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Utilizing Paper-Based Devices for Antimicrobial-Resistant Bacteria Detection

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Abstract: *Antimicrobial resistance (AMR), the ability of a bacterial species to resist the action of an antimicrobial drug, has been on the rise due to the widespread use of antimicrobial agents. Per the World Health Organization, AMR has an estimated annual cost of USD 34 billion in the US and is predicted to be the number one cause of death worldwide by 2050. One way AMR bacteria can spread, and by which individuals can contract AMR infections, is through contaminated water. Monitoring AMR bacteria in the environment currently requires that samples be transported to a central laboratory for slow and labor intensive tests. We have developed an inexpensive assay using paper-based analytical devices (PADs) that can test for the presence of β-lactamase-mediated resistance. To demonstrate viability, the PAD was used to detect β-lactam resistance in wastewater and sewage and identified resistance in individual bacterial species isolated from environmental water sources.*

The introduction of antimicrobial agents in the early 20th century revolutionized medicine, significantly decreasing morbidity and mortality. However, owing to the widespread use of antimicrobial agents and the genetic plasticity of bacteria, more pathogens have developed the ability to resist these drugs, giving rise to antimicrobial resistant (AMR) bacteria.[1] According to the World Health Organization (WHO), AMR costs approximately USD 21 to 34 billion annually within the United States alone and is predicted to surpass heart disease as the number one cause of death worldwide by 2050.[2] Contaminated water is a significant source of infection and outlet for the spread of AMR bacteria. AMR propagation in water is further advanced through contamination by antimicrobial agents, which results in the selective proliferation of AMR bacteria, and the horizontal gene transfer of resistance from AMR bacteria to non-AMR bacteria.[3] Because of the significant role of water in the spread of AMR, many sources of water have been studied for the presence of AMR bacteria including urban wastewater,[4] irrigation water,[5] and drinking water in China[6] to name a few.[7]

Growth inhibition assays, the assessment of bacterial growth in the presence of antimicrobial agents, is the gold standard for detecting AMR bacteria.[8] While growth inhibition assays provide reliable results, they also require samples to be sent to a central laboratory to complete testing. In addition to transportation time, these methods require at least overnight (12–16 h) incubation, trained laboratory personnel to execute the procedure and analyze results, and expensive instrumentation.[8] Alternative methods for detecting AMR bacteria have also been developed, including expanded microarrays,[9] microfluidic devices fabricated with polydimethysiloxane (PDMS),[10] and paper-based culture devices.[11] While these are all promising systems, they also require expensive equipment, long times, or trained personnel. To monitor AMR bacteria in the field and diagnose AMR infections at the point of care, a rapid, disposable, and inexpensive device that does not require instrumentation or trained laboratory personnel for analysis is still needed.

Paper-based analytical devices (PADs) have shown significant promise as an alternative platform for diagnostics since the initial publication in 2007.[12] PADs have been developed for a variety of applications, including point-of-care (POC) diagnostics and environmental monitoring. Because of AMR concerns in both developed and developing countries, the WHO specifically mentions in their *Global Action Plan for Antimicrobial Resistance* the need for portable and inexpensive diagnostic tools.[13] PADs offer a cost effective platform because the starting substrate materials are inexpensive (often less than USD 0.01), the manufacturing techniques are well established, and the reagents (the most expensive part) are deposited in small amounts (microgram–picogram).[14] Many diagnostic motifs exist for PADs, but few have detected naturally-produced enzymes. Our group reported colorimetric and electrochemical assays to detect bacteria from food and water sources using the enzymes they produce.[15] This same detection motif can be used for detecting AMR, as some antimicrobial properties can be traced back to enzymes responsible for deactivating antibiotics.[16]

β-lactam antibiotics are the most widely used class of antibiotics. Bacterial resistance to these antibiotics is the most commonly acquired resistance classified as a serious threat by

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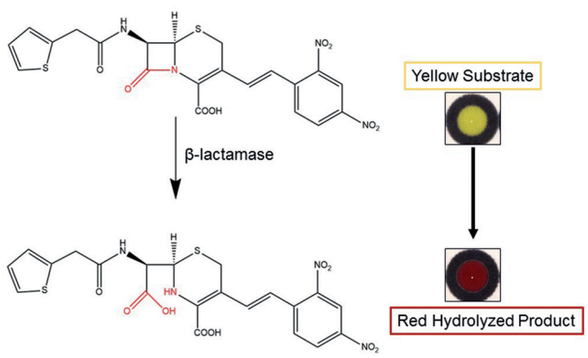
the Center for Disease Control (CDC).[17] Resistance can be a result of bacterial expression of β-lactamase enzymes,[18] which inactivate β-lactams by hydrolyzing the β-lactam ring in the antibiotic.[16b] Several ways exist to detect β-lactamase activity[19] including reactions with nitrocefin, a chromogenic cephalosporin.[20] The reaction results in the hydrolysis of the carbon–nitrogen bond in the β-lactam ring, causing a distinct color change from yellow to red (Scheme 1). Using this



Scheme 1. Reaction overview of β-lactamase and nitrocefin. Hydrolysis of the β-lactam ring in nitrocefin, mediated by β-lactamase, results in a distinct color change from yellow to red, making a visually detectable and user-friendly test.

straightforward detection method, we have developed a PAD that can detect β-lactamase-expressing bacteria in real-world samples. The platform is inexpensive, costing approximately USD 0.20 per test, compared to USD 10–22 for antibiotic susceptibility testing, and provides sensitivity that matches that of a microtiter plate.[8]

Reaction optimization between β-lactamase and nitrocefin was performed using arrays of 8-mm-diameter paper wells fabricated with Whatman grade 4 filter paper. The devices were photographed with a smartphone camera and analyzed with ImageJ software. Phosphate buffered saline (PBS) was used in solution and the optimal reaction pH was determined to be pH 7.5. The optimal nitrocefin concentration was 0.5 mM to maximize product signal, and the limit-of-detection (LOD) of lyophilized β-lactamase was 10 mU mL^{-1}. More information on reaction optimization can be found in the Supporting Information. The Michaelis–Menten kinetics of β-lactamase and nitrocefin were calculated for reactions on paper at circa 22 °C. Using a Lineweaver–Burk plot, the calculated V_{max} was 0.0285 ± 0.0012 mM min^{-1} and K_m was 0.293 ± 0.013 mM (Supporting Information, Figure S2 E). Literature searches have not generated published Michaelis–Menten values for β-lactamase reacting with nitrocefin, but the obtained values were similar to other reported values for β-lactamase.[21] This similarity in Michaelis–Menten values shows promise for the paper-based assays.

To demonstrate detection of β-lactamase in live bacteria, the optimized reaction conditions were used to analyze *E. coli* without culturing. Serial dilutions of β-lactamase-expressing *E. coli* and control *E. coli* were reacted with 0.5 mM nitrocefin at room temperature directly on the paper devices. No color change was observed unless the bacteria expressed β-lactamase (Figure 1 A). The color change in the assay occurred with more than 3.8×10^6 CFU mL^{-1} bacteria but not with lower concentrations. To determine whether non-β-lactamase producing bacteria would interfere with the detection of β-lactamase-producing *E. coli*, different ratios of β-lactamase-expressing bacteria to control bacteria were analyzed. The color intensities were the same with or without non-β-lactamase producing bacteria present in the sample (Figure 1 B). *E. coli* that do not express β-lactamase do not interfere with the reaction, as indicated by the similar color intensities that were observed in pure or mixed cultures. β-lactamase is produced within bacteria; therefore, we attempted to increase the sensitivity by repeating the assay with lysing. For DH5α *E. coli* cells expressing β-lactamase, lysing the cells using probe sonication improved the intensity

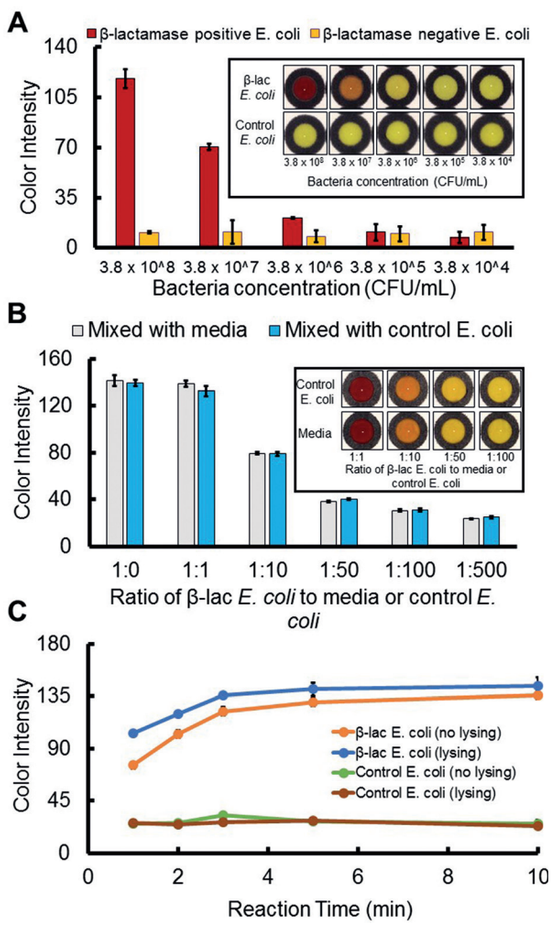


Figure 1. Optimization of β-lactam-resistant bacteria detection. A) The paper-based tests were used for serial dilutions of bacteria that were both positive and negative for expressing β-lactamase to demonstrate specificity. B) β-lactamase-expressing bacteria were mixed with either non-β-lactamase-expressing bacteria or pure media to determine if non-resistant bacteria would interfere with the reaction. C) To determine if bacteria lysis would result in more sensitive detection, the reaction rate of sonicated bacteria was compared to that of intact bacteria. Error bars denote s.d. ($n = 3$).

of the signal and the reaction time, but only marginally compared to no lysing (Figure 1C). After 10 min of reaction, the color intensity of lysed cells was approximately 5% higher than intact cells. These results indicate that the cells either secrete β-lactamase or that nitrocefin is cell permeable. Several studies support bacteria translocating β-lactamase from the cytoplasm across the bacteria's inner membrane into the periplasm, but not outside the cell entirely, supporting the latter hypothesis.[22]

Because nitrocefin is a colorimetric substrate, it has been speculated that using UV/visible spectrophotometry would result in more sensitive bacterial detection. Serial dilutions of laboratory *E. coli* expressing β-lactamase were reacted in a microtiter plate with nitrocefin and the absorbance was measured using a plate reader. Using a microtiter plate and plate reader compared to a PAD and smartphone did not yield a lower LOD (Figure 2A). This demonstrates that using a PAD and smartphone is a cost-effective way to detect bacteria using nitrocefin, without the need for expensive instrumentation. Because the goal of point-of-need devices is to have a final product that can be taken into the field with minimal supplies for testing, it was also investigated whether nitrocefin could be dried on the paper beforehand. It was determined that the ideal concentration to dry onto paper was 5 μL of 1 mM nitrocefin (more in the Supporting Information). Adding 40 μL of bacteria sample to the PAD test with dried nitrocefin was compared to PAD tests that held 20 μL of 0.5 mM nitrocefin solution and 20 μL of bacteria sample.

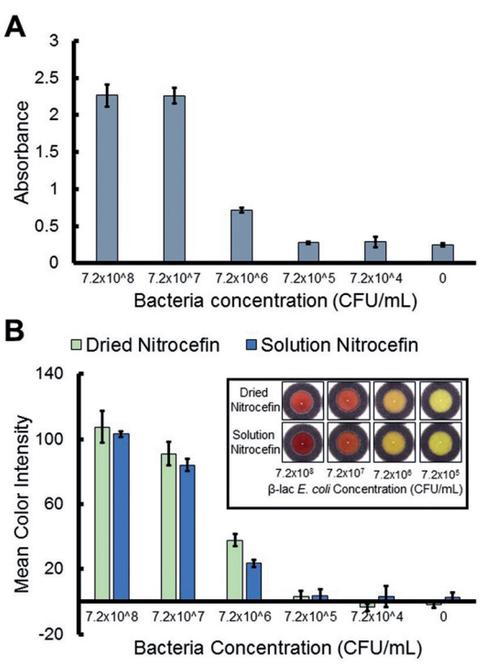


Figure 2. Comparing nitrocefin detection methods. A) Detecting color change using UV/vis spectrophotometry in a plate reader yielded the same limit of detection of 10^6 CFU mL^{-1} as observed on paper. B) Drying nitrocefin on paper before adding sample yielded similar or slightly more sensitive results compared to adding nitrocefin solution to the bacterial sample on paper. Error bars denote s.d. ($n=3$).

Drying nitrocefin onto the paper before adding the sample showed slightly more sensitive results compared to nitrocefin solution (Figure 2B). This is likely because nitrocefin did not need to be added to the total solution volume, therefore more sample could be added. Nitrocefin's long-term stability on paper is unknown but will be the subject of future studies.

To confirm the new method would work with real-world samples, influent and effluent water was obtained from the Drake Water Reclamation Facility located in Fort Collins, Colorado, United States. In the influent, β-lactamase was detected after only 2 h of sample incubation in media. The signal continually increased until reaching a maximum at circa 10 h of incubation (Figure 3). Similar results could be

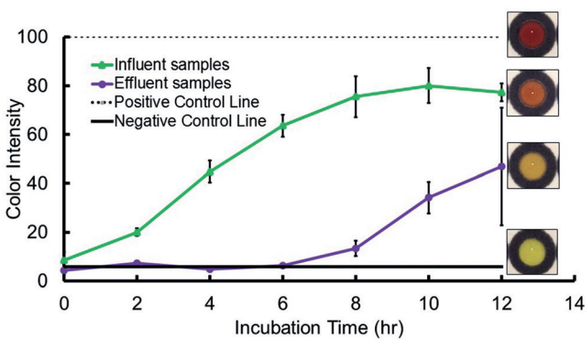


Figure 3. Detecting β-lactam resistance in urban sewage water. Samples of influent and effluent water were obtained and incubated in medium for 12 h. Samples were obtained every 2 h for testing and both the influent and effluent tested positive for β-lactam resistance, which was confirmed by traditional culture methods. Error bars denote s.d. ($n=3$).

obtained with a microtiter plate but at much higher costs. The effluent, which should contain less bacteria, did not show a signal until 8 h of incubation. These results were confirmed using dilution and plating methods, which gave a concentration of 4.50×10^6 CFU mL^{-1} of total bacteria in the influent, and 5.08×10^3 CFU mL^{-1} of total bacteria in the effluent. AMR bacteria were confirmed using commercially available extended-spectrum-β-lactamase (ESBL) plates from CHROMagar. On these plates, concentrations of 4.96×10^4 CFU mL^{-1} of total ESBL-containing bacteria in the influent and 1.30×10^1 CFU mL^{-1} in the effluent were determined. This correlates to 1.1% and 0.257% ESBL bacteria in the influent and effluent, respectively. There could be several reasons for such a high signal in the influent sample, considering the 1:99 ratio of β-lactam-resistant bacteria to non-resistant bacteria. Bacteria resistant to β-lactam antibiotics could be growing at a faster rate than the non-resistant bacteria, therefore occupying more of the sample once it was concentrated enough to detect resistance. This variance in growth rate was also observed in the effluent between samples as demonstrated by the large error bars at 12 h. The sewage sample bacteria also had to react for over an hour with nitrocefin to obtain a detectable signal, compared to 2–5 min of reaction for samples that were composed entirely of resistant bacteria. However, this slower reaction rate could

also be due to chemicals in the sewage water interfering with the enzymatic reaction.

To determine how many different bacterial species were detected in the sewage samples, several bacterial species were isolated and cultured from the original sewage and other environmental samples. The bacterial cultures were given to the tester blind to ensure no biases when using the paper-based tests. Of 10 different bacterial isolates tested from a variety of species and environmental sources, there were no false positives and one false negative (Figure 4). Bacterial

Nitrocefin Test	Bacteria Species	ESBL Status	Isolation Source	Nitrocefin Test	Bacteria Species	ESBL Status	Isolation Source
	**Positive Control** *Escherichia coli*	(+)	New England Biolabs (DH5α cells)		**Negative Control** *Escherichia coli*	(-)	New England Biolabs (DH5α cells)
	*Enterobacter (cloacae/ absurdium)*	(+)	Effluent		*Citrobacter werkmanii*	(+)	City Sewage
	*Chryseobacterium gleum*	(+)	Effluent		*Serratia liquefaciens*	(+) (weak)	Effluent
	*Pseudomonas alcaligenes*	(+) (weak)	Effluent		*Pseudomonas putida*	(+)	City Sewage
	*Escherichia coli*	(+)	Influent		*Escherichia coli*	(+) (weak)	River Water
	*Chromobacterium violaceum*	(+)	Influent		*Serratia fonticola*	(-)	River Water

Figure 4. Detecting β-lactam resistance in bacterial isolates. Different bacterial species were isolated from environmental samples and tested for individual resistance using the paper-based test. There have been no false positives and one false negative (*Chromobacterium violaceum* isolated from the influent of urban sewage water).

solutions were kept intact and not lysed for consistency. When using the paper-based test on intact bacteria, results indicate that the assay could also quantify resistivity for different bacteria species. The "slightly positive" paper tests corresponded to "weak positives" that were confirmed by using CHROMagar ESBL plates. "Slightly positive" was defined as having a color intensity change of 20%–80% compared the positive control laboratory *E. coli*, and "weak positive" was defined as reduced bacteria growth on ESBL plates compared to a non-antibiotic plate.

One bacterial isolate, *Chromobacterium violaceum,* tested negative using the paper-based test but tested positive using a CHROMagar ESBL plate. This same species did not grow on an ampicillin-containing agar plate, indicating that it is likely susceptible to penicillins. To confirm which test was correct, the minimum inhibitory concentration (MIC) of different β-lactam antibiotics was tested. The isolate was resistant to cephalosporins like cefazolin and cephalothin but was susceptible to penicillins, such as amoxicillin and ticarcillin. The bacteria were also susceptible to imipenem, a carbapenem β-lactam antibiotic that is used as a last resort in clinical cases. Overall, this resistance profile is inconclusive but similar to a previously published profile on resistance to cephalosporins combined with susceptibility to penicillins.[23] The reason that nitrocefin would not react with *C. violaceum's* β-lactamase is unknown. Nitrocefin is defined as a chromogenic cephalosporin, so in theory should be reactive with a β-lactamase that protects the cell against cephalosporin antibiotics.

While ESBL-selecting plates are a common method to determine β-lactamase expression, it is more common in medicine to subject bacteria to antibiotic susceptibility testing. To compare the PAD to this method, 32 different environmental *E. coli* isolates were subjected to antibiotic susceptibility testing of different β-lactam antibiotics as well as plating the isolates on ESBL-selecting plates. The PAD test was compared to these methods for accuracy, and no false negatives were observed (Figure S3). When comparing the PAD test to ESBL-selecting plates, two false positives occurred (isolate #7 and #20). However, when comparing to antibiotic susceptibility testing, these isolates were resistant to at least two penicillin antibiotics. When comparing the PAD test to antibiotic susceptibility testing, the tests were negative when the bacteria were susceptible to all of the tested antibiotics and were positive when resistant to at least one of the tested antibiotics. As further confirmation, the *E. coli* isolates were also tested for the presence of ESBL genes blaTEM and blaCTX-M by using polymerase chain reaction (PCR). Isolates #7 and #20 had the blaTEM gene present in their genome, also corresponding to the PAD results (Figure S3). With 42 tested isolates and one true false negative, this test has so far shown 97.6% accuracy.

A straightforward and accurate paper-based colorimetric assay to detect bacteria resistant to β-lactam antibiotics has been developed that costs circa USD 0.20 per test but gives similar sensitivity to more expensive microtiter plate methods. We have also optimized the enzymatic reaction between nitrocefin and β-lactamase on paper and demonstrated that non-AMR bacteria do not interfere with the assay performance and cell lysis is not required. Detection of β-lactamase-expressing bacteria in community sewage water and identification of resistance in various species of bacterial isolates have demonstrated the practicality of this method. All tests were confirmed and compared to traditional culturing methods, antibiotic susceptibility testing, and PCR gene analysis. Although a laboratory was necessary to concentrate the sewage samples, this method still reduced the laboratory-process time by 14–20 h. It is also possible to ultimately integrate this test into a field-ready module by creating a more sensitive test or concentrating samples in the field. Bacterial samples were shown to react with nitrocefin whether in solution or dried onto the paper, also demonstrating its potential for a field-ready module. It was confirmed that using a paper-based test and a camera phone for quantification yielded the same LOD as using an expensive and non-portable plate reader and microtiter plate. While traditional methods can also quantitatively determine resistance, our paper-based method would be a rapid, cost-effective surveillance tool with a yes/no informed decision outcome prior to establishing a need for additional testing.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: antimicrobial resistance · nitrocefin · paper-based analytical devices · β-lactam antibiotics · β-lactamase

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