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Toward Decentralizing Antibiotic Susceptibility Testing via Ready-to-Use Microwell Array and Resazurin-Aided Colorimetric Readout

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Author Contributions

F.E.C.: Conceptualization, validation, visualization, writing original draft, and editing. A.K.: Writing original draft, review, and editing. K.H.: writing, review, and editing. E.C.: Validation and visualization. L.C. and P.Z.: Resources. T.W.: Conceptualization, funding acquisition, supervision, and writing, review, and editing.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.analchem.0c04095>.

Experimental section: Chip design and fabrication, preparation of ready-to-use microwell array with preloaded antibiotics, preparation of bacteria samples, data analysis of marcAST result, evaluation of rehydration process of the microwell array device, and clinical isolate testing. Figures: Microwell device structure, demonstration of operation procedure with a 30 s step prior to AST, time course of marcAST with ciprofloxacin and *E. coli* ATCC 25922, MIC determination using benchtop in-tube broth dilution method, ready-to-use microwell array device shelf life, MIC determination of *S. aureus* ATCC 23922 with gentamicin, and classification of antimicrobial susceptibilities for additional clinical isolates. Tables: Interpretive categories and MIC breakpoints of four antibiotics for Enterobacteriaceae species based CLSI guidelines, and AST profiles of clinical isolates ($n = 7$). (PDF)

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Abstract

In the face of the global threat from drug-resistant superbugs, there remains an unmet need for simple and accessible diagnostic tools that can perform important antibiotic susceptibility testing against pathogenic bacteria and guide antibiotic treatments outside of centralized clinical laboratories. As a potential solution to this important problem, we report herein the development of a microwell array-based resazurin-aided colorimetric antibiotic susceptibility test (marcAST). At the core of marcAST is a ready-to-use microwell array device that is preassembled with custom titers of various antibiotics and splits bacterial samples upon a simple syringe injection step to initiate AST against all antibiotics. We also employ resazurin, which changes from blue to pink in the presence of growing bacteria, to accelerate and enable colorimetric readout in our AST. Even with its simplicity, marcAST can accurately measure the minimum inhibitory concentrations of reference bacterial strains against common antibiotics and categorize the antibiotic susceptibilities of clinically isolated bacteria. With more characterization and refinement, we envision that marcAST can become a potentially useful tool for performing AST without trained personnel, laborious procedures, or bulky instruments, thereby decentralizing this important test for combating drug-resistant superbugs.

Graphical Abstract

The emergence and propagation of multidrug-resistant “superbugs” that cause life-threatening infections and imperil modern medicine have created an urgent need for advancing diagnostic methods in clinical microbiology.¹ Among these diagnostic methods, antimicrobial susceptibility testing (AST) remains one of the most important and informative² because it reveals bacterial susceptibilities that are not only crucial for appropriate antimicrobial treatments but also useful for community-based surveillance of antimicrobial resistance,³ leading to better patient outcomes while also reducing the potential of antibiotic resistance. Ideally, this critical diagnostic procedure would be the most beneficial if it is accessible to patients in decentralized settings such as

local clinics. Unfortunately, AST is predominantly performed by coculturing the target pathogenic bacteria with antibiotics at varying concentrations in culture media (e.g., broth microdilution) or agar plates (e.g., E-test), which requires trained personnel to operate in laboratories and more than 16 h to detect bacterial growth/inhibition and reveal bacterial susceptibilities.^{4,5} Commercial AST instruments from industry leaders like Becton Dickinson and BioMérieux, which have become the standard for performing AST in centralized clinical microbiology laboratories, provide solid solutions for scaling up ASTs to batch processes, but these are costly and have bulky footprints that render them laboratory bound.^{6,7} Consequently, there remains an unmet need for reducing the reliance of AST on trained personnel, laborious procedures, time-consuming incubation, and expensive and bulky instruments, thus allowing this important diagnostic method to be decentralized and accessible to more patients in need.

Recent advances in microfluidics-empowered AST have shed light on a potentially useful approach for advancing AST, although most of these have emphasized miniaturization of assay volume and shortening of assay time rather than simplifying the assay. For example, several devices implemented intricate features and operational schemes for partitioning samples and reagents into multiple microchambers containing distinct concentrations of antibiotics for AST. In developing these platforms, researchers have put forth a variety of strategies including PDMS microvalves,⁸ antibiotic gradient generators,⁹ and vacuum-driven “self-loading” designs.^{10,11} However, all these strategies invoke cumbersome user interfaces (e.g., solenoid valves and vacuum pumps), complex fabrication schemes, and expensive fluorescence detectors, limiting their portability, ease-of-fabrication, and ultimately ease of operation. As such, an AST device that not only leverages microfluidics to shorten assay time but also can be operated easily without trained personnel and bulky instruments still needs to be developed.

We are thus motivated to develop microwell array-based resazurin-aided colorimetric AST (marcAST) as a potential solution toward decentralizing AST. At the core of marcAST is a stamp-sized ready-to-use microwell array device that is preassembled with custom titers of various antibiotics and splits the bacterial sample upon a single syringe injection step to initiate AST against all antibiotics, leading to a significantly simplified procedure. We also employ resazurin, which changes from blue to pink in the presence of growing bacteria,^{12,13} to accelerate and enable colorimetric readout in our AST without sophisticated optical detection apparatuses. Despite its simplicity, marcAST retains reliable AST, as it can measure the minimum inhibitory concentrations (MICs) for reference bacterial strains against common antibiotics and distinguish bacteria strains with distinct antibiotic susceptibilities. We demonstrate the overall utility of marcAST by generating antibiograms for seven clinical isolates against four first-line antibiotics and achieve 96% categorical agreements with standard clinical microbiology reports.

2. MATERIALS AND METHODS

Microwell array devices housing 12 AST microwells were designed and fabricated via PDMS soft lithography and PDMS glass bonding. Prior to performing marcAST, antibiotic titers were preloaded and dried in the microwells. For initiating marcAST, the sample

mixture containing Mueller–Hinton (MH) broth (Sigma-Aldrich), resazurin viability reagent (AlamarBlue Cell Viability Reagent, Invitrogen), and 5×10^5 CFU/mL of bacteria (either clinical isolates or reference strains from ATCC) followed by mineral oil (Sigma-Aldrich) were syringe injected into the device through its central inlet. The sample mixture was evenly split into the 12 microwells and surrounded by mineral oil, thus establishing 12 separate AST reactions. The device was incubated at 37 °C for 5 h unless otherwise noted. Immediately after incubation, a bright-field image was acquired for each device using a cell-phone camera. Experimental procedures are detailed in the Supporting Information.

3. RESULTS

3.1. Overview of marcAST.

We have designed marcAST with the aim toward decentralizing AST by reducing its reliance on trained personnel, laborious procedures, time-consuming incubation, and bulky instruments. Integral to marcAST is the ready-to-use microwell array device—a stamp-sized microfluidic device that is preloaded with preselected antibiotics at defined titrations for assessing the susceptibilities of the sample bacteria (Figure 1, step1). As an example, we demonstrate in this work simultaneous AST against four antibiotics within a single device. To perform AST in our device, the sample containing both bacteria and resazurin dye followed by a lower density companion oil are injected into the device using a common laboratory syringe without complicated tubings, vacuum/pressure pumps, or syringe pumps (Figure 1, step 2). The sample and resazurin assay mixture are passively split into up to 12 distinct chambers for parallelized resazurin-amplified broth microdilution testing. The oil effectively separates the samples and reduces sample evaporation during AST. The fully portable device can then be immediately incubated at 37 °C to initiate AST. With the viability assay, blue resazurin is reduced to pink resorufin in the presence of growing bacterial cells, and the microwells can be visually inspected to interpret the susceptibility or resistance of the bacterial cells to the antibiotics and generate the AST report known as antibiogram akin to standard AST performed in centralized clinical microbiology laboratories (Figure 1, step 3). The simple sample loading, partitioning, and detection scheme in marcAST thus obviates trained personnel, laborious procedures, and bulky instruments.

3.2. Operation of Ready-to-Use Microwell Array Device.

We first illustrate the simple operation of the ready-to-use microwell array device, which is the core of marcAST. The device houses 12 peripheral AST microwells that are 4 mm in diameter, open to the atmosphere for convenient antibiotic deposition, and connected to a single central access port at the center for inputting the sample and the oil. We fabricate the devices out of PDMS and glass via standard PDMS soft lithography and PDMS glass bonding (Figure S-1).¹⁴ To realize the ready-to-use simplicity for performing AST in our device, we prepipette antibiotic titrations into the 12 open microwells and air dry them. Doing so simplifies the operation procedure prior to AST to a 30 s step, in which the bacterial sample is drawn into a syringe that is partially prefilled with an immiscible oil, injected into the device, and partitioned by the oil (Figure S-2).

As uniform splitting of the bacterial sample into the 12 microwells is crucial to reliable AST in our device, we next verify that its radial channel design indeed facilitates uniform sample splitting. Here, we employ fluorescein dye as mock antibiotics so that we can measure fluorescence intensities via imaging to quantitatively characterize sample splitting in our device. We preload the 12 microwells with linearly increasing concentrations of fluorescein from 0 to 100 μM (Figure 2A, left), air dry them, then syringe inject water and oil to rehydrate and partition them in their respective microwells, and finally take a fluorescence image of the microwells (Figure 2A, right) and quantify the fluorescence intensities in the microwells. We observe linearly increasing fluorescence intensities in the microwells (Figure 2B, $R^2 = 0.98$), indicating the restoration of linearly increasing concentrations of fluorescein and therefore equal volumes of water in all microwells because of uniform splitting.

3.3. Validation of marcAST.

We validate marcAST with several standard AST experiments. First, we test the ATCC reference *E. coli* (ATCC 25922) against a widely used antibiotic, ciprofloxacin (CIP), and ensure that the minimum inhibitory concentrations (MICs) measured by our device with a naked-eye readout are comparable to the MIC defined by the Clinical Laboratory Standards Institute (CLSI) guidelines.¹⁵ Here, we predeposit and dry 0–0.06 $\mu\text{g/mL}$ of CIP in 2-fold increments in the 12 microwells of our device, then load and partition the sample containing 5×10^5 CFU/mL *E. coli* (per CLSI guidelines) and 10% alamarBlue (a commercially available resazurin dye), and incubate the device at 37 °C for 5 h—a sufficient culture time that we have empirically determined (Figure S-3). After incubation, we clearly see that samples with 0.008 $\mu\text{g/mL}$ CIP have turned pink, which indicate significant *E. coli* growth, whereas samples with 0.015 $\mu\text{g/mL}$ CIP remain blue, which correspond to inhibition of *E. coli* growth (Figure 3A). The MIC determined via marcAST is therefore 0.015 $\mu\text{g/mL}$ and agrees not only with the CLSI MIC but also the results from benchtop in-tube AST (Figure S-4)—indicative of the robustness of using the sample to rehydrate dried antibiotics and performing AST in our device. Moreover, consistent MICs are measured from dried CIP up to 14 days prior to marcAST (Figure S-5). We can also obtain supplemental quantitative readouts via RGB analysis of the device image. The resulting red-to-blue (R/B) ratios are greater than 0.75 for samples with 0.008 $\mu\text{g/mL}$ ciprofloxacin but less than 0.75 for samples with 0.015 $\mu\text{g/mL}$ ciprofloxacin (Figure 3A). We can therefore quantitatively define marcAST MIC as the titration at which the R/B ratio falls below 0.75. We subsequently subject the resistant *E. coli* strain (ATCC BAA-2471) to the same CIP titrations in a microwell array device. After incubation, samples in all 12 microwells have turned pink and register greater than 0.75 R/B ratios (Figure 3B). These results indicate that even the higher CIP titrations are insufficient to inhibit the growth of this *E. coli* strain, which corroborate with its resistance to CIP. Finally, when we test the ATCC reference *S. aureus* (ATCC 29213) against another common antibiotic gentamicin (GEN), our marcAST MIC again agrees with the CLSI MIC (Figure S-6). These results collectively provide strong validation for marcAST.

3.4. Classification of Antimicrobial Susceptibilities for Clinical Isolates via marcAST.

Having verified that marcAST can measure MICs of reference strains and differentiate bacterial strains with distinct antibiotic susceptibilities, we demonstrate that marcAST can also be utilized in the clinically relevant scenario of categorizing the susceptibilities of clinically isolated bacteria to multiple antibiotics. Here, AST is performed by challenging clinically isolated bacteria at specific interpretive breakpoint concentrations that have been defined by the CLSI guidelines to categorize the bacteria as susceptible (S), intermediate (I), or resistant (R).¹⁶ This categorical readout, rather than the MIC, is ultimately used toward treatment guidance. We test seven clinical isolates obtained from the Johns Hopkins Hospital Clinical Micro-biology Laboratory against four frontline antibiotics: CIP, GEN, trimethoprim-sulfamethoxazole (SXT), and tobramycin (TOB). The seven isolates—three isolates of *E. coli*, one isolate of *K. aerogenes*, two isolates of *C. freundii*, and one isolate of *E. cloacae*—all belong to the Enterobacteriaceae family, which is highly prevalent in infectious diseases such as urinary tract infections.²² The CLSI-defined interpretive breakpoint concentrations for Enterobacteriaceae against these antibiotics are 1, 2, and 4 $\mu\text{g/mL}$ for CIP; 4, 8, and 16 $\mu\text{g/mL}$ for GEN; 2/38 and 4/76 $\mu\text{g/mL}$ for SXT; and 4, 8, and 16 $\mu\text{g/mL}$ for TOB (Table S-1),¹⁵ which conveniently fit in a single microwell array device along with one no-antibiotic control. marcAST provides clear and simple readouts for interpretive breaking testing (Figure 4A and Figure S-7). For clinical isolates #1 and #2, only their no-antibiotic controls turn pink, indicating that both isolates are susceptible to all four antibiotics. On the other hand, for clinical isolate #3, all but sample with TOB 8 $\mu\text{g/mL}$ turn pink, indicating that this isolate is resistant to CIP, GEN, and SXT and intermediate to TOB (Figure 4A). When compared to the standard clinical laboratory results (Table S-2), marcAST achieved 96% (27 out of 28) categorical agreements for the seven clinical isolates and four antibiotics (Figure 4B). These results demonstrate the potential of marcAST in classifying the antibiotic susceptibilities of multiple species of clinically isolated bacteria.

4. CONCLUSION

In summary, we have developed a microwell array-based colorimetric readout workflow that enables decentralizing AST. Compared to conventional time-consuming and resource-intensive culture-based platforms, our platform leverages an easy-to-operate microwell array device and couples it with a resazurin-aided broth microdilution assay for rapid results which can be useful in point-of-care or resource-limited settings. Our device can be easily assembled with customizable titers of antibiotics, dried, and stored for on-demand operation. Furthermore, our device may be operated with a common syringe and does not require complicated instrumentation or microfluidic expertise. We demonstrate facile differentiation of susceptible and resistant bacteria, as well as resource-free visual interpretation of MIC for clinical isolates against four distinct antibiotics using our platform. The antibiograms that our platform helps generate can be immensely useful in guiding antimicrobial therapy as well as inferring antimicrobial resistance patterns which can be useful for community-based surveillance and antimicrobial stewardship.

We envision several paths for improving the utility of marcAST. First, the antibiotic titer can be expanded beyond the current four antibiotics by adding more microwells in each device. Second, the assay time can be reduced by tuning the bacteria and resazurin concentrations, similar to previous works.^{18,20,21} Third, marcAST currently generates antibiograms from bacteria following clinical single-colony isolation. Appending a device that can filter and concentrate bacterial cells from patient samples^{17–19} before marcAST may obviate clinical isolation and enable direct AST from samples with monomicrobial infections, such as the plurality of samples from uncomplicated UTIs.²² Finally, colorimetric identification of bacteria^{23–25} may be implemented within our microwell array device and be performed in tandem with marcAST. Such an addition can further enhance the utility and accuracy of marcAST. With current performance and potential for improvement, we foresee a future iteration of marcAST becoming an easy-to-operate and reliable solution for performing AST in decentralized settings toward both clinical and research applications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Overview of microwell array-based resazurin-aided colorimetric antibiotic susceptibility testing (marcAST). (1) At the heart of marcAST is the ready-to-use microwell array device, which is preloaded with dried antibiotic titers. (2) AST is initiated upon injecting the bacterial sample mixed with resazurin and an oil through the central access port of the device. Upon injection, the bacterial sample readily splits, flows into microwells, and rehydrates the antibiotics in the microwells for AST, while the oil subsequently splits to partition the split samples. Thus, using one device and one injection step, the sample can be tested against a no-antibiotic positive control (Pos ctrl) and four antibiotics (Abx 1–4) at predefined concentrations for categorizing bacteria as resistant, intermediate, or susceptible (C^R, C^I, C^S). (3) After incubation, the presence of growing bacteria reduces blue resazurin into pink resorufin, thus enabling colorimetric readouts for yielding an antibiogram that categorizes the bacteria as either resistant (R), intermediate (I), or susceptible (S) against the four antibiotics.

Figure 2.

Operation of ready-to-use microwell array device. (A) Uniform splitting of samples in the device via a single syringe injection is evaluated by preloading fluorescein dyes in the microwells (left), drying the dyes, and subsequently rehydrating the dyes with water injected through the central port (right). (B) Strong linearity between the fluorescence signals and the rehydrated fluorescein dyes in the microwells indicates uniform splitting of water via a single syringe injection.

Figure 3.

AST performance of marcAST. (A) When testing *E. coli* ATCC 25922 against ciprofloxacin (CIP) titrations in duplicate via marcAST (left), samples with 0.008 $\mu\text{g/mL}$ CIP become pink due to *E. coli* growth, whereas samples with 0.015 $\mu\text{g/mL}$ CIP remain blue. The MIC measured by marcAST, at 0.015 $\mu\text{g/mL}$ (red arrow), matches that in the CLSI guidelines. Quantitative readouts based on a red-to-blue (R/B) ratio from the colorimetric readout can also be acquired (right), where the MIC corresponds to less than 0.75 in the R/B ratio. (B) When testing the multidrug resistant *E. coli* via marcAST, all samples become pink (left) and register greater than 0.75 R/B ratios (right), which corroborate with CIP resistance.

B

Clinical Isolate #	ID	marcAST Readout/Clinical Readout			
		CIP	GEN	TOB	SXT
1	<i>E. coli</i>	S/S	S/S	S/S	S/S
2	<i>E. coli</i>	R/R	R/R	I/I	R/R
3	<i>E. coli</i>	S/S	S/S	S/S	S/S
4	<i>K. aerogenes</i>	S/S	S/S	S/S	S/S
5	<i>C. freundii</i>	S/S	S/S	S/S	S/S
6	<i>E. cloacae</i>	S/I	S/S	S/S	R/R
7	<i>C. freundii</i>	S/S	R/R	R/R	R/R

Figure 4.

Classification of antimicrobial susceptibilities for clinical isolates via marcAST. (A) marcAST provides clear and simple readouts for classifying the susceptibilities to ciprofloxacin (CIP), gentamicin (GEN), tobramycin (TOB), and trimethoprim-sulfamethoxazole (SXT) for clinically isolated bacteria. For example, clinical isolates #1 is classified as susceptible to all four antibiotics because only the no-antibiotic control is pink, while clinical isolate #2 is classified as resistant to CIP, GEN, and SXT and intermediate to TOB because all titers but TOB 8 $\mu\text{g/mL}$ are pink. (B) For seven clinical isolates against the four antibiotics, marcAST achieves 27 out of 28 (96%) categorical agreements with standard clinical laboratory results.