Journal of Antimicrobial Chemotherapy (2009) **63**, 745–757
doi:10.1093/jac/dkn554
Advance Access publication 13 February 2009

JAC

Functional relationship between bacterial cell density and the efficacy of antibiotics

Klas I. Udekwu[1], Nicholas Parrish[1], Peter Ankomah[1], Fernando Baquero[2] and Bruce R. Levin[1]*

[1]Department of Biology, Emory University, Atlanta, GA 30307, USA; [2]Department of Microbiology, FIBio-RYC, Ramón y Cajal University Hospital, and CIBERESP, Madrid, Spain

Received 31 October 2008; returned 10 December 2008; revised 17 December 2008; accepted 18 December 2008

***Objectives*: To determine the functional relationship between the density of bacteria and the pharmacodynamics of antibiotics, and the potential consequences of this inoculum effect on the microbiological course of antibiotic treatment of *Staphylococcus aureus* infections.**

***Methods*: *In vitro* time–kill, MIC estimation and antibiotic bioassay experiments were performed with *S. aureus* ATCC 25923 to ascertain the functional relationship between rates of kill and the MICs of six classes of antibiotics and the density of bacteria exposed. The potential consequences of the observed inoculum effects on the microbiological course of antibiotic treatment are explored with a mathematical model.**

***Results*: Modest or substantial inoculum effects on efficacy were observed for all six antibiotics studied, such as density-dependent declines in the rate and extent of antibiotic-mediated killing and increases in MIC. Although these measures of antibiotic efficacy declined with inoculum, this density effect did not increase monotonically. At higher densities, the rate of kill of ciprofloxacin and oxacillin declined with the antibiotic concentration. For daptomycin and vancomycin, much of this inoculum effect is due to density-dependent reductions in the effective concentration of the antibiotic. For the other four antibiotics, this density effect is primarily associated with a decrease in per-cell antibiotic concentration. With parameters in the range estimated, our mathematical model predicts that the course of antibiotic treatment can be affected by cell density; treatment protocols based on conventional (density-independent) MICs can fail to clear higher density infections.**

***Conclusions*: The MICs used for pharmacokinetic/pharmacodynamic indices should be functions of the anticipated densities of the infecting population.**

Keywords: density, pharmacodynamics, MIC, inoculum

Introduction

The MIC of antibiotics is employed both as part (or all) of the criteria for susceptibility (resistance) of bacteria to these drugs and as the single, quantitative pharmacodynamic (PD) parameter formally used for the rational design of antibiotic treatment protocols, the denominator of the pharmacokinetic PK/PD index.[1–9]

In accordance with the CLSI guidelines,[10] MICs are estimated over a short period (e.g. 18 h), from optical density (OD) data in cultures inoculated with $\sim 5 \times 10^5$ cells/mL of bacteria growing exponentially, at temperatures and in media optimal for their growth and the action of the antibiotic.[10,11] It is obvious that MICs estimated in this way do not consider a number of the realities of bacteria in infected hosts including: (i) the physiological state of the bacteria—most antibiotics are ineffective against cells that are not actively growing;[12–14] (ii) persistence—minority populations of non-growing bacteria in otherwise exponentially growing cultures;[15–17] (iii) the possible physical structure of the bacterial population—e.g. biofilms;[18–24] (iv) the relationship between the rate of growth (or death) of the bacteria and the concentration of the antibiotic—the shape of the PD function;[25,26] (v) the bacteria-driven local changes in the pH or other environmental conditions resulting in levels that are not optimal for the action of the antibiotic;[11,27] and (vi) potential post-antibiotic effects (PAEs).[28,29] Moreover, as shown by Mouton and Vinks,[30,31] MICs as measured by standard protocols may not be equal to the concentration of the antibiotic at which bacteria are neither killed nor grow [the stationary concentration (SC)]. In practice as well as in

*Corresponding author. Tel: +1-404-727-2826; Fax: +1-404-727-2880; E-mail: blevin@emory.edu

theory, the above-described factors can affect the course of antibiotic treatment[32–34] in ways not anticipated from estimates of conventional MICs.

In this report, we revisit another long-standing concern about the limitations of conventional MICs, the density (cells per volume unit) of bacteria being treated. There are at least two reasons to anticipate that the efficacy of antibiotics will decline with the density of the bacteria exposed. One would be a reduction in the effective concentration of the antibiotic (free active drug) in the medium, A_E, due to antibiotic-denaturing enzymes or by binding of the antibiotic to the cell structures of killed as well as viable bacteria and/or their structural chemical components and debris. The rate at which these processes occur would be proportional to the density of the bacteria exposed, D,[35–37] thus a declining $[A_E/D]$ ratio.

The second general reason to anticipate that the efficacy of antibiotics will decline with density when its effective concentration in the medium, A_0, is constant and unaffected by the bacteria is, at this juncture, less easy to explain mechanistically. For PD and physiological reasons that are likely to vary among species of bacteria and antibiotics, the rate of killing or inhibition of the growth of the bacteria can be proportional to the amount of antibiotic available to *each* bacterium at the time of exposure, the $[A_0/D]$ ratio. For example, for the same concentration of an antibiotic in the medium (let us say, 1 mg/L), for a culture containing 10^8 bacteria/mL, the amount of drug available *per bacterium* is 1/1000 as great as that in culture of 10^5 bacteria/mL. To be sure, at both densities the number of antibiotic molecules per bacterium would be enormous, but for probabilistic or physiological reasons the ability of antibiotics to kill or inhibit the growth of bacteria may require a massive number of molecules of the drug per cell or per target.

For whatever reason mechanistically, empirically, ever since the early days of antibiotics, it has been known that the relative amount of drug needed to inhibit the growth of a bacterial population increases with the density of that population (for example, see refs 11, 38–41) and for some antibiotics, the relationship between the MIC and the SC changes with the density of bacteria exposed.[31] The magnitude of this 'inoculum' effect is frequently recommended for inclusion among the files (*in vitro* microbiological data) submitted when approval is sought for new antimicrobial agents or systems used for clinical antimicrobial susceptibility testing.[42–44] Despite this, the potential consequences of the density of the target bacteria on the course of treatment are given little consideration in the rational design of antibiotic treatment protocols,[8,45] save possibly for the cases where drug-inactivating enzymes are anticipated, e.g. β-lactamases.[46] Beyond these predictable cases,[9,47] the basic question remains poorly explored: how do changes in bacterial density influence the efficacy of antibiotics? It has been suggested that there is no evidence for inoculum effects confounding predictions of clinical outcomes of treatment protocols determined by conventional MICs.[47] Is this the case?

Using time–kill experiments and MIC determinations, we consistently observed moderate to substantial effects of the density (inoculum) of *Staphylococcus aureus* on the efficacy of six different classes of antibiotics: ciprofloxacin, daptomycin, gentamicin, linezolid, oxacillin and vancomycin. For vancomycin and daptomycin, our bioassays indicate that a major component of this effect can be attributed to a density-dependent decline in the effective concentration of the drug in the medium, the $[A_E/D]$ ratio. For the other four antibiotics, the effect could best be attributed to the decrease in the per-cell antibiotic concentration, the $[A_0/D]$ ratio. Using a mathematical model, we explore the potential consequences of the observed density effects on the course of a hypothetical antibiotic treatment regimen. We discuss the implications of the results of this (*in vitro* and mathematical modelling) study, as well as those of earlier studies of density effects, for the rational design of antibiotic treatment protocols.

Methods

Bacterial strains and culture conditions

The β-lactamase negative, methicillin-sensitive *S. aureus* strain ATCC 25923 was used in all of our studies. Bacterial cultures were incubated overnight at 37°C with shaking at 200 rpm in cation-adjusted Mueller–Hinton (MHII) medium alone or supplemented with 50 mg/L $CaCl_2$ for the experiments with daptomycin. Unless otherwise noted, all assays were performed with cultures growing aerobically with constant agitation at 37°C.

Antibiotics

Daptomycin (Cubist Pharmaceuticals, Lexington, MA, USA) and linezolid (Pfizer, New York, NY, USA) were purchased commercially. Gentamicin, oxacillin, ciprofloxacin and vancomycin were all from Sigma (St Louis, MO, USA). Stock solutions were prepared by dissolving the antibiotics in sterile water or 0.9% (w/v) NaCl, stored at −20°C and used within 2 weeks of preparation. The concentrations reported are the amount of active antibiotic in milligrams per unit volume.

MIC determination

The MICs of the different antibiotics employed here for this strain were estimated by both the broth microdilution and the Etest methods (AB Biodisk, Solna, Sweden). For each antibiotic, we estimated the MIC via broth microdilution using the densities and protocols recommended by the CLSI guidelines except that cultures were incubated at 37°C with shaking at ∼200 rpm. For this and estimates of higher density MICs, we used both optical density (OD, 630 nm) and colony forming unit (cfu) data. The MIC was the dilution at which the 18 h OD or the number of cfu was equal to or less than that at time 0. The Etest method was carried out according to the manufacturer's guidelines specific for the antibiotic and organism at 37°C.

Time–kill experiments

Overnight cultures of *S. aureus* ATTC 25923 were diluted 250-fold in fresh medium and incubated at 37°C for 180 min (high density culture, HD). From this, an aliquot was taken and diluted 1000-fold (low density culture, LD) in fresh pre-warmed MHII and both cultures were allowed to continue growth for 1 h more. Growth rate was estimated from OD measurements to ensure that the cultures were in the linear phase of exponential growth. The HD and LD cultures were then challenged with antibiotics in pre-warmed medium at a 1:1 (v:v) ratio. Aliquots of 100 μL were sampled from each culture at the times indicated, diluted and plated on Luria–Bertani agar and incubated at 37°C overnight. To minimize antibiotic carryover, cells from dilutions of $\leq 1 \times 10^2$ were washed twice in phosphate-buffered saline (PBS) prior to plating. The lower limit

of detection for these studies was set at 10^2 bacteria/mL. Antibiotic efficacy was assayed at various multiples of the conventional or baseline MIC (bMIC) as indicated in the Figures, and as obtained by the above protocol.

Assay for the effective residual concentration of antibiotics in the culture medium

Exponentially growing high density ($\sim 5 \times 10^8$) *S. aureus* cultures were challenged with each antibiotic for 18 h in liquid culture. At 3, 6 and 18 h, sample aliquots of these cultures were filtered (0.22 μm) to remove the cells. In order to ensure that we were measuring the residual effective (biologically active) antibiotic activity in the filtrates, a bioassay test was used; an overnight culture of *S. aureus* ATTC 25923 was diluted to $\sim 5 \times 10^5$ cfu/mL and the MICs of the filtered media estimated by a higher resolution (1.25-fold dilution versus 2-fold) broth microdilution assay modified from the CLSI protocol. The results were then compared with the bMICs estimated for those antibiotics from bacteria-free, antibiotic-containing controls.

A model of antibiotic treatment density-dependent PD

To explore the potential clinical implications of the density-dependent declines in antibiotic efficacy, we used an extension of the mathematical model of antibiotic treatment used by Regoes *et al*.[25] that takes into account the preceding experimental results. In this model, the relationship between the rate of population growth (death) and the concentration of the antibiotic, A, is described as a Hill function. As in Regoes *et al*.,[25] we assumed that three of the four parameters of this function are constants: Ψ_{MAX} (h^{-1}), the maximum exponential growth rate; Ψ_{MIN} (h^{-1}), the maximum rate of antibiotic-mediated killing; and the Hill coefficient, κ, which determines the shape of the function. Unlike the model used in Regoes *et al*.[25] but consistent with what we observed here (Figure 3), we assume that the fourth parameter, the MIC, is an increasing function of the density of the bacteria, D, $M(D)$, that levels off as the density increases,

$$M(D) = M_{MIN} + \mathrm{pd}\left(M_{MAX}\frac{D}{D + k_M}\right) \quad (1)$$

where M_{MIN} is the minimum MIC and is the baseline MIC as defined in our experiments, and M_{MAX} is the maximum MIC, k_M is the density of bacteria, D, at which the MIC is half its maximum value and pd, a coefficient for the density effect, $0 < \mathrm{pd} < 1$. With this modification, the rate of growth or death of the bacteria is now:

$$\Psi(A, D) = \Psi_{MAX} - \left[\frac{(\Psi_{MAX} - \Psi_{MIN})(A/M(D))^{\kappa}}{(A/M(D))^{\kappa} - \Psi_{MIN}/\Psi_{MAX}}\right] \quad (2)$$

As in our previous work[25] for the PK, we assume that the antibiotic is added at defined intervals and at its effective concentration, A, and decays exponentially at a rate d per hour. With these definitions and assumptions, at any given time, the rates of change in the density of bacteria are given by

$$\frac{dD}{dt} = \varphi(A, D) * D \quad (3)$$

$$\frac{dA}{dt} = -dA \quad (4)$$

It should be noted that this simple model of the PD of antibiotics does not account for persistence[16,17] or PAEs.[28,48]

To explore the properties of this model, we use numerical solutions, simulations, to the differential equations (1) and (2). These simulations were programmed in Berkeley Madonna and this program can be obtained online at www.eclf.net.

Results

Experimental results

Baseline MICs. The baseline or bMICs for these antibiotics and *S. aureus* ATCC 25923 were estimated with the CLSI protocol;[10] $\sim 5 \times 10^5$ bacteria/mL of medium with antibiotics serially diluted by factors of two, using OD and cfu data (no net increase or decline). These bMICs are consistent with those published for these antibiotics for susceptible *S. aureus* ATCC 25923.[37,49,50] The bMICs estimated by OD and cfu data were, respectively, in mg/L: 0.5 and 0.5 for oxacillin; 0.5 and 1 for ciprofloxacin; 0.5 and 0.5 for gentamicin; 0.5 and 1 for daptomycin; 1 and 1 for vancomycin; and 2 and 1 for linezolid. Although the estimated bMICs obtained from OD and cfu are not identical, the differences between these estimates are within a factor of two of each other and thereby consistent with the variation anticipated by this dilution protocol. In the following, the bMIC estimates used are those obtained with OD data.

Time–kill dynamics with different initial densities of bacteria. In Figure 1, we present the results of time–kill experiments with low-density (solid lines) and high-density (broken lines) inocula in MHII media with 20× the bMICs reported above. At both initial densities, in the absence of antibiotics, the bacteria grow exponentially. As measured by the rate at which bacteria are killed using cfu data at low initial densities, daptomycin and gentamicin are the most effectively bactericidal antibiotics followed by ciprofloxacin (Figure 1a). At this lower density, the rate of kill by vancomycin results in an approximately three order of magnitude decline in 6 h. Also at this low initial density, oxacillin displays the weakest bactericidal activity, being only slightly better than linezolid, which is anticipated to be bacteriostatic,[51] a two and one order of magnitude decline in viable cell density, respectively, in the course of 6 h.

Save for linezolid, the relative rate of killing of the higher density cultures (dotted lines, $\sim 1 \times 10^8$ cells/mL) is lower than that for the corresponding cultures initially containing fewer bacteria (solid lines, $\sim 5 \times 10^5$ cells/mL). It can be consistently shown that the low-density performance of these antibiotics is not necessarily a good predictor of their relative performance when confronting higher densities of bacteria. When $\sim 10^8$ cells are exposed to 20× bMIC of these antibiotics, gentamicin is the most effective, showing a six orders of magnitude decline in 6 h. Unlike what it is obtained at the lower densities, when confronting a high density of bacteria, daptomycin initially kills at a lower rate than gentamicin, and the population appears to recover, returning to its initial density by 7 h. When vancomycin was confronted with high densities of bacteria, killing appears to be delayed for the first 200 min but by 6 h the viable cell density of bacteria is three orders of magnitude less than the initial density. At high densities, the rate and extent of killing over 6 h by ciprofloxacin and oxacillin are also substantially less

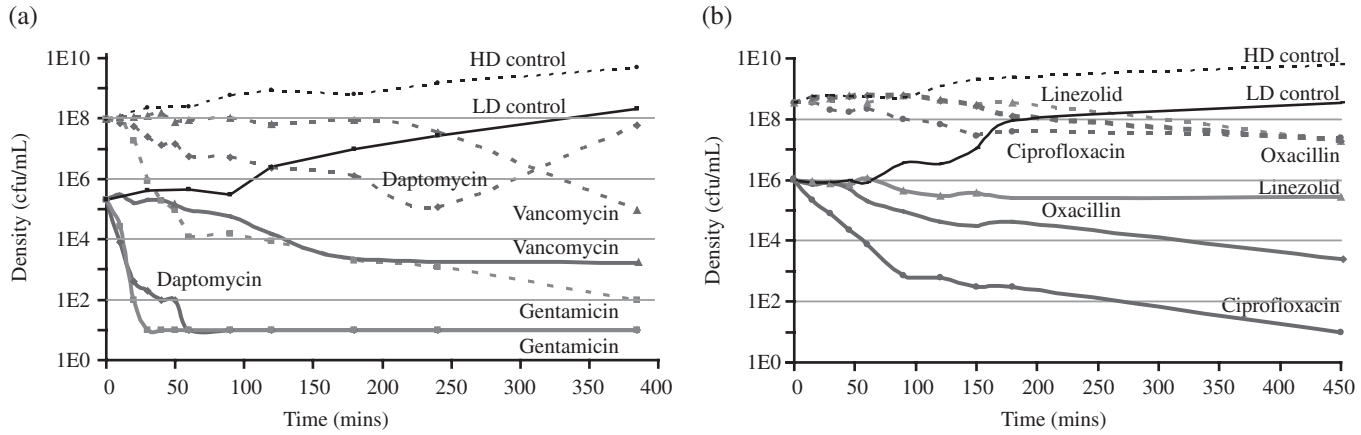

Figure 1. Short-term time–kill experiments with high and low initial densities at 20× the bMIC of each antibiotic. (a) and (b) Viable cell densities (cfu data) estimated at different times for exponentially growing cultures of *S. aureus* exposed to antibiotics relative to the initial (time 0) densities. Controls are for antibiotic-free media. Low densities are represented by solid lines and high densities by broken lines.

than when these drugs confront a lower density of bacteria. Linezolid kills at approximately the same rate and over the course of 6 h to the same extent at this high density as it did at low densities.

In Figure 2, we plot the density of viable cells (cfu data) for a low ($\sim 5 \times 10^5$) and high ($\sim 1 \times 10^8$) initial density after 3 and 18 h of exposure to various antibiotics. At both these densities, the bacteria were still growing exponentially as determined by OD measurements (data not shown). With the low-density inoculum, after 3 h all six of these antibiotics were at least bacteriostatic at 5× and 20× bMIC, i.e. the number of recovered cells was less than or equal to the number inoculated and substantially less than that of the growth control. At 5× bMIC, daptomycin was the only one of these antibiotics that was bactericidal by 3 h (>2 logs decrease). However, at 18 h, at 5× bMIC, the cfu estimate of the density of bacteria exposed to this drug was an order of magnitude greater than the initial inoculum density, and the density of cells in the 5× bMIC culture was greater than what it was at 3 h. At 20× bMIC, daptomycin, gentamicin and ciprofloxacin were clearly bactericidal at 3 h. At 18 h, in the low-density cultures at both 5× and 20× bMIC, linezolid, which is anticipated to only be bacteriostatic,[51] as well as gentamicin, ciprofloxacin, vancomycin and oxacillin had bactericidal effects. In addition to this time-dependent effect, the extent to which vancomycin and oxacillin killed increased with the concentration of these drugs. Since the viable cell density was below the detection threshold at both 5× and 20× bMIC of gentamicin and ciprofloxacin, these data do not provide information about a concentration-dependent effect on killing by these drugs.

For daptomycin, the increase in viable cell density between 3 and 18 h does not appear to be due to the ascent of resistant mutants. If it were, the vast majority of the bacteria on the sample plates would be resistant to the respective antibiotic in the culture. This was not the case; the four independently isolated surviving colonies tested from the LB sample plates had the same bMICs as their ancestors. Although some form of phenotypic resistance, such as persistence,[15–17] could account for the surviving bacteria in these antibiotic-containing liquid cultures, that phenotype would have been lost upon subculture and thereby is excluded by this experiment.

Considering the high initial density (Figure 2b), at 3 h, gentamicin at 5×, 20× and 100× MIC as well as daptomycin at 20×, 40× and 100× bMIC are the most potent of these six antibiotics and their efficacy appears to be proportional to their concentration. The 5× bMIC data for daptomycin are not presented in Figure 2 because other results (data not shown) indicate that at this density, the viable cell density of bacteria at 18 h is no different from that of the antibiotic-free control. At 3 h, at least at this higher density, ciprofloxacin is somewhat bactericidal, but its efficacy is inversely proportional to the drug concentration. This is the same, although to a lesser extent, in the case of oxacillin (more will be said of this apparent paradox in the Discussion). On the other hand, vancomycin and linezolid are only bacteriostatic at this high density, even at 100× bMIC. At 18 h, daptomycin and gentamicin are the most bactericidal antibiotics and are roughly equally so. However, it should be noted that, as anticipated from Figure 1(a), at 24 h the bacteria in the 20× bMIC daptomycin culture had recovered and the turbidity of the culture was not different from that in antibiotic-free medium (data not shown). The viable cell density of the culture with vancomycin at 5× bMIC is not significantly different from that in the antibiotic-free control and is only slightly better than bacteriostatic (<1 log decline) at 20× and 100× bMIC at 18 h. Although it is not apparent by 3 h, oxacillin reduces the viable cell density by between one and three orders of magnitude by 18 h, with the extent of kill appearing to be inversely proportional to antibiotic concentration. In the case of ciprofloxacin, the extent of kill is evident at 3 h and is also inversely proportional to its concentration, with the maximum kill at 18 h being nearly four orders of magnitude at 5× bMIC and only two orders of magnitude at 100× bMIC.

At these high initial densities, mutants that are resistant or partially resistant to antibiotics could well be present in the inoculum and, in the case of bactericidal antibiotics, might increase to dominate the culture after exposure to the antibiotic. To control for this contribution of inherited resistance, four independent colonies were taken from the 18 h sample plates where bacteria were recovered, cultured in antibiotic-free medium MHII, and their bMICs estimated. The bMICs of recovered colonies did not, in any of the six cases, significantly differ from that of the ancestral cell or the antibiotic-free control (data not

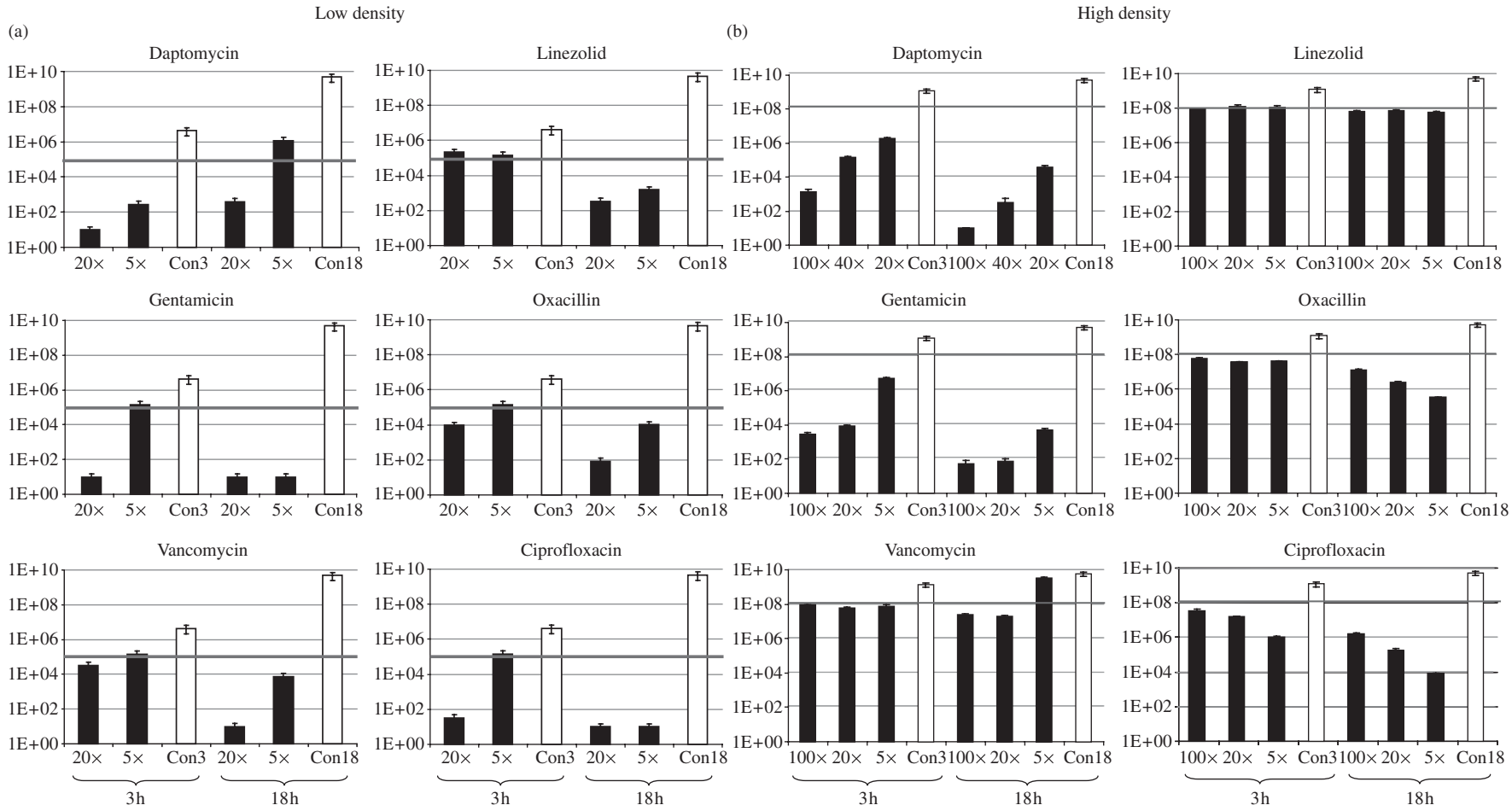

Figure 2. Concentration and time effects of antibiotic-mediated killing and growth inhibition in a (a) low- and (b) high-density culture: viable cell density (cfu data) at 3 and 18 h. The initial ($t = 0$) density is indicated with heavy horizontal line. The unshaded bar is the antibiotic-free control. Each bar represents the mean of four independent samples from the same culture with the standard error of those estimated.

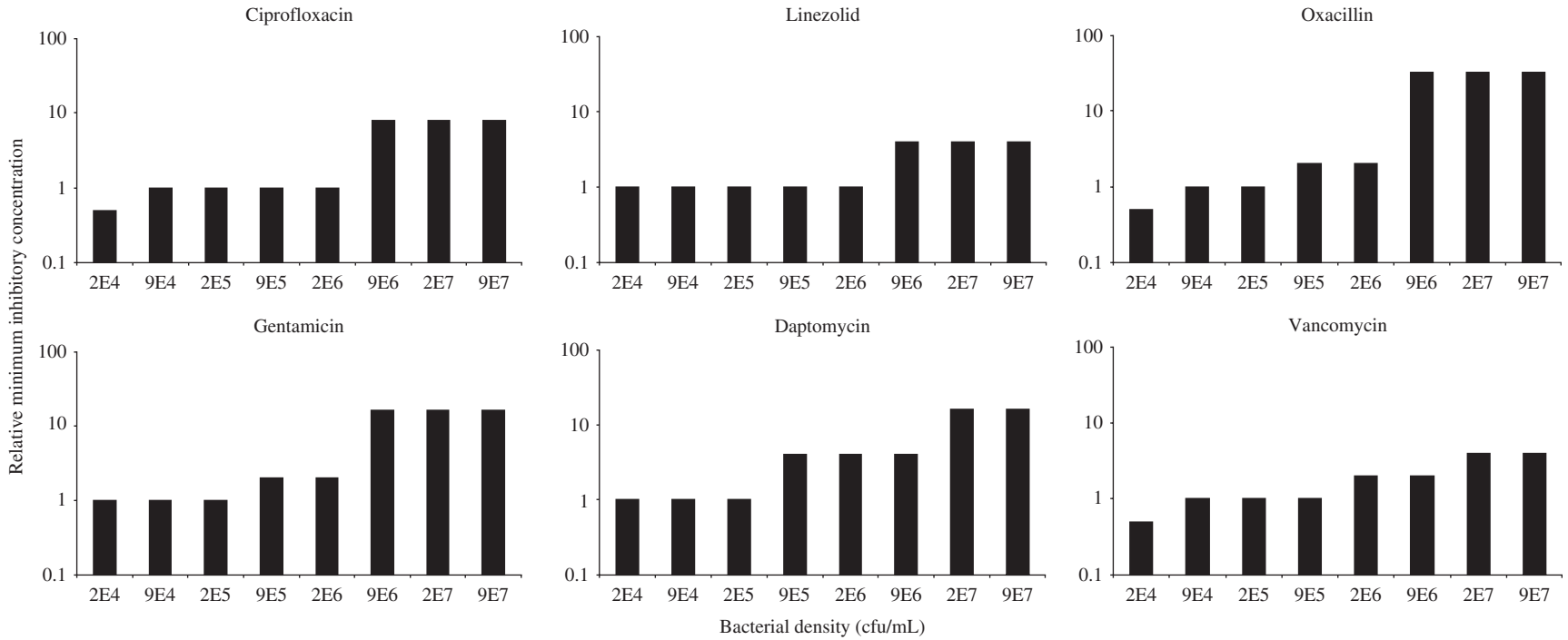

Figure 3. MICs estimated with different inoculum densities, relative to that MIC at 2×10^5. These estimates were obtained from cfu data; when the viable cell density at 18 h was approximately equal to that in the initial inoculum.

shown), indicating the absence of mutants dominating the culture after antibiotic exposure.

MIC as a function of cell density. In Figure 3, we plotted the MICs estimated for various inoculum densities of susceptible *S. aureus* exposed to each of the antibiotics. As our estimator of MIC in this experiment, we used cfu rather than OD data. The reason for this is the inability to accurately estimate relative densities of $<10^7$ from the OD of the culture. Moreover, since ODs measure the size and to some extent the shape of the bacteria, it is not always clear how OD reflects cell density. Nevertheless, to facilitate our cfu estimates of densities, we used OD data to select which wells we sampled and the dilutions needed to obtain these estimates. In these experiments, MIC is defined as the minimum concentration of the antibiotic needed to maintain the cell density after 18 h of exposure at a level approximately equal to the initial density, i.e. the minimal antibiotic concentration preventing growth.

As can be seen in the figure, for all six of these antibiotics, the estimated MICs increased with the density of bacteria exposed, albeit to extents that varied with these drugs. The greatest relative increase between the highest and baseline density, 9.2×10^7 and 1.9×10^5 bacteria/mL respectively, was for oxacillin, 32-fold, and the lowest, 4-fold for both vancomycin and linezolid (Figure 3). These increases in relative MIC with increasing density are in the range similar to that reported by LaPlante and Rybak[41] for three of the four drugs considered in both studies, respectively, daptomycin (32×), linezolid (2×) and vancomycin (4×), with gentamicin being the exception (LaPlante and Rybak reported a 2-fold rather than the 16-fold increase observed here). It should be noted, however, that the strains of *S. aureus* used in these two studies and the methods employed for these experiments differed; these authors used OD data and considered only two inoculum densities, 5×10^5 and 5×10^9, the latter some 50 times greater than the maximum considered here.

Save for daptomycin, inocula of densities ranging between $\sim 2 \times 10^4$ and $\sim 2 \times 10^6$ cells/mL have no significant effect on the estimated MICs. In the case of daptomycin, there is a 4-fold increase in MIC at $\sim 10^6$ cells/mL that levels off and is sustained between 10^6 and $\sim 10^7$ cells/mL. For most of these antibiotics, the most significant increase in estimated MIC does not occur until the inoculum density is in the order of 10^7. This increase in MIC with inoculum density is not continuous but rather levels off; for example, for oxacillin, a 32-fold increase relative to the baseline MIC was observed over the entire range of inoculum densities, $\sim 10^4 - 10^8$. In our experiments, we did not test for further increases in MIC with density, because of confounding with changes in physiological state as the bacteria approached and entered stationary phase. As noted earlier, at the highest inocula, $\sim 10^8$ in the absence of antibiotics, the bacteria grew exponentially at the same rate at which they did at lower densities.

Density-dependent decline in antibiotic activity. To ascertain the extent to which the effective concentration of the antibiotics in the medium declines due to confrontation with high densities of bacteria, we used the bioassay described in the Methods section. For this, $\sim 5 \times 10^8$ bacteria/mL of susceptible bacteria were incubated for 18 h in MHII medium containing 20× bMIC daptomycin (with $CaCl_2$), 10× bMIC vancomycin, or 5× bMIC for the other four antibiotics. The reason for using greater multiples of the bMICs for daptomycin and vancomycin are the results of preliminary experiments suggesting that the effective concentrations of these antibiotics were more strongly affected by the density of bacteria exposed than they were for the other antibiotics. As a control for bacteria-independent degradation or inactivation of the antibiotic in the medium, we incubated bacteria-free cultures for the same amount of time. Following incubation, all of the above-described samples were passed through 0.22 μm filters to remove the bacteria and the bMIC of the filtrates was estimated.

In Table 1, we list the bMICs estimated from this bioassay for each of these antibiotics at 3, 6 and 18 h. If there were no reductions in the effective concentrations of the antibiotic with or without bacteria, the bMICs of the filtrates of antibiotic-containing medium exposed to high bacterial densities would be the same as their CLSI estimate. If the effective concentration of the antibiotic declined over time, the bMICs estimated for filtrates of the bacteria-containing or bacteria-free controls would be higher than that of the original estimate. In accordance with this criterion and the sensitivity of this assay, there is no evidence for the effective concentrations of any of the antibiotics declining in the course of 18 h in the absence of bacteria. The

Table 1. Estimated bMICs of filtrates of cultures bearing $\sim 5 \times 10^8$ cfu/mL *S. aureus* following exposure to different antibiotics

Antibiotic	Maximum concentration in multiples of bMICs (mg/L)	MIC of cell-free control at 18 h (mg/L)	MIC of filtered medium (mg/L)		
			3 h	6 h	18 h
Ciprofloxacin	5× (2.5)	0.25	0.25	0.25	0.25
Daptomycin	20× (10)	0.5	1.0	NA[a]	NA
Gentamicin	5× (2.5)	0.38	0.38	0.38	0.38
Linezolid	5× (10)	1.5	1.5	1.5	1.5
Oxacillin	5× (2.5)	0.38	0.25	0.25	0.38
Vancomycin	10× (10)	1	5	NA	NA

[a]NA—no activity, bacterial growth in the filtered medium was consistent with that anticipated for those bacteria in antibiotic-free broth.

MICs obtained from these filtrates were essentially the same as those presented in the section Baseline MICs above. The apparent increase in the activity of oxacillin (decline in MIC) upon incubation with cells at 3 and 6 h is assumed to be spurious and a reflection of the error anticipated for a biological assay of this sort.

No decline in the effective concentrations of drug in the filtrate was observed for ciprofloxacin, gentamicin, linezolid and oxacillin when incubated with $\sim 5 \times 10^8$ cells. For vancomycin, the bMIC of the medium after 3 h of incubation with these bacteria was 5-fold greater than that in the cell-free control, and by 6 and 18 h there was no detectable antibiotic activity as estimated by the bioassay. In the presence of cells, the effective daptomycin concentration in the medium at 3 h is at best slightly less than that in the bacteria-free controls (a 2-fold increase in bMIC). However, by 6 and 18 h, there is no evidence for the filtrates of the daptomycin-containing media being at all inhibitory to bacterial growth. In interpreting all of these results, it is critical to consider that at time 0, the concentration of the antibiotics in the media for vancomycin and daptomycin was 10× and 20× MIC, respectively, while that for the other antibiotics was 5× MIC.

Theoretical results

Computer simulations. In our numerical analysis of the properties and predictions of this model, we used parameter values that roughly mimic the PD and density effect observed in our experiments. With respect to the rate of antibiotic-mediated killing, there are roughly three groups of drugs: (i) high rates, gentamicin and daptomycin; (ii) moderate rates, ciprofloxacin, oxacillin, and vancomycin; and (iii) a low rate, linezolid. In Figure 4(a and b), we plotted the Hill functions and anticipated 8 h time–kill curves for bacteria exposed to antibiotics of these three groups. In Figure 4(c), we present these hypothetical MIC functions for two situations; one where there is a substantial increase in MIC with density of the sort observed for oxacillin, gentamicin and daptomycin, and one with a more modest density effect seen with the other three antibiotics. For the PK, in all cases we assumed 20 mg/L of the antibiotic is added every 8 h and the antibiotic decays at a rate $d = 0.5\ \mathrm{h}^{-1}$. With these PK parameters, the concentration of the antibiotic at the end of each dosing period is effectively 0. The peak antibiotic concentration is that added, $A_\mathrm{d} \sim 20$; the time above the CLSI or baseline MIC is $T \sim \ln(A_\mathrm{d}/\mathrm{MIC})/d \sim 6$ h, and the area under the PK curve, $\mathrm{AUC} \sim A_\mathrm{d}/(d - \mathrm{MIC}) = 19$. These PKs are illustrated in Figure 4(d).

Although the MIC, A_d, $T > \mathrm{MIC}$ and AUC of these antibiotic–bacteria associations are identical, because of differences in their maximum rate of kill, Ψ_MIN, the PD (Hill) functions (Figure 4a) and anticipated kill curves of these antibiotics are different (Figure 4b). This difference is reflected in both the predicted microbiological course of treatment for both low- and high-density infections, 10^5 and 10^8 bacteria/mL, respectively. With the parameter values used, all of these antibiotics are able to clear an infection with a density of 10^5 bacteria/mL and do so at rates that reflect their respective rates of kill (Figure 5a and b).

However, when the density of the treated bacteria is 10^8 bacteria/mL, even with the modest density effect of antibiotic efficacy depicted in Figure 4(c), 'LOW', the antibiotic with the low rate of kill is unable to clear the infection (Figure 5a). This situation is even worse when the MIC is yet more sensitive to increases in density, when only the antibiotic with the highest rate of kill is able to clear an infection of high density (Figure 5b).

Although it is unlikely that the PD or PK of these antibiotics can be changed, the maximum dose and or dosing regimen could be modified to deal with these refractory high-density infections. We illustrate this in the simulations depicted in Figure 5(c), where the antibiotic fails to control the infection with a density of 10^8 bacteria/mL with a dose of 20 mg/L, but can do so when the dose is raised to 40 mg/L. The point is that if the dosing regimen was based solely on the conventional MIC or bMIC and the density of the infection were not considered, the antibiotic treatment could fail.

Discussion

It has been known for some time that the relative efficacy of some antibiotics decline with the density of bacteria exposed.[11,38–41] However, to our knowledge the quantitative or functional relationship between the density of bacteria exposed and the efficacy of antibiotics as measured by time–kill data or MICs has not been determined for any bacteria or antibiotic. In this report, we explored this functional relationship and how it varies among drugs in parallel experiments with antibiotics from six different families to which a single strain of *S. aureus* ATCC 25923 was exposed.

The results of our *in vitro* experiments with *S. aureus* ATCC 25923 provide evidence that all six antibiotics examined display some density-dependent reductions in their efficacy, as measured by declines in the rates of, and/or extent of, kill over a defined period, and increases in MICs. Both the rate and extent of kill of all six antibiotics decline with the density of bacteria exposed. At higher densities, however, increasing the concentrations of linezolid and vancomycin does not increase the extent of kill over a defined period. More paradoxically, at higher densities for ciprofloxacin and oxacillin, the extent of kill becomes inversely related to the concentration of the antibiotic. At densities of less than $\sim 10^6$ cells/mL, estimates of the MICs appear to be relatively independent of the inoculation density and consistent with that anticipated from the standard CLSI protocol, their conventional or baseline MICs. However, the observed increase in MIC appears to level off above a certain threshold density ($\sim \geq 1 \times 10^7$ bacteria/mL).

The functional relations proposed in the two models of density-dependent antibiotic action, $[A_\mathrm{E}/D]$ and $[A_0/D]$, are not mutually exclusive. From the experiments performed, we cannot say with assurance that only one of these mechanisms (or possibly others not considered) is responsible for the observed density-dependent declines in the efficacy of any of these antibiotics. We can, however, say with some assurance that for both daptomycin and vancomycin, declines in the absolute or effective concentrations of the antibiotic in the medium play a major role in this density effect (decrease in $[A_\mathrm{E}/D]$). Using our bioassay, after 6 h of incubation with 5×10^8 cells/mL, we were unable to detect any effective daptomycin or vancomycin in cultures containing, respectively, 20× and 10× the bMIC of these drugs. It should be noted that this does not rule out the possibility that more sensitive detection methods such as high performance liquid chromatography would detect substantial quantities of these drugs, despite their biological inactivity. For

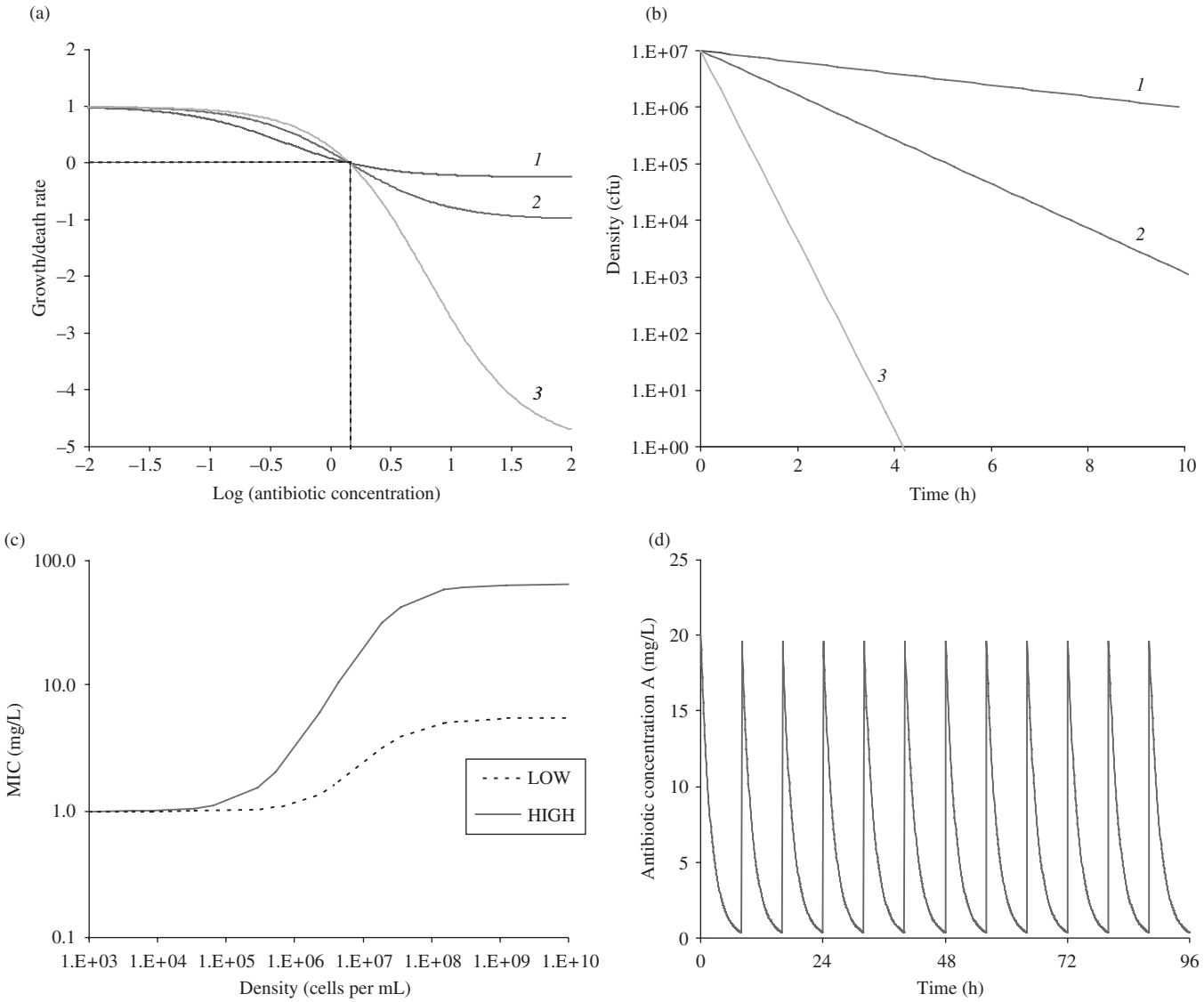

Figure 4. PD and PK models of antibiotic treatment: (a) rate of bacterial growth as a function of antibiotic concentration (a Hill function). Maximum growth rate, $\Psi_{MAX} = 1$, MIC = 1, $\kappa = 1$ for all, and three values for the maximum kill, $\Psi_{MIN} = -0.25$, -1.0 and -5.0, respectively, for lines 1, 2 and 3; (b) decline in viable cell density for Hill functions with the noted parameters, 1, 2 and 3, respectively; (c) MIC as a function of density (see text), $M_{MIN} = 1$, $k_M = 5 \times 10^7$ for all $1 - M_{MAX} = 100$, pd = 0.5, $2 - M_{MAX} = 20$, pd = 0.20; (d) PK, temporal changes in antibiotic concentration. Every 8 h, 20 mg/L of the antibiotic is added and its concentration declines at a rate of $d = 0.5\ h^{-1}$.

the other antibiotics, no declines in effective activity were observed at 18 h in cultures containing this density of bacteria and these antibiotics at 5× their respective conventional MICs.

As noted earlier in our high-density inoculum experiments, ciprofloxacin at 5× MIC is more effective than it is at 20× MIC, which in turn is more effective than this fluoroquinolone is at 100× MIC. This seemingly paradoxical inverse relationship between antibiotic concentration and efficacy of a quinolone was originally observed for nalidixic acid-mediated killing of *Escherichia coli*,[52,53] but is also observed for other quinolones and other bacteria.[54,55] It has been suggested that this inverse concentration effect is a consequence of reduced RNA synthesis (which is required for bactericidal activity) due to quinolone-induced relaxation of DNA negative supercoiling.[54–56] The precise mechanism(s) for this, however, have yet to be fully elucidated. A similar inverse relationship is observed for oxacillin, which to our knowledge is the first report of such a paradox in relation to this drug and the bacterium *S. aureus*. Although it is tempting to speculate on the reasons for this effect, we do not consider this to be worth the readers' time.

In this report, we have focused on the functional effect of density on the PD of different classes of antibiotics and *S. aureus*, but have not really addressed the mechanisms responsible for these effects. That is not to say that we are not interested in these mechanisms. Indeed, the biochemical, physiological and molecular mechanisms responsible for the above-described relationships between the density of *S. aureus* and the PD and PK of antibiotics are intriguing. This is particularly so for situations where the density effect on antibiotic efficacy is observed when there is no apparent decline in the effective concentration of the drug in the medium, the $[A_0/D]$ model. Although for a given concentration of the drug there may be

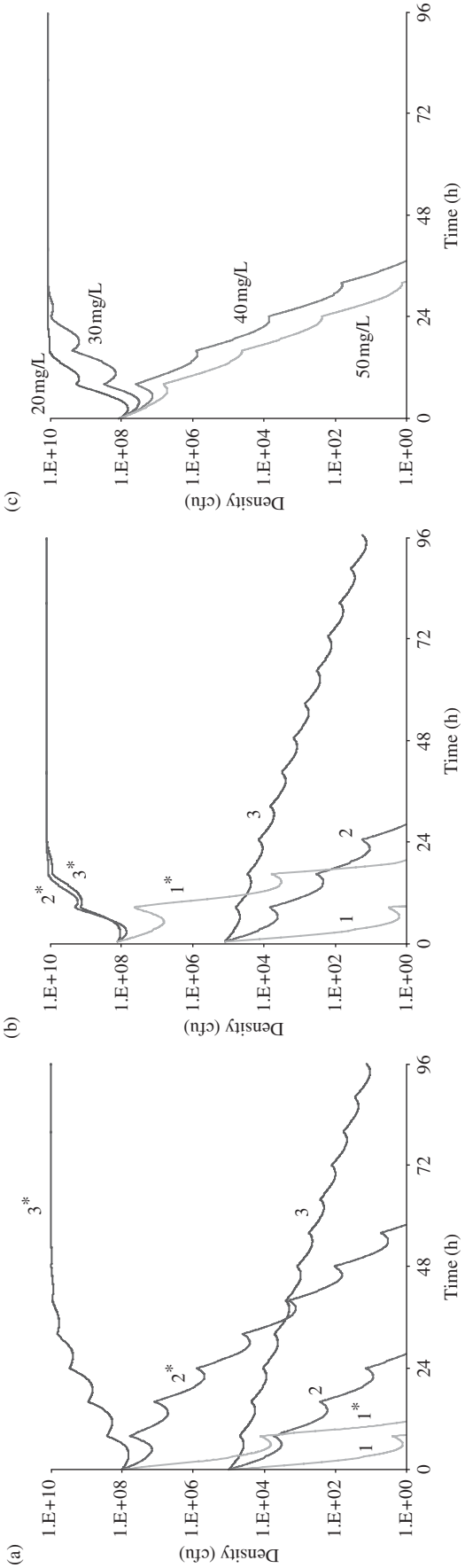

Figure 5. Simulation of antibiotic treatment; changes in the density of viable bacteria for different initial densities. The PK of this antibiotic treatment regimen is depicted in Figure 4(d), a dose of 20 mg/L administered every 8 h. In (a) and (b), the same set of parameters designated by the number (*n*) is used for different initial densities (n^* = high density). (a) Modest density effect, $M_{MAX} = 20$, pd = 0.20 (Figure 4c 'LOW') with three maximum kill rates, Ψ_{MIN}, −5, −1.0 and −0.25 h^{-1}, lines 1, 2 and 3, respectively; (b) strong density effect, $M_{MAX} = 100$, pd = 0.50, three maximum kill rates, $\Psi_{MIN} = -5$, −1.0 and −0.25 h^{-1}, lines 1, 2 and 3, respectively; (c) effect of increasing the dose of an antibiotic that failed to control the infection with 20 mg/L, Ψ_{MIN}, −5 h^{-1}, $M_{MAX} = 100$, pd = 0.50.

1000-fold fewer antibiotic molecules per bacterial cell in a culture containing 10^8 bacteria/mL than one with a density of 10^5 bacteria/mL, the total number of antibiotic molecules per bacterium remains enormous. While it has been suggested that different classes of bactericidal antibiotics kill by the same mechanism, the intrabacterial production of reactive oxygen species,[57] the relationship between this 'suicide' induction and the ratio of the concentration of antibiotic to the density of bacteria has not been explored. To be sure, elucidating the mechanism(s) responsible for these density effects is certainly important from a practical as well as an academic perspective. However, we see them as secondary to the clinical implications of these *in vitro* PD results at this juncture.

We have tried to control for the physiological state of the bacteria and other factors contributing to the susceptibility of the population to antibiotics by restricting the densities examined to a range where the population is growing exponentially at its maximum rate, where the change in the log of the density is linear. Nevertheless, it is not possible to rule out a role of density-associated difference in physiological state, age-structure (distribution of times since division) persistence, or the accumulation of metabolic by-products, wastes and biofilms (flask wall growth) to observed density effects. Indeed, we cannot say that these kinds of factors would not contribute to differences in the response to antibiotics in populations of 10^3 and 10^5 bacteria/mL. However, from the perspective of antibiotic treatment, whether density itself is the sole reason for the decline in the efficacy of antibiotics with inoculum size or other density-associated factors contribute is almost irrelevant as long as those factors also contribute to the density effect in treated hosts.

Potential clinical implications of density-dependent antibiotic action

We interpret the results of our simulation experiments with the PK/PD antibiotic treatment model equations (1)–(4) as support for the proposition that the density of the infection should be taken into account in the design of an antibiotic treatment regimen. Treatment regimens based on the classic PK/MIC index that may be effective in treating low-density infections may fail to, or require extensive amounts of time to, clear higher density infections. To be sure, this model is a simplistic caricature of the dynamics of antibiotic treatment in a human host. In addition to not considering persistence and PAEs, it assumes that the infection is homogeneous, and that the density of the bacteria and concentration of the antibiotic are the same throughout all infected sites. In reality, there is likely to be a distribution of bacterial densities in an infected host and the PK of the antibiotic will almost certainly vary among these sites as well as among hosts. The antibiotic may readily eliminate the lower but not higher density subpopulations of the infecting bacteria, which could remain as a reservoir in the infected organism. Also working against the clearance of high-density infections by antibiotics for which resistance can be generated by mutation (or the acquisition of a resistance encoding plasmid from other colonizing bacteria) is a greater likelihood of resistant mutants (or transconjugants) being present and not eliminated by the drug. For a more detailed consideration of the relationship between bacterial density (really total numbers) and the

emergence of resistant mutants, see refs 58–60, or for a recent review of the mutation prevention literature, see ref. 61.

The arguments for considering the density of the infection in the design of antibiotic treatment protocols are not restricted to theoretical considerations. Starting with the classical investigations of Eagle *et al*. (see ref. 62, for example), experimental studies of antibiotic prophylaxis and treatment with laboratory animals support the proposition that *in vitro* studies of the PD of antibiotics can provide good predictions of their *in vivo* performance. To our knowledge, there have not been quantitative animal model experiments that explore the functional relationship between the *in vitro* density (inoculum) effects on the PD of antibiotics and the outcome of treatment. There are, however, animal model experiments that support the proposition that a density-dependent decline in the *in vitro* efficacy of some antibiotics can substantially affect their efficacy *in vivo*. Nearly 20 years ago Soriano *et al*.[38,39] presented evidence that antibiotics that have a marked inoculum effect with *E. coli in vitro* require vastly greater concentrations (as measured by multiples of their MIC) to have the same clinical efficacy (rates of survival) in treating *E. coli* infections in laboratory rats than antibiotics with more modest *in vitro* inoculum effects.

One can also interpret in a similar light some of the observations from experiments with penicillin treatment of streptococcus infections in laboratory mice and *Treponema pallidum* infections in rabbits made by Eagle *et al*.[62] Their data provide compelling evidence that the concentrations of penicillin needed to abort infections when treated immediately or prophylactically are not much greater than those necessary to kill these bacteria *in vitro*.[62] The number of bacteria inoculated in these immediate treatment experiments was low: 100 in mice and 20 in rabbits. On the other hand, the dose of penicillin required to abort more established infections was substantially greater. We quote: 'It is true that when mice or rabbits were treated a number of hours after inoculation with either pneumococci or streptococci or when rabbits were treated 6 weeks after inoculation with *T. pallidum*, the single curative dose (CD_{50}) of aqueous penicillin was as much as 1000 times greater than when they were treated immediately after inoculation'[62] (p. 635), but also see ref. 63. While a greater density of the bacteria in longer-term infections may not be the sole reason that vastly greater doses of penicillin were needed for cure, a substantial increase in the density of these bacteria is anticipated and was observed.

To be sure, the results of these animal model experiments may not mean that similar density effects would be seen in treated humans. Indeed, it may well be that a similar density effect accounts for the profound increase in the mortality rate in patients with septic hypotension with increase in the time between diagnosis and treatment, 20% mortality with immediate treatment versus 60% when treatment is initiated 6 h later.[64] It also seems worth raising the question that appears to have motivated Soriano *et al*.'s animal model inoculation effects experiments. Why are so many antibiotics administered in clinical practice at 50 or more times their conventional MICs? Dosing schedules used in experimental animal models and clinical trials are initially derived from PD/PK studies employing the conventional estimates of the MICs of these drugs and these schedules are then frequently corrected as a result of clinical experience. Our models and experiments suggest that PK/PD indices would be more predictive of the efficacy of antibiotics if, instead of using conventional estimates of the MIC, density-dependent functions of MICs were employed as the denominators of these indices.

Acknowledgements

We wish to thank the *Ec*LF Collective for useful comments and suggestions and Sergei Pilyugin for help with some of the real mathematics behind the models used in this study.

Funding

This investigation was supported by grants from the US National Institutes of Health AI40662, and also from Pfizer Inc. K. I. U. acknowledges stipend support from the Swedish research council.

Transparency declarations

Although we received part of the funding for this investigation from Pfizer Inc., there are no financial or other conflicts of interest involved in this study by any of the authors. Pfizer played no role in the design of this investigation, in the decisions made about the antibiotics studied or the interpretation of the results obtained, nor did they request a review of the results or the manuscript before its submission.

References

1. Blaser J, Stone BB, Groner MC *et al*. Comparative study with enoxacin and netilmicin in a pharmacodynamic model to determine importance of ratio of antibiotic peak concentration to MIC for bactericidal activity and emergence of resistance. *Antimicrob Agents Chemother* 1987; **31**: 1054–60.

2. Fantin B, Leggett J, Ebert S *et al*. Correlation between in vitro and in vivo activity of antimicrobial agents against gram-negative bacilli in a murine infection model. *Antimicrob Agents Chemother* 1991; **35**: 1413–22.

3. Drusano GL, Johnson DE, Rosen M *et al*. Pharmacodynamics of a fluoroquinolone antimicrobial agent in a neutropenic rat model of *Pseudomonas* sepsis. *Antimicrob Agents Chemother* 1993; **37**: 483–90.

4. Forrest A, Nix DE, Ballow CH *et al*. Pharmacodynamics of intravenous ciprofloxacin in seriously ill patients. *Antimicrob Agents Chemother* 1993; **37**: 1073–81.

5. Craig WA. Interrelationship between pharmacokinetics and pharmacodynamics in determining dosage regimens for broad-spectrum cephalosporins. *Diagn Microbiol Infect Dis* 1995; **22**: 89–96.

6. Vinks AA. The application of population pharmacokinetic modeling to individualized antibiotic therapy. *Int J Antimicrob Agents* 2002; **19**: 313–22.

7. Mouton JW. Impact of pharmacodynamics on breakpoint selection for susceptibility testing. *Infect Dis Clin North Am* 2003; **17**: 579–98.

8. Drusano GL. Antimicrobial pharmacodynamics: critical interactions of 'bug and drug'. *Nat Rev* 2004; **2**: 289–300.

9. RxKinetics. antibiotic pk pd. http://www.rxkinetics.com/antibiotic_pk_pd.html (16 December 2008, date last accessed).

10. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing. Fifteenth Informational Supplement*. CLSI, Wayne, PA, 2005.

11. Amsterdam D. Susceptibility testing of antimicrobials in liquid media. In: Lorian V, ed. *Antibiotics in Laboratory Medicine*. Baltimore: Lippincott Williams & Wilkins, 2005; 61–144.

12. Hobby G, Dawson M. Effect of growth rate of bacteria on action of penicillin. *Proc Soc Exp Biol* 1944; **56**: 181–4.

13. Tuomanen E, Cozens R, Tosch W *et al*. The rate of killing of *Escherichia coli* by beta-lactam antibiotics is strictly proportional to the rate of bacterial growth. *J Gen Microbiol* 1986; **132**: 1297–304.

14. Eng RH, Padberg FT, Smith SM *et al*. Bactericidal effects of antibiotics on slowly growing and nongrowing bacteria. *Antimicrob Agents Chemother* 1991; **35**: 1824–8.

15. Bigger J. Treatment of Staphylococcal infections with penicillin. *Lancet* 1944; **2**: 497–500.

16. Balaban NQ, Merrin J, Chait R *et al*. Bacterial persistence as a phenotypic switch. *Science* 2004; **305**: 1622–5.

17. Wiuff C, Zappala RM, Regoes RR *et al*. Phenotypic tolerance: antibiotic enrichment of noninherited resistance in bacterial populations. *Antimicrob Agents Chemother* 2005; **49**: 1483–94.

18. Brooun A, Liu S, Lewis K. A dose–response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* 2000; **44**: 640–6.

19. Ashby MJ, Neale JE, Knott SJ *et al*. Effect of antibiotics on non-growing planktonic cells and biofilms of *Escherichia coli*. *J Antimicrob Chemother* 1994; **33**: 443–52.

20. Gilbert P, Collier PJ, Brown MR. Influence of growth rate on susceptibility to antimicrobial agents: biofilms, cell cycle, dormancy, and stringent response. *Antimicrob Agents Chemother* 1990; **34**: 1865–8.

21. Brown MRW, Allison DG, Gilbert P. Resistance of bacterial biofilms to antibiotics: a growth-rate related effect? *J Antimicrob Chemother* 1988; **22**: 777–80.

22. Stewart PS. Mechanisms of antibiotic resistance in bacterial biofilms. *Int J Med Microbiol* 2002; **292**: 107–13.

23. Keren I, Shah D, Spoering A *et al*. Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *J Bact* 2004; **186**: 8172–80.

24. Lewis K. Riddle of biofilm resistance. *Antimicrob Agents Chemother* 2001; **45**: 999–1007.

25. Regoes RR, Wiuff C, Zappala RM *et al*. Pharmacodynamic functions: a multiparameter approach to the design of antibiotic treatment regimens. *Antimicrob Agents Chemother* 2004; **48**: 3670–6.

26. Wiuff C, Andersson DI. Antibiotic treatment in vitro of phenotypically tolerant bacterial populations. *J Antimicrob Chemother* 2007; **59**: 254–63.

27. Barry AL, Badal RE, Hawkinson RW. Influence of inoculum growth phase on microdilution susceptibility tests. *J Clin Microbiol* 1983; **18**: 645–51.

28. Craig WA. Post-antibiotic effects in experimental infection models: relationship to in-vitro phenomena and to treatment of infections in man. *J Antimicrob Chemother* 1993; **31**: 149–58.

29. Lowdin E, Odenholt I, Cars O. In vitro studies of pharmacodynamic properties of vancomycin against *Staphylococcus aureus* and *Staphylococcus epidermidis*. *Antimicrob Agents Chemother* 1998; **42**: 2739–44.

30. Mouton JW, Vinks AA. Pharmacokinetic/pharmacodynamic modelling of antibacterials in vitro and in vivo using bacterial growth and kill kinetics: the minimum inhibitory concentration versus stationary concentration. *Clin Pharmacokinet* 2005; **44**: 201–10.

31. Mouton JW, Vinks AA. Relationship between minimum inhibitory concentration and stationary concentration revisited: growth rates and minimum bactericidal concentrations. *Clin Pharmacokinet* 2005; **44**: 767–8.

32. Eagle H. Experimental approach to the problem of treatment failure with penicillin. *Am J Med* 1952; **13**: 389–99.

33. McDermott W. Microbial Persistence. *Yale J Biol Med* 1958; **30**: 257–91.

34. Levin BR, Rozen DE. Non-inherited antibiotic resistance. *Nat Rev* 2006; **4**: 556–62.

35. Garrod LP, Waterworth PM. Effect of medium composition on the apparent sensitivity of *Pseudomonas aeruginosa* to gentamicin. *J Clin Pathol* 1969; **22**: 534–8.

36. Grayson M, Eliopoulos G, Ferraro M *et al*. Effect of varying pH on the susceptibility of *Campylobacter pylori* to antimicrobial agents. *Eur J Clin Microbiol Infect Dis* 1989; **8**: 888–9.

37. Baudoux P, Bles N, Lemaire S *et al*. Combined effect of pH and concentration on the activities of gentamicin and oxacillin against *Staphylococcus aureus* in pharmacodynamic models of extracellular and intracellular infections. *J Antimicrob Chemother* 2007; **59**: 246–53.

38. Soriano F, Santamaria M, Ponte C *et al*. In vivo significance of the inoculum effect of antibiotics on *Escherichia coli*. *Eur J Clin Microbiol Infect Dis* 1988; **7**: 410–2.

39. Soriano F, Ponte C, Santamaria M *et al*. Relevance of the inoculum effect of antibiotics in the outcome of experimental infections caused by *Escherichia coli*. *J Antimicrob Chemother* 1990; **25**: 621–7.

40. Craig W, Ebert S. Killing and regrowth of bacteria in vitro: a review. *Scand J Infect Dis Suppl* 1990; **74**: 63–70.

41. LaPlante KL, Rybak MJ. Impact of high-inoculum *Staphylococcus aureus* on the activities of nafcillin, vancomycin, linezolid, and daptomycin, alone and in combination with gentamicin, in an in vitro pharmacodynamic model. *Antimicrob Agents Chemother* 2004; **48**: 4665–72.

42. Agency TEM. *Summary of Product Characteristics for Antimicrobial Medicinal Products: Directive 75/318/EEC*. 1996.

43. Thrupp LD, Cleeland R, Jones RN *et al*. General guidelines for clinical bacteriology. Infectious Diseases Society of America and the Food and Drug Administration. *Clin Infect Dis* 1992; **15** Suppl 1: S339–46.

44. US Department of Health and Human Services. *Class II Special Controls Guidance Document: Antimicrobial Susceptibility Test (AST) Systems; Guidance for Industry and the FDA*. Washington, DC: US DHHS, 2007.

45. Gunderson BW, Ross GH, Ibrahim KH *et al*. What do we really know about antibiotic pharmacodynamics? *Pharmacotherapy* 2001; **21**: 302–18S.

46. Queenan AM, Foleno B, Gownley C *et al*. Effects of inoculum and beta-lactamase activity in AmpC- and extended-spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* clinical isolates tested by using NCCLS ESBL methodology. *J Clin Microbiol* 2004; **42**: 269–75.

47. Craig WA, Bhavnani SM, Ambrose PG. The inoculum effect: fact or artifact? *Diagn Microbiol Infect Dis* 2004; **50**: 229–30.

48. Gudmundsson S, Einarsson S, Erlendsdottir H *et al*. The post-antibiotic effect of antimicrobial combinations in a neutropenic murine thigh infection model. *J Antimicrob Chemother* 1993; **31** Suppl D: 177–91.

49. Hanberger H, Nilsson LE, Maller R *et al*. Pharmacodynamics of daptomycin and vancomycin on *Enterococcus faecalis* and *Staphylococcus aureus* demonstrated by studies of initial killing and postantibiotic effect and influence of Ca^{2+} and albumin on these drugs. *Antimicrob Agents Chemother* 1991; **35**: 1710–6.

50. Barcia-Macay M, Seral C, Mingeot-Leclercq M-P *et al*. Pharmacodynamic evaluation of the intracellular activities of antibiotics against *Staphylococcus aureus* in a model of THP-1 macrophages. *Antimicrob Agents Chemother* 2006; **50**: 841–51.

51. Clemett D, Markham A. Linezolid. *Drugs* 2000; **59**: 815–27.

52. Winshell EB, Rosenkranz HS. Nalidixic acid and the metabolism of *Escherichia coli*. *J Bact* 1970; **104**: 1168–75.

53. Crumplin GC, Smith JT. Nalidixic acid: an antibacterial paradox. *Antimicrob Agents Chemother* 1975; **8**: 251–61.

54. Lewin CS, Morrissey I, Smith JT. The mode of action of quinolones: the paradox in activity of low and high concentrations and activity in the anaerobic environment. *Eur J Clin Microbiol Infect Dis* 1991; **10**: 240–8.

55. Phillips I, Culebras E, Moreno F *et al*. Induction of the SOS response by new 4-quinolones. *J Antimicrob Chemother* 1987; **20**: 631–8.

56. Smith JT. Mutational resistance to 4-quinolone antibacterial agents. *Eur J Clin Microbiol* 1984; **3**: 347–50.

57. Kohanski MA, Dwyer DJ, Hayete B *et al*. A common mechanism of cellular death induced by bactericidal antibiotics. *Cell* 2007; **130**: 797–810.

58. Lipsitch M, Levin BR. The population dynamics of antimicrobial chemotherapy. *Antimicrob Agents Chemother* 1997; **41**: 363–73.

59. Dong Y, Zhao X, Kreiswirth BN *et al*. Mutant prevention concentration as a measure of antibiotic potency: studies with clinical isolates of *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2000; **44**: 2581–4.

60. Zhao X, Drlica K. Restricting the selection of antibiotic-resistant mutant bacteria: measurement and potential use of the mutant selection window. *J Infect Dis* 2002; **185**: 561–5.

61. Drlica K, Zhao X. Mutant selection window hypothesis updated. *Clin Infect Dis* 2007; **44**: 681–8.

62. Eagle H, Fleischman R, Musselman A. The bactericidal action of penicillin in vivo: the participation of the host, and the slow recovery of the surviving organisms. *Ann Intern Med* 1950; **33**: 544–71.

63. Eagle H. The effect of the size of the inoculum and the age of the infection on the curative dose of penicillin in experimental infections with streptococci, pneumococci, and *Treponema pallidum*. *J Exp Med* 1949; **90**: 595–607.

64. Kumar A, Roberts D, Wood KE *et al*. Duration of hypotension before initiation of effective antimicrobial therapy is the critical determinant of survival in human septic shock. *Crit Care Med* 2006; **34**: 1589–96.