectra, rifampicin and streptomycin.

The study was performed on four Gram-positive and four Gram-negative bacteria (ATCC collection) commonly used to investigate antimicrobial potential. The ratio between the optical density and number of bacteria in cultures was determined for each strain, and a strong linear correlation was detected. However, it was evident that different bacteria have different cell numbers at the same OD_{600} value. Based on the obtained results, inoculums for selected strains were prepared to obtain final cell numbers of 10^3, 10^4, 10^5 and 10^6 /well in the microdilution assay. Two different approaches were used in determining the MIC for rifampicin and streptomycin: approximation of MIC with IC_{90} and the resazurin reduction assay.

Our results indicated that the ratio between optical density and cell numbers is not constant and use of the same OD for inoculums for all strains can therefore lead to misinterpretation of the MIC values. We also observed influence of cell numbers in inoculums in determination of MIC values. For both approaches used (approximation of MIC with IC_{90} and the resazurin reduction assay), the same trend was detected: antibiotics had the highest potency in experiments with the lowest bacteria cell number (10^3/well). The lowest cell number (10^3/well) is not recommended, as it can lead to false susceptibility results and to partial reduction of resazurin, which further complicates MIC determination. A final cell number of 10^4/well can therefore be recommended as optimal.

Keywords: MIC, minimum inhibitory concentration, antibacterial activity, microdilution, resazurin

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*correspondence: stoimirk@bio.bg.ac.rs

INTRODUCTION

Due to the growing occurrence of microbial resistance to currently available antimicrobials, there is a continuous need for new agents, such as biologically active substances of plant origin, which can serve as a potential alternative. It is therefore necessary to develop and optimise tests that will reliably show the actual antimicrobial activity of selected substances.

In general, assessment of antimicrobial activity is performed by three basic types of tests: diffusion, dilution and bioautography (Fennell *et al.* 2004). The diffusion and bioautography tests only provide general information about the possible existence of antimicrobial activity, while the dilution method provides information on the lowest concentration of the compound capable of inhibiting growth – the minimum inhibitory concentration (MIC) (Mann & Markham 1998).

Determinations of MIC values can be used to follow the development of resistance to antibiotic drugs (Wiegand *et al.* 2008). In theory, MICs could be a valuable tool for comparing different susceptibility. Unfortunately, however, MICs obtained for various substances in different studies cannot be compared due to variations in the protocols that are applied in different laboratories (Rios *et al.* 1988).

One of the variables which could significantly influence the determination of MIC values is preparation of the bacterial inoculum used in the microdilution method. In most cases, authors for multiple strains use inoculum prepared by McFarland standard 0.5 (Karaman *et al.* 2003; Sahin *et al.* 2003; Schuurmans *et al.* 2009). In this case the approximation of optical density (OD) is very subjective and difficultly reproducibile between two laboratories (Sarker *et al.* 2007). Moreover, use of this approach is questionable, as the same McFarland standard 0.5 for different strains does not mean the same concentration of bacteria. Since the microdilution method is currently the most appropriate way to determinine MIC values (taking into account speed of execution and price), it is essential to standardise protocols, which will permit comparison of the data obtained in different laboratories.

The main objective of this study was to investigate the influence of inoculum preparation in terms of the number of bacteria in the microdilution method for assessment of MIC values when using two antibiotics of broad spectra, rifampicin and streptomycin. First of all, since the size, shape and grouping of bacteria can affect the values of optical density, we wanted to investigate the indirectly assessed relation between optical density and bacterial numbers. The study was performed on four Gram-positive and four Gram-negative bacteria commonly used to investigate antimicrobial potential. Afterwards, based on data obtained from the calibration curves, inoculums with different numbers of bacteria were prepared for the microdilution method to test the impact of bacterial cell numbers on the assessment of MIC values for antibiotics.

MATERIALS AND METHODS

2.1. Bacteria and media. The study was performed on four strains of Gram-negative bacteria (*Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC15442, *Salmonella enteritidis* ATCC12076, *Shigella flexneri* ATCC9199) and four strains of Gram-positive bacteria (*Enterococcus fecalis* ATCC 29212, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633 and *Listeria innocua* ATCC 33090). Bacterial strains were cultivated in Mueller-Hinton broth (MHB) or brain-heart infusion broth (BHI) (*L. innocua*) for 24 h at 37 °C. Streak plates on Mueller-Hinton agar (MHA) or brain-heart infusion agar (BHA) (*L. innocua*) were incubated for 24 h at 37°C and stored at 4°C for up to one month.

2.2. Assessment of the relation between optical density and bacterial cell numbers. For each strain, one colony was picked from an agar plate, inoculated in 50 mL of MHB (BHI for *L. innocua*) and incubated at 37°C with aeration at 185 rpm. At different time intervals, optical density of cultures was read at 600 nm (OD_{600}). The number of viable bacteria at 0.1, 0.2, 0.3 and 0.4 OD_{600} was determined using the indirect method, 5 mL of culture was centrifuged at 4,000 rpm, supernatants were discharged, the pellets resuspended in 0.01 M $MgSO_4$ and 0.1 mL of appropriate dilution was plated on MHA (BHA for *L. innocua*) and incubated for 24 h at 37 °C. Experiments were performed in triplicate in three individual trials.

2.3. Determination of the minimum inhibitory concentration (MIC). For this study two antibiotics of broad spectra were chosen: streptomycin for *E. coli*, *S. enteritidis*, *S. flexneri*, *P. aeruginosa*, *S. aureus* and *B. subtilis*; and rifampicin for *E. faecalis* and *L. innocua*. The microtitre plate-based antibacterial assay was performed according to the protocol of Sarker *et al.* (2007) with slight modifications. Bacterial cultures were prepared as described (section 2.2), incubated until they reached the cell numbers

Table 1. Values of OD_{600} and corresponding cell numbers.

Bacteria	OD_{600} of bacterial culture	Cell number
Staphylococcus aureus	0.3	2.3×10^8
Enterococcus faecalis	0.2	2.5×10^8
Bacillus subtilis	0.3	2.4×10^7
Listeria innocua	0.2	2.2×10^8
Escherichia coli	0.3	1.2×10^8
Shigella flexneri	0.2	2.6×10^8
Salmonella enteritidis	0.3	1.5×10^8
Pseudomonas aeruginosa	0.4	1.7×10^8

indicated in Table 1, and diluted in 0.01 M $MgSO_4$ to obtain final cell numbers of 10^5, 10^6, 10^7 and 10^8/mL.

A concentration gradient of each antibiotic was created in 96 sterile well plates. In the first row, 200 µL of antibiotic in MHB (or BHI for *L. innocua*) was added to the wells, while 100 µL of MHB (or BHI for *L. innocua*) was added to the remaining wells. Serial dilutions were performed by pipetting 100 µL of antibiotic solution from row to row in serially decreasing concentrations. To obtain the final cell number (10^3, 10^4, 10^5 and 10^6 /well), 80 µL of media and 20 µL of bacteria were added to each well. The plates also included a negative control (media only) and a bacteria growth control (media and bacteria). They were wrapped with parafilm to prevent dehydration, incubated for 24 h at 37°C and read at 600 nm (Multiscan FC, Thermo Scientific, USA). The concentration of antibiotics which reduced bacterial growth by 90% was declared to be the MIC value. For the resazurin reduction assay, microtitre plates were additionally incubated for 3h with 22 µL of an aqueous solution of resazurin [0.675 mg of resazurin sodium salt (TCI Europe, Belgium) per mL].

The results were analysed visually by observing the changes in colour of resazurin, changes from purple to pink or colourless being recorded as positive. The lowest concentration of antibiotic with no change of resazurin colour was taken as the MIC value. Effects of antibiotics were tested in triplicate in two individual experiments.

2.4. Statistical analyses. Statistical analysis of results obtained in the experiments was carried out using Statistica 6.0 software (StatSoft, Inc.). The Kolmogorov-Smirnov test for distribution normality was used prior to statistical analysis. Since the data were in line with the requirements for the application of parametric tests, correlation analyses were carried out using the Pearson correlation test with a significance level $p < 0.05$.

RESULTS

3.1. Assessment of the relation between optical density and bacterial cell numbers. For each of the selected optical densities of bacterial culture, the number of viable cells

Table 2. Values of MICs (µg/mL) determined by approximation with IC_{90} value and by resazurin reduction assay; values represent mean ± SD of two individual experiments.

Bacterial strain	Approach in MIC assessment	Number of bacteria/well			
		10^3	10^4	10^5	10^6
S. aureus	IC_{90}	2.8	3.0 ± 0.1	3.0 ± 0.1	6.0
	resazurin	1.6	2.3 ± 1.1	3.1	6.3
E. faecalis	IC90	1.2 ± 0.3	1.4	1.1 ± 0.6	9.4 ± 4.4
	resazurin	3.1	3.1	25	ND
B. subtilis	IC_{90}	0.6 ± 0.3	0.8 ± 0.1	2.0 ± 0.6	NT
	resazurin	0.8	1.2 ± 0.6	3.1	NT
L. innocua	IC_{90}	0.2 ± 0.1	0.3 ± 0.1	0.5 ± 0.4	5.6
	resazurin	0.2 ± 0.2	0.2	0.3 ± 0.1	0.6 ± 0.3
E. coli	IC_{90}	2.8	5.8 ± 3.8	11.5 ± 12.8	12.8 ± 5.9
	resazurin	3.1	4.7 ± 2.2	14.1 ± 15.5	18.8 ± 8.8
S. enteritidis	IC_{90}	1.5 ± 0.1	1.5 ± 0.1	5.0 ± 3.3	14.0 ± 3.5
	resazurin	1.6	1.5	2.3 ± 1.1	6.3
S. flexneri	IC_{90}	1.3 ± 0.1	2.4 ± 0.3	2.1 ± 0.8	2.6 ± 0.4
	resazurin	1.6	4.7 ± 2.2	4.7 ± 2.2	3.1
P. aeruginosa	IC_{90}	19.3 ± 3.2	21.5 ± 8.5	46.5	95.0
	resazurin	18.8 ± 8.8	18.8 ± 8.8	25	100

ND – not detected
NT – not tested

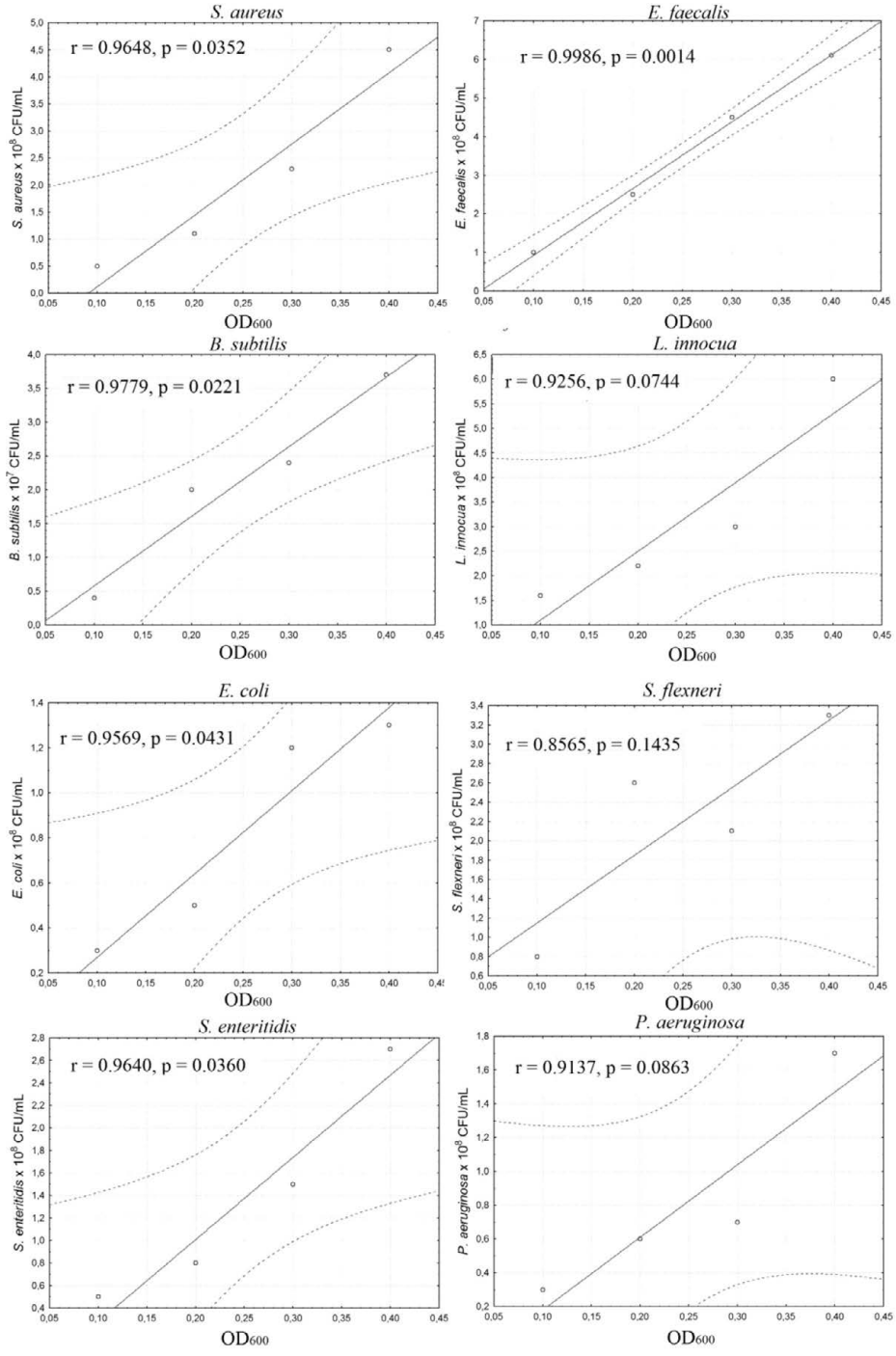

Figure 1. Relation between optical density and cell numbers determined using the indirect method; full line – linear regression line, dashed line – 95% confidence interval.

was assessed using the indirect method. A strong linear correlation between OD_{600} and cell numbers was detected for all tested bacteria (Fig. 1). However, it is evident that different bacterial strains showed different cell numbers at the same OD_{600} value. For further work we used bacteria in the exponential phase of growth with a similar cell number (10^8/mL), except in the case of *B. subtilis* (10^7/mL). The values are summarised in Table 1.

3.2. Determination of the minimum inhibitory concentration (MIC). The effect of different numbers of bacteria in inoculums on antibiotic potency was judged from inhibition of growth measured at 600 nm (Fig. 2). As expected, the tested antibiotics had the highest potency as inhibitors of bacterial growth in cultures with low cell numbers (10^3/well) and the lowest potency in ones with high cell numbers (10^6/well for all strains

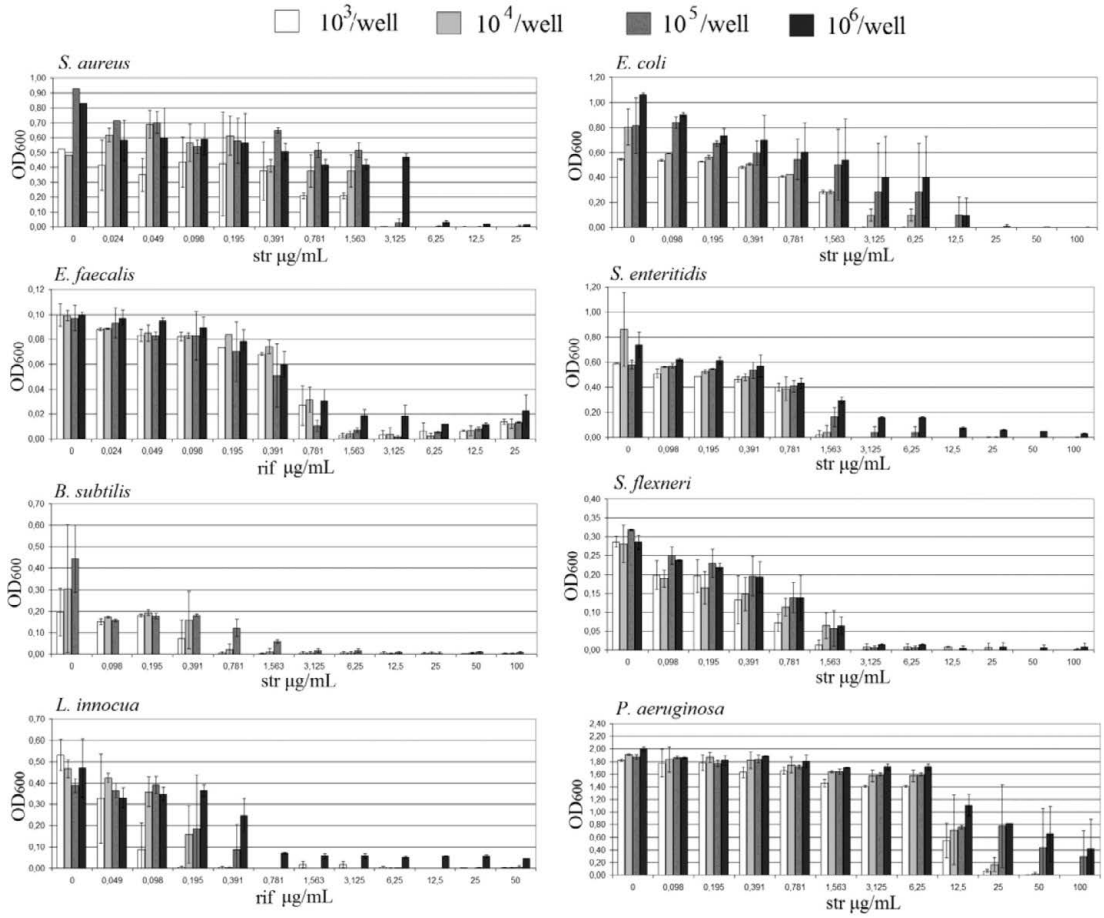

Figure 2. Impact of streptomycin (str) and rifampicin (rif) on growth in bacterial cultures with different cell numbers (10^3 - 10^6/well) assessed spectrophotometrically (OD_{600}); values represent mean ± SD of two individual experiments.

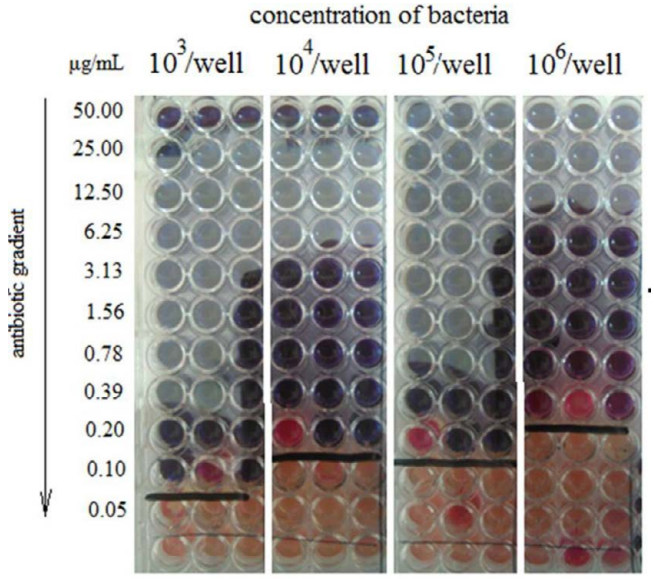

Figure 3. Impact of cell numbers (*L. innocua*) in detection of MICs for rifampicin in the resazurin reduction assay.

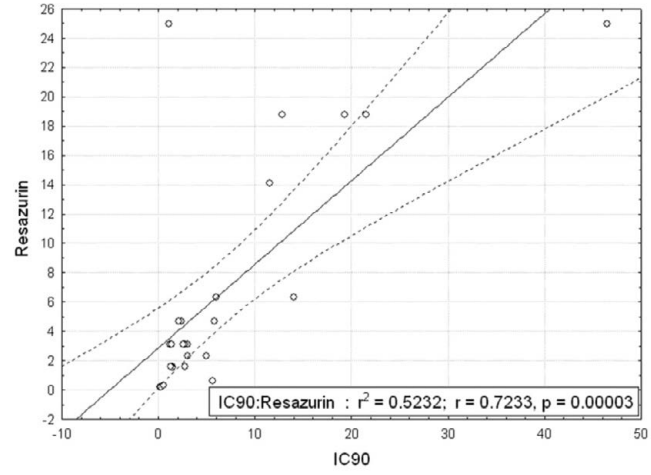

Figure 4. Relation between the mean values of MIC (µg/mL) determined by approximation with IC_{90} value and by reduction of resazurin.

except *B. subtilis*, where 10^5/well was the highest tested concentration).

Results of the resazurin assay also indicated that assessment of MICs is influenced by cell numbers. It follows from the data presented in Fig. 3 that the gradient of MICs depended on cell numbers in the case of *L. innocua*.

Values of MICs (μg/mL) determined by approximation with the IC_{90} value (the antibiotic concentration that inhibits 90% of bacterial growth) and by the resazurin reduction assay are shown in Table 2. It can be seen that in both cases the same trend was detected: antibiotics had the highest potency in experiments with the lowest bacteria cell numbers. Moreover, when the MICs obtained by approximation with IC_{90} were plotted against MICs obtained by the resazurin reduction assay (Fig. 4), a significant positive correlation was detected ($r = 0.7233$, $p = 0.00003$).

DISCUSSION

In this study we have taken a step toward the optimisation of the microdilution protocol for MIC assessment. The focus was placed on preparation of the bacterial inoculum, as this variable can significantly influence the assessment of MIC values. Moreover, we used two different approaches in determining MIC: (1) approximation of MIC on the basis of IC_{90} values obtained with OD; and (2) the resazurin reduction assay.

We have here primarily questioned the approach employed in the majority of studies, where the same optical density is used for preparation of inoculums for different bacterial strains. Our results indicate that this approach can lead to misinterpretation of the obtained data. For the investigated strains, the ratio between optical density and bacterial numbers is not constant. For example, at OD_{600} 0.4 the number of viable bacteria for *E. faecalis* was 6.1×10^8/mL, while for *B. subtilis* the number at the same OD was only 3.7×10^7/mL. Based on our calibration experiments, we selected for each of the tested bacteria an appropriate OD that represented the starting point for inoculums used in the microdilution assay.

The approximation of IC_{90} values as possible MICs was based on the studies of Kronvall *et al.* (2006) and Okeleye *et al.* (2013), while the resazurin assay relies on an oxidation–reduction indicator that is commonly used for evaluation of cell growth and MIC assessment. The indicator in question is a blue non-fluorescent and non-toxic dye which converts to pink and fluorescent resorufin on being reduced by oxidoreductases in viable cells (Sarker *et al.* 2007). In view of the correlation between the approaches used, it is difficult to point out one of them as more efficient. The only drawback to which we can call attention is related to the resazurin reaction, in which the occurrence of transitional wells with partial reduction of resazurin was observed, so it could not be said with certainty whether or not growth was inhibited.

Further, we wanted to investigate whether the number of bacteria in inoculums can affect assessment of the MIC value. We investigated the influence of cell numbers ranging from 10^3 to 10^6/well, and for each of the tested strains it was evident that antibiotics have the highest potency in experiments with the lowest cell number used (10^3/well). The best example is *E. faecalis*, in which case the MIC for rifampicin at the lowest cell number (10^3/well) was detected at 3.1 μg/mL, while for the highest cell number (10^6/well) no MIC was detected at all. One possible way of explaining increased resistance is by attributing it to the protective effect that results from grouping of bacteria in cultures with high numbers. In this case, the antibiotic has a weaker effect simply because of inferior penetrability (Stewart 2002). A high cell number in the inoculum can lead to an increase of MIC, particularly if the tested bacterium produces an enzyme capable of destroying the antibiotic, as is frequently seen when testing beta-lactam susceptibility for isolates which produce beta-lactamases that inactivate beta-lactam antibiotics (Wiegand *et al.* 2008).

However, choosing the lowest cell number for inoculum preparation is not recommended. In the first place, use of inoculums with cell numbers of less than 5×10^5 mL can lead to false susceptibility results (Chambers 1988; Granier *et al.* 2002; Wiegand *et al.* 2008). Low cell numbers in inoculums can also lead to partial reduction of resazurin, which further complicates determination of MIC values (Mann *et al.* 1998). This is a problem we faced in the case of *E. faecalis*. On the other hand, if the cell number in the inoculum is too high, complete reduction of resazurin may occur. A final cell number of 10^4/well can therefore be recommended as the optimum, which is in compliance with the guidelines of Wiegand *et al.* (2008).

CONCLUSIONS

It can be concluded from the results of this study that preparation of bacterial inoculums for the microdilution assay can influence the assessment of MICs. Use of the same optical density for preparation of inoculums for different bacterial strains can lead to misinterpretation, since the ratio between optical density and cell numbers is not the same for different strains. It is evident that the cell number in inoculums also affects MIC assessment, seeing as how the antibiotics had the highest potency in experiments with the lowest cell number used (10^3/well). Nevertheless, use of a final cell number of 10^4/well in the microdilution assay is recommended because numbers lower than that can lead to additional complications in MIC assessment, complications such as false susceptibility results and partial reduction of resazurin.

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Botanica SERBICA

REZIME

Optimizacija protokola mikrodilucione metode za određivanje vrednosti minimalnih inhibitornih koncentracija na odabranim bakterijama

Stoimir Kolarević, Dragana Milovanović, Merisa Avdović, Mariana Oalđe, Jovana Kostić, Karolina Sunjog, Biljana Nikolić, Jelena Knežević-Vukčević i Branka Vuković-Gačić

U ovom radu praćen je uticaj broja bakterija u inokulumu za mikrodilucionu metodu na minimalnu inhibitornu koncentraciju (MIK). Preispitan je pristup u pripremi inokuluma koji se koristi u većini studija a koji podrazumeva korišćenje iste optičke gustine za inokulume različitih bakterijskih vrsta. Takođe, ispitivan je uticaj različitog broja bakterija u inokulumu na dobijeni MIK za dva antibiotika širokog spektra, rifampicina i streptomicina.

Studija je rađena na 4 grampozitivne i 4 gramnegativne bakterijske vrste (ATCC kolekcija) koje se uobičajeno koriste u detekciji antimikrobnog potencijala. Za svaku vrstu, prvo je određen odnos optičke gustine i broja bakterija u kulturi. Zabeležena je jaka linearna korelacija između OD_{600} i broja živih ćelija. Međutim, za istu vrednost OD_{600} primećena je razlika u broju živih ćelija kod različitih bakterijskih vrsta. Na osnovu dobijenih rezultata, pripremljeni su inokulumi za mikrodilucionu metodu tako da finalna koncentracija bakterija u bunarima mikrotitarske ploče iznosi 10^3, 10^4, 10^5 i 10^6. Dva različita pristupa su korišćena za određivanje MIK za rifampicin i streptomicin - aproksimacijom MIK sa IC_{90} i redukcijom resazurina. Naši rezultati pokazuju da odnos optičke gustine i broja ćelija varira kod ispitivanih vrsta i da korišćenje iste optičke gustine za pripremu inokuluma za različite sojeve može dovesti do pogrešne interpretacije MIK. Isti trend je dobijen za oba pristupa (aproksimacija MIC s IC_{90} i redukcija resazurina) - antibiotici su imali najveći efekat u eksperimentima s najmanjim brojem ćelija (10^3/bunaru). Međutim, odabir najmanjeg broja ćelija (10^3/bunaru) može dovesti do lažno-pozitivnih rezultata i delimične redukcije resazurina što još više otežava određivanje MIK. Zbog toga, finalni broj ćelija 10^4/bunaru se preporučuje kao optimalan.

Ključne reči: MIK, minimalna inhibitorna koncentracija, antibakterijska aktivnost, mikrodilucija, resazurin