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Rapid antibiotic sensitivity testing in microwell arrays

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Abstract

The widespread bacterial resistance to a broad range of antibiotics necessitates rapid antibiotic susceptibility testing before effective treatment could start in the clinic. Among resistant bacteria, *Staphylococcus aureus* is one of the most important, and Methicillin-resistant (MRSA) strains are a common cause of life threatening infections. However, standard susceptibility testing for *S. aureus* is time consuming and thus the start of effective antibiotic treatment is often delayed. To circumvent the limitations of current susceptibility testing systems, we designed an assay that enables measurements of bacterial growth with higher spatial and temporal resolution than standard techniques. The assay consists of arrays of microwells that confine small number of bacteria in small spaces, where their growth is monitored with high precision. These devices enabled us to investigate the effect of different antibiotics on *S. aureus* growth. We measured the Minimal Inhibitory Concentration (MIC) in less than 3 hours. In addition to being significantly faster than the 48 hours needed for traditional microbiological methods, the assay is also capable of differentiating the specific effects of different antibiotic classes on *S. aureus* growth. Overall, this assay has the potential to become a rapid, sensitive, and robust tool for use in hospitals and laboratories to assess antibiotic sensitivity.

Keywords

Staphylococcus aureus; Tetracycline; Carbenicillin; Nafcillin; Penicillin G; Microfluidics

INNOVATION

Current methods for testing bacterial antibiotic sensitivity are time-consuming and expensive. Recently, use of microfluidic approaches has been investigated as an alternative. Although these methods have facilitated parallel testing of many samples and significantly shortened assay times, many suffer from complicated and time-consuming pre-loading steps. Here we describe a microwell bacterial culture system optimized for live imaging approaches that is low-cost, easy to set up, and provides MIC values and antibiotic sensitivity testing in three hours.

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INTRODUCTION

Bacterial infections with *Staphylococcus aureus* often result in abscess formation, furuncles, and cellulitis. Although most skin infections caused by *S. aureus* heal without medical attention, many can progress into serious conditions that require antibiotic treatment. If treatment is ineffective, potentially life-threatening complications can develop. Over the past decade, the increased prevalence of antibiotic-resistant *S. aureus* strains has emerged as a major threat to public health. Antibiotic-resistant *S. aureus* now represents a leading cause of morbidity and mortality worldwide[1]. New technologies for rapid antibiotic sensitivity testing (AST) are needed to help address this issue[2].

Traditionally, AST is performed by *broth dilution* or *disk diffusion* techniques[3]. These tests are based on visual observation of bacterial growth inhibition in the presence of increasing concentrations of antibiotics[4]. They characterize the bacteria as resistant, intermediate, or susceptible. However, both tests are time consuming. The results from disk diffusion assays require at least 24 hours, often even longer, and their results are semi-quantitative[5]. Serial dilutions provide quantitative results but are more expensive[5].

Microfluidic technologies have recently started entering the AST field, with the goal of providing assays that would be easier to use and provide results faster[6–26]. The new microfluidic assays take advantage of miniaturization approaches, which have also been utilized for other clinical applications e.g. hemostasis, clinical biomarker analysis, cancer diagnosis, and nanoparticle sensors[27–32]. Campbell *et al.* reviewed the most recent advances in microfluidic devices for AST and identification four major strategies: 1) microfluidic incubator platforms; 2) gradient generators; 3) combined assays for identification and AST; and 4) AST based on bacterial death[33]. Several common features emerge from this classification. The volume of fluids within these devices is generally in the low nanoliter range, they require small amounts of sample[9,16]. Multiple tests could be simultaneously performed on multiple samples[5,6,10,11]. Imaging to quantify microbial growth in the presence and absence of antibiotics was implemented using several techniques, including the monitoring of optical density[6,8,9,11], bright field/phase contrast[11] and fluorescence measurements[6,8–10,13,14,18]. Several unconventional methods for monitoring AST have also been proposed, including RNA specific electrochemical biosensors[34], pH changes of culture media during cell growth[10,35], or asynchronous magnetic beads[36]. However, several limitations of current microfluidic systems also become apparent. Microscale systems usually require long and elaborated bacteria pre-loading and preparation steps[6] and thus remain time-consuming[6]. Some of the devices require complex infrastructure, such as use of pumps and syringes[9,11,13,14,16–18]. Many require the immobilization of bacteria with agarose within microchannels[11,15] or on agarose microparticles[37].

Here, we describe a low-cost, time-effective open microwell assay that enables rapid measurements of Minimal Inhibitory Concentration (MIC) and evaluations of AST for *S. aureus*. We probed the effect of four antibiotics (Tetracycline, Carbenicillin, Nafcillin, and Penicillin G) at six concentrations each on *S. aureus* proliferation. One interesting finding was the transient increase of proliferation rates of *S. aureus* in the presence of Penicillin G and Nafcillin at concentrations below MIC, compared to no-antibiotic controls. Our assay

provides quantitative measurements from low-density bacterial samples and enables parallel AST of up to 24 drugs in three hours.

MATERIALS AND METHODS

Antibiotic preparation

Stock solutions of 10 mg/mL of Tetracycline, Carbenicillin disodium, Nafcillin sodium salt, and Penicillin G sodium salt (Sigma Aldrich, Saint Louis, Missouri) were prepared in water (WFI, Sigma Aldrich). Working solutions were then prepared for each antibiotic, at 100 μg/mL. Subsequently, 1:10 dilution series were made in heart infusion broth (BHI, Sigma Aldrich), to encompass antibiotic concentrations ranging from 100 μg/mL down to 1 ng/mL.

Fabrication of microwell array

Devices were fabricated using standard soft-lithography techniques on four-inch wafers. Photoresist (SU-8, Michrochem, Newton, MA) was spin-coated onto a silicon wafer and exposed to ultraviolet (UV) light, through a photolithography mask. The silicon master wafer with photo-patterned structures was employed to mold arrays of microwells that were 40 μm in diameter, 100 μm in depth, and were spaced at 20 μm. Using this technique, 70 arrays, each with 1,164 microwells, were molded at one time from a single silicon wafer. Polydimethylsiloxane (PDMS, Sylgard 184, Dow Corning, Midland, MI) was mixed with cross-linking agent in a ratio of 10:1 and poured onto wafers. A 100 μm layer of PDMS was created by pressing a flat plastic sheet on top of the wafer using a 0.5 Ib weight, for 12 hours. The PDMS was cured overnight at 65°C, after which the PDMS layer was peeled off the wafer and the arrays of wells were cut using a scalpel. The microwell-arrays were bonded to glass-bottom 24-well plates after treating the bonding surface of PDMS and plate with oxygen plasma (1,164 microwells per well). The plates were heated to 85°C for 10 minutes to complete the PDMS-to-glass bonding.

Bacterial cell culture

The SH1000-GFP *S. aureus* strain, which constitutively expresses green fluorescent protein (GFP), was received as a generous gift from the laboratory of Mary Mullins at the University of Sheffield (Sheffield, UK). Bacterial cultures were routinely cultivated in brain heart infusion (BHI) Agar (Remel, Lenexa, KS, USA). Single colonies from agar plates were picked and suspended in 5 mL of BHI broth medium and then incubated at 37°C in aerobic incubator with shaking overnight. After overnight incubation, bacterial suspensions were sub-cultured by adding 1 mL of the overnight culture into 49 mL of BHI broth for 4 hours. Bacterial concentrations were determined using a hemocytometer and the final concentration of bacteria was adjusted to 1×10^6 cells/mL and diluted with BHI broth.

Device loading

To facilitate the loading of the bacterial suspension into open microwells, we treated the devices with oxygen plasma to restore the hydrophilic surfaces. Approximately 100 μL of bacterial suspension was then loaded and the plate placed under vacuum for 10 minutes to remove any gas trapped within the PDMS microwells. After vacuum, antibiotics at a range of concentrations in BHI, were added to each well of the 24-well plate.

Off-chip MIC measurements

To validate our microwell assay, off-chip MIC determination was performed using the standard broth dilution (SBD) method according to CLSI protocols[3]. We prepared four different antibiotics stock solutions of 10 mg/mL for Carbenicillin, Nafcillin, Penicillin G, and Tetracycline in water. The working solutions were then prepared for each antibiotic at 100 μg/mL. A serial dilution series with a final volume of 1 mL was created by preparing a 1:10 dilution series in *S. aureus* suspension at concentration of 1×10^6 cells/mL diluted with BHI, such that the final solutions contained antibiotic concentrations ranging from 100 μg/mL to 1 ng/mL. We then incubated the culture for 20 hours in an incubator at 37°C according to CLSI protocols, and the MIC determined after incubation.

Image processing, data acquisition, quantification and analysis

During the experiments, a 24-well plate with microwells was placed on a fully automated Nikon TiE microscope (Micro Device Instruments, Avon, MA, USA) with an incubator heated to 37°C. Images were acquired through a 10× objective in fluorescence or phase contrast settings. Growth of bacteria was recorded using time-lapse imaging, with individual frames recorded at an interval of 10 minutes for a minimum of 250 minutes. The total number of experimental repeats for this analysis was eight for Penicillin G and six for Carbenicillin, Nafcillin and Tetracycline. Error bars represent mean ± SEM. Time lapse image sequences were analyzed by FIJI (Fiji Is Just ImageJ, NIH). Results were plotted using Sigma Plot version 12.

RESULTS

Bacterial growth is usually measured either by visual inspection of colony growth on solid media or by optical density measurements of liquid cultures. Growth of visible colonies often takes 12 hours, while liquid culture measurements can be complicated by contamination with faster growing bacteria. We hypothesized that microscale approaches may combine aspects of these two methods, while avoiding some of their inherent shortcomings.

Microwell array design and optimization

We evaluated bacterial growth in the presence of various antibiotics at a range of concentrations inside arrays consisting of 12 groups of 97 microwells (1164 microwells per condition). The hexagonal layout of the array was optimized for microscopy using a 10× lens with a field of view of at least 700 μm × 700 μm without requiring rotational alignment (Fig. 1). Each microwell has 40 μm diameter. We compared the efficacy of bacterial capture in microwells with depths between 20 and 50 μm, and found that all exhibited unwanted washing of bacteria from the device. This issue was resolved by increasing the microwell depth to 100 μm. We found that a 2:5 ratio of microwell width to depth allowed efficient washing of the device and addition of antibiotics without any loss of bacteria from the microwells.

Application of growth assay for calculating MIC

We tested the ability of four antibiotics commonly used for *S. aureus* infections: Carbenicillin, Nafcillin, Penicillin G, and Tetracycline, to inhibit the growth of bacteria. Carbenicillin, Nafcillin, and Penicillin G are known to which inhibit bacterial cell wall formation, while Tetracycline prevents bacterial protein biosynthesis. We hypothesized that by measuring bacterial growth over time at different doses, detailed information about antibiotic sensitivity and corresponding growth responses could be obtained.

We measured *S. aureus* growth by time-lapse imaging performed on bacteria constitutively expressing EGFP. Images were acquired every 10 minutes for a minimum of 250 minutes using fluorescence microscopy (Fig. 2). We measured bacterial growth in a range of antibiotic concentrations and calculated the MIC for the four different antibiotics.

As expected, bacteria growth was altered dependent on antibiotic concentration (Fig. 3a–d). The inhibitory concentrations were comparable for the four antibiotics. We found that *S. aureus* was resistant to Carbenicillin for concentrations up to 100 ng/mL. At lower concentrations, between 1 ng/mL and 10 ng/mL, the bacteria growth curves reached a plateau at approximately 220 minutes, consistent with a bacteriostatic effect. *S. aureus* became susceptible to killing by Carbenicillin at concentration of 1 μg/mL and higher. Bacterial growth in the presence of Tetracycline and Nafcillin concentrations below 100 ng/mL did not reach a stationary phase within the observation window of 250 minutes. For Penicillin G, concentrations higher than 10 ng/mL killed the bacteria. From the *S. aureus* growth measurements using our assay, we estimated MIC values of 1 μg/mL for Carbenicillin, Tetracycline and Nafcillin, and 100 ng/mL for Penicillin G (Fig. 3).

Distinct bacterial growth signatures with different antibiotics

We observed differences in growth curves for *S. aureus* in the presence of different antibiotics. These differences suggest that the growth rate changes over time may be due to different mechanisms of action by different antibiotic classes. We observed a plateau in the growth rate above threshold concentrations for *S. aureus* incubated with antibiotics that target the bacteria cells wall (Carbenicillin, Penicillin and Nafcillin). We did not observe a plateau phase state in the presence of an antibiotic that targets protein synthesis (Tetracycline — Fig. 3a–d). These differences are consistent with the known mechanisms by which each antibiotic inhibits bacterial growth.

A comparison of *S. aureus* growth curves in the presence of the four antibiotics at the same 100 ng/mL concentration confirmed that the wildtype *S. aureus* was susceptible to Penicillin G, which blocked growth and reduced bacterial numbers, but was resistant to Tetracycline, Carbenicillin disodium, and Nafcillin sodium salt, which allowed bacterial growth (Fig. 4).

Selection of viable clones occurs in the first round of bacterial replication

By seeding the device such that only one or two bacteria were present in each microwell, we hypothesized that it should also be possible to identify emerging antibiotic-resistant clones. Analysis of the rate of change of the number of *S. aureus* in the wells in response to specific antibiotics revealed a transient decrease in bacteria numbers in the first 30 minutes followed

by a peak in growth (Fig. 5a–d). However, a lag phase was noted also for control samples, suggesting that a proportion of bacterial death may be due to sensitivity to the change of culture media or the procedures during the device priming. Interestingly, selective killing was observed in response to Penicillin and Nafcillin, but not to Tetracycline or Carbenicillin. At later times, bacteria growth resumed at antibiotic concentrations below MIC. A summary of bacterial growth rate characteristics at different concentrations of antibiotics is presented in Table 1.

Low doses of antibiotic boost rates of bacterial proliferation

Strikingly, we observed higher growth rates in the presence of Penicillin and Nafcillin at concentrations below the MIC (Fig. 5). These were preceded by the large initial dips in bacterial growth, suggesting that the killing of the most sensitive bacteria may select for clones with enhanced proliferative capacity, which grow faster in the absence of competition for nutrients from other bacteria. For antibiotic concentrations that eventually terminated almost all bacteria, the rate decreased, and after a sharp change in rate (corresponding to a sharp bend in the graph), the rate approached zero. This sharp change in growth rate occurred 60–80 minutes into the experiment, indicating that several rounds of bacterial division occurred before the antibiotic became fully effective. Interestingly, for intermediate concentrations, at which the bacteria resist the antibiotic, there were two or three peaks in the rate graph within the time range of the experiment. The position and the amplitude ratio of peaks appeared to be specific for each antibiotic, and may indicate the progressively effective activity of the antibiotic noted above, coupled with the emergence of progressively resistant clones.

Bacterial proliferation in microwells can be observed for non-fluorescent strains

This device was designed with practical clinical applications in mind, which would clearly exclude the use of transgenic markers. To test whether bacterial growth could be measured with bacteria strains that do not express fluorescent proteins, we conducted experiments that relied on bright field imaging and compared the results with those obtained by fluorescent imaging. The results show that it is possible to distinguish between susceptibility, intermediate sensitivity, and antibiotic resistance for bacteria that are not fluorescent (Fig. 6). Results from bright field imaging are largely consistent with those obtained using fluorescent imaging (Fig. 6d). Some differences could be noted, likely due to the variability of growth rate in the middle range of antibiotics concentrations (Fig. 6e).

Comparing the MIC of different antibiotics against bacteria using off-chip measurements

We validated our on-chip assay by comparing our MIC measurements to off-chip broth dilution approaches, using *S. aureus* and four different antibiotics: Carbenicillin, Nafcillin, Penicillin G, and Tetracycline. The broth dilution approach obtained an MIC for Carbenicillin, Nafcillin, Penicillin G, and Tetracycline of 1 μg/mL, 1 μg/mL, 100 ng/mL, and 100 ng/mL, respectively. We found that the results of our MIC assay on-chip closely matched those obtained by broth dilution approaches for Carbenicillin, Nafcillin, and Penicillin G. Interestingly the only exception was the MIC of Tetracycline against *S. aureus*, in which the MIC on-chip assay was 1 μg/mL vs. 100 ng/mL in the broth assay. This result

suggests that a cumulative effect of protein synthesis inhibition may require incubation longer than 3 hours for accurate results.

DISCUSSION

This study presents open-microwell arrays system for faster evaluation of antibiotic sensitivity for *S. aureus*. The system reduces the AST time from 48 hours for traditional assays, down to 3 hours. The high-aspect ratio of the wells is critical for our ability to hold in place and monitor small numbers of *S. aureus* while replacing the media and adding antibiotics around them. Each microwell array is placed in a well of a multiwell plate. Thus, we were able to measure MIC for four antibiotics at six concentrations each (Tetracycline, Carbenicillin, Nafcillin, and Penicillin G, at concentrations from 1 ng/mL up to 100 μg/mL).

The new assay is faster than other AST assays (reviewed recently in Ref. 38). One significant time-saving feature are the deep microwells, which enable loading bacteria in liquid media and circumvents the need for longer protocols for pre-loading and holding bacteria in fixed positions e.g. encapsulating bacteria in various gels[8–10]. Only two other studies investigated antibiotic sensibility without preloading steps. Hou *et al.,* performed experiments in less than 3 hours[11]. However, their approach requires pre-encapsulation of bacteria in agarose-based gel prior to the assay. He *et al.* measured MIC and antibiotic susceptibility in 4 to 8 hours[6] and the whole process, including the preparation of the assay, takes 8.5 hours. Overall, our approach allows fast and simple loading of bacteria and antibiotics directly into the open microwells and reduced the time of the assay to 3 hours.

The assay provides large amount of data regarding *S. aureus* growth, through the use of time-lapse microscopy. These allow us to monitor bacterial growth and record changes in growth rate. These measurements revealed distinct variations of the growth rate for different bacteria at various concentrations, starting with the initial lag phase in the 30 minutes after loading the bacteria[39], and continuing with the exponential growth. In addition to discrimination of growth phases, our device enables distinction between cell wall inhibitory antibiotics (Carbenicillin, Nafcillin, and Penicillin G), and antibiotics that inhibited protein synthesis (e.g. Tetracycline) by observing the time when bacterial growth reached the stationary phase. The growth rate was faster for cell wall inhibitors and slower for protein synthesis inhibitors. One surprising result from our work was the higher growth rate of the *S. aureus* at concentrations lower than the MIC. This effect is consistent with the observation of an increased bacterial density at the edge of antibiotic zones of inhibition in classical antibiotic sensitivity testing, noted before to occur only in the presence of nutrient rich media and sub-lethal concentration of antibiotic[40]. This effect may be relevant to the acquisition of antibiotic resistance and the higher precision of our assay may help design new approaches to study the molecular mechanisms involved. Overall, our approach has the potential to be a rapid, sensitive, and reliable technology for use at hospitals and laboratories for testing bacteria for antibiotic sensitivity.

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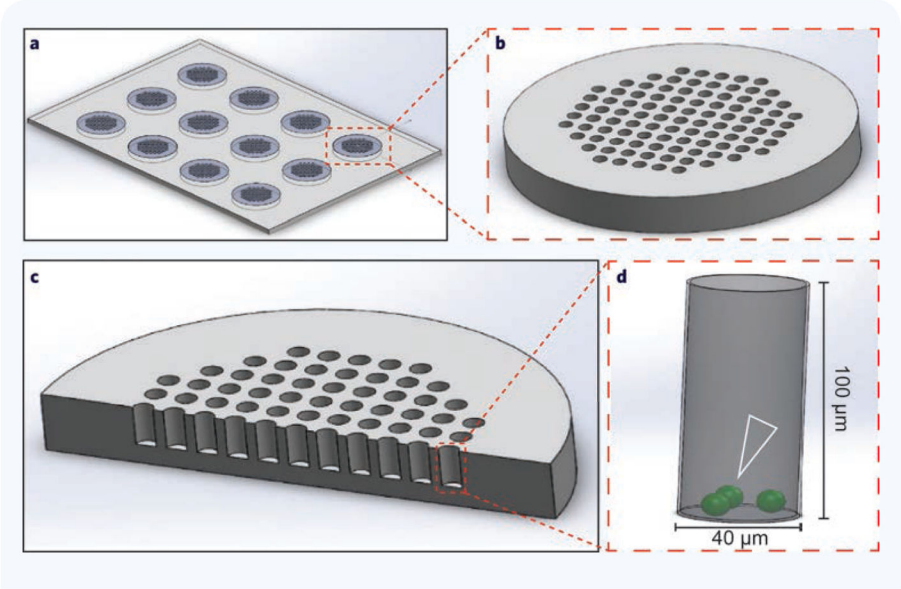

Figure 1. Schematic of the microwell devices for bacterial antibiotic sensitivity testing

(**a**) Overview of devices with 12 viewing fields. The devices were designed such that one device fits in one well of a multi-well plate. (**b**) A single viewing field magnified to show the open microwell array. (**c**) Cross-section through array shows microwell geometry. (**d**) Magnification of one microwell. Open white arrowhead indicates bacterial positioning following loading.

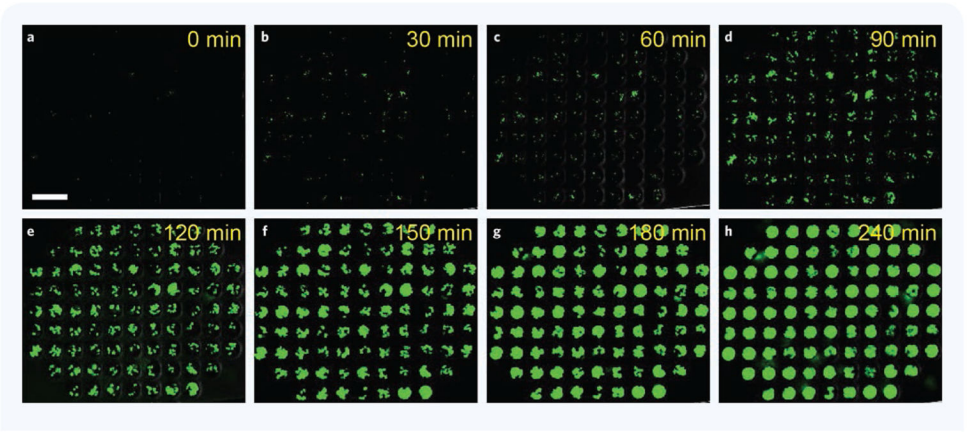

Figure 2. *S. aureus* growth inside microwell arrays in the presence of 1 ng/mL Tetracycline

(**a–h**) Time-lapse images show *S. aureus* growth in the presence of bellow MIC, 1 ng/mL Tetracycline. Most microwells are filled with green fluorescent *S. aureus* at 180 minutes; 87 of the 97 microwells in one array can be captured in one image. Scale bar: 100 μm.

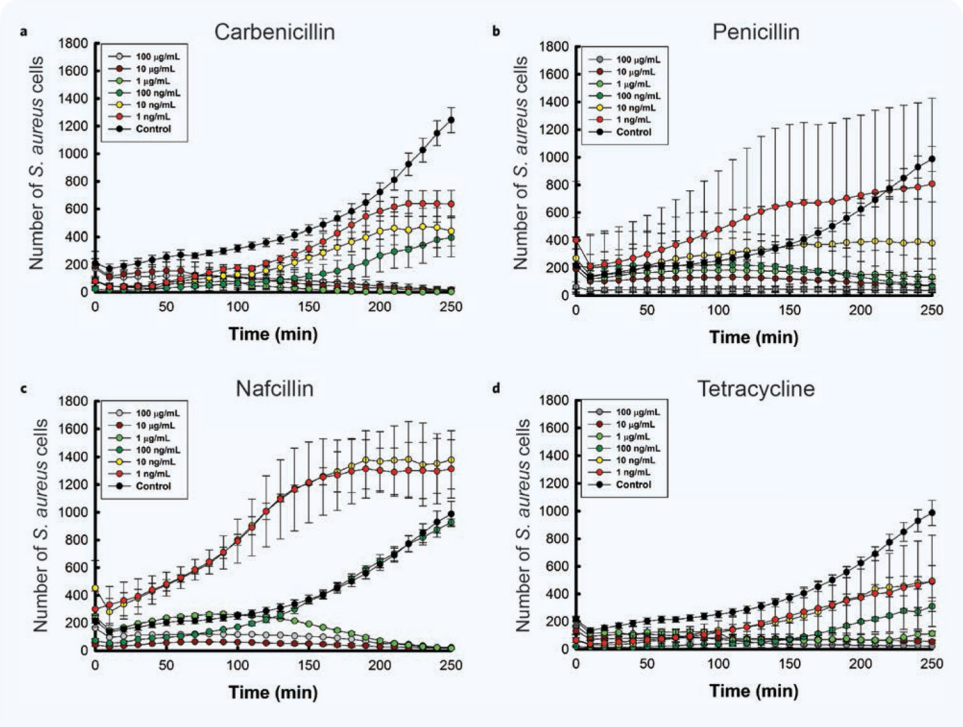

Figure 3. Correlation between antibiotic concentration and bacterial growth over time

(**a–d**) *S. aureus* growth curves are shown at different concentrations of various antibiotics. The total number of observations for this analysis was 8 for Penicillin G and 6 for the remaining groups. Data is presented as mean and standard error of the mean number of individual cells counted in the 97 wells of an array.

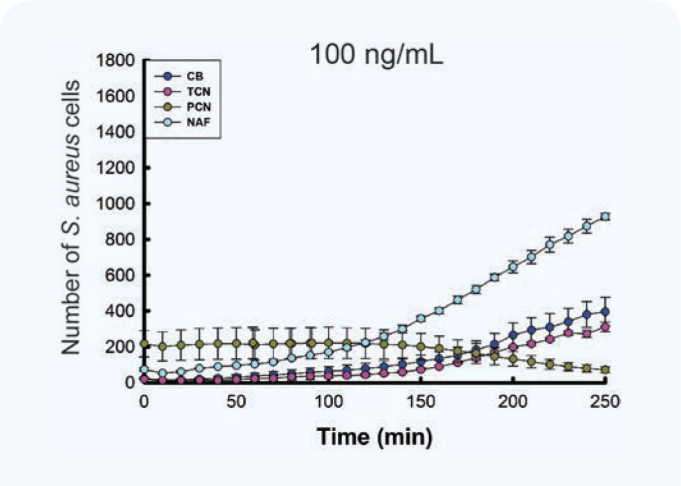

Figure 4. Different effect of different antibiotic on bacterial growth

S. aureus is susceptible to Penicillin G (PCN) but resistant to Tetracycline (TCN), Carbenicillin disodium (CB), and Nafcillin sodium salt (NAF) at 100 ng/mL. The total number of observations for this analysis was 8 for Penicillin G and 6 for the remaining groups. Data is presented as mean and standard error of the mean number of cells counted in the 97 wells of an array.

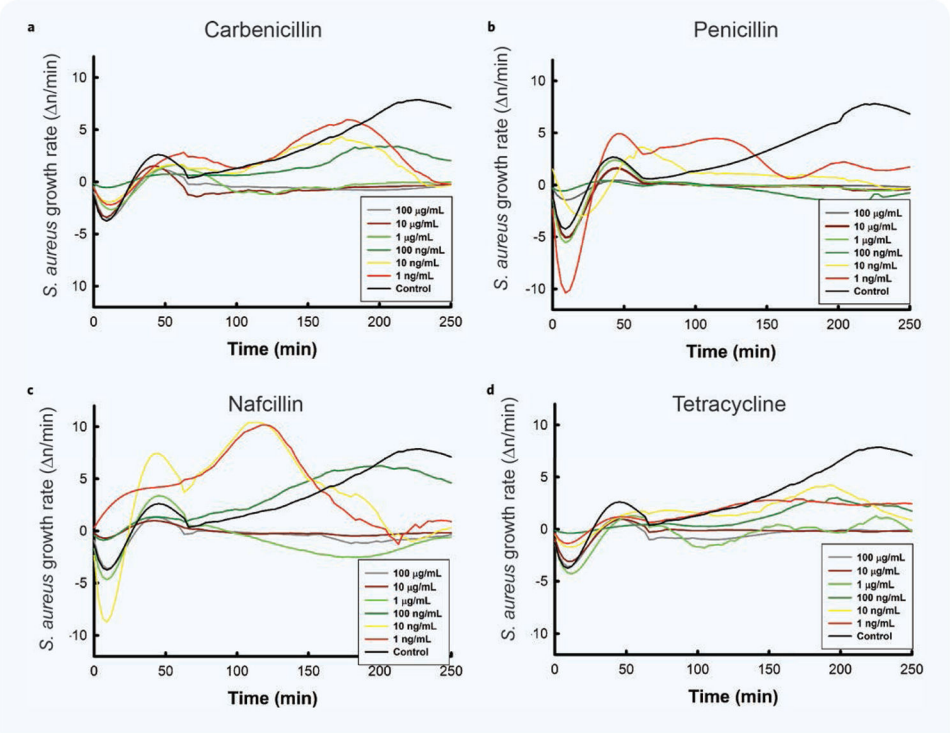

Figure 5. Changes of bacterial growth rate over time

(**a–d**) *S. aureus* average growth rates are shown at different concentrations of different antibiotics. "Negative" growth rates represent bacteria death rates. $N = 8$ experimental repeats for Penicillin G and $N = 6$ for all other conditions.

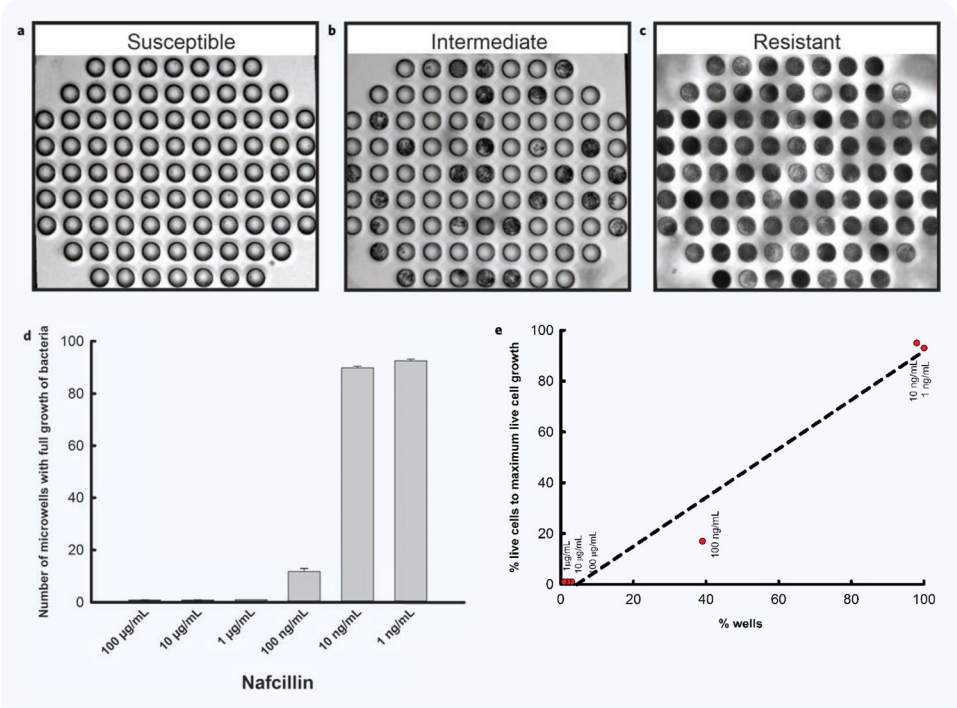

Figure 6. Representative micrographs depicting different categories of bacteria sensitivity in experiments to investigate effect of antibiotics on bacterial growth

(**a–c**) Micrographs of three microwell arrays, showing an image of time lapse imaging following 3 hours incubation with Nafcillin. (**a**) An example of the antibiotic susceptible category. There is no evidence of bacteria growth inside the wells. (**b**) An example of the intermediate antibiotic sensitivity category. There is evidence of bacteria growth inside some but not all microwells, and many wells are only partially filled. (**c**) An example of the antibiotic resistant category. There is evidence of bacteria inside all microwells. (**d**) Data captured from time lapse imaging following 3 hours incubation with Nafcillin. *S. aureus* is susceptible to Nafcillin at concentrations above 1 µg/mL. *S. aureus* has intermediate antibiotic sensitivity to Nafcillin at 100 ng/mL. *S. aureus* shows resistance to Nafcillin at concentrations below 10 ng/mL. (**e**) Comparison of BF imaging results with fluorescent imaging results.

Table 1

Summary of bacterial growth rate observation at different concentrations of antibiotics.

Antibiotic	Condition	Observations from growth rate graphs
No antibiotic	Control — no antibiotic	• A transient ~30 min of substantial death • followed by a ~30 min growth burst • followed by a longer, ~150 min period of sustained growth at increasing rate
All antibiotics	Concentrations above MIC	• A transient ~30 min of substantial death • followed by a ~30 min growth burst comparable to control • followed by persistent death starting at ~60 min
Carbenicillin	Concentrations below MIC	• A transient ~30 min of substantial death • followed by a longer, 60 min growth burst • followed by a second, 120 min period of fast growth
Penicillin G	Concentrations below MIC	• Sustained growth at 1 ng/mL, sub-lethal concentration, at rates higher than control, for 120 min • transient death followed by sustained growth for 120 min at 10 ng/mL concentration
Nafcillin	Concentrations below MIC	• Sustained growth rates, higher than control, at 1 and 10 ng/mL concentrations, for 120 and 90 min, respectively
Tetracycline	Concentrations below MIC	• A second growth peak at 90–120 min after exposure to antibiotic • growth rates below the control for all concentrations