Low-Cost Point of Care Assay for Detecting ESBL-Expressing Bacteria Infections

Executive Summary

Antimicrobial resistance (AMR), the ability for a pathogen to resist the action of an antimicrobial drug, has been on the rise due to the widespread use of antimicrobial agents. To currently diagnose AMR in bacterial infections, a central diagnostic laboratory using slow, expensive, and labor intensive tests is required. We aim to develop a low-cost, fast, specific, user-friendly point-of-care assay that can identify a bacterial infection and determine their susceptibility to β-lactam antibiotics. Our vision is to create an integrated, easy to use two-stage assay that first identifies whether bacteria are causing the infection, and then determines the bacteria's susceptibility to penicillins and cephalosporins. In unpublished work, we have demonstrated the essential elements for our proposed assay. A low-cost microfluidic paper-based analytical device (μPAD) that can test for the presence of β-lactamase-mediated resistance without external instrumentation has been developed. The μPAD demonstrated rapid and selective visual detection of β-lactam-resistant bacteria in environmental water, serum, and urine samples, and has also identified resistance of clinically significant bacterial species, such as *E. coli* and *Klebsiella*. μPAD detection validation using standard culture methods showed good statistical agreement and accuracy when detecting bacterial resistance. In related work, we demonstrated immunomagnetic separation (IMS) and colorimetric detection of *Salmonella typhimurium* without culturing and a detection limit of 100 colony forming units (CFU)/mL. Our proposed approach will combine inexpensive μPADs using naked eye colorimetric detection with fast, selective IMS to provide sensitive, timely detection of AMR. In this pilot project, we will expand upon our β-lactamase-detection motif by incorporating IMS to increase assay sensitivity and specificity. By increasing sensitivity and specificity, we will eliminate the need for culturing steps, cutting hours off sample-to-result time. *Our goals for this pilot stage are; 1) Expand the sample matrices and bacterial isolates tested with our assay to determine the extent of its accuracy when combined with our IMS method for enhanced sensitivity and assay timeliness, and 2) Incorporate the entire assay into an easy-to-use, automated paper-based device that does not require additional instrumentation.* By incorporating antibodies specific to target pathogens during the IMS step, we can identify AMR active bacterial species. This assay addresses two serious-level pathogens as identified by the Center for Disease Control and Prevention, including *Enterobacteraceae* that express extended-spectrum-β-lactamases (ESBL) and methicillin-resistant *Staphylococcus aureus*. The final assay will be user-friendly and require little to no instrumentation, making it ideal for outpatient services.

Outcomes. The team will develop a low cost and sensitive paper-based device for outpatient services capable of rapid detection of β-lactamase-mediated resistance in both human urine and blood. The presence of which is considered a serious threat and can exist across a spectrum of bacterial species of concern.

Description of proposed in-vitro diagnostic (Confidential)

The proposed diagnostic device will use a paper-based format due to paper's low cost and natural liquid wicking properties, which make it a good platform for instrument-free diagnostics that do not require electricity-powered instrumentation. Paper-based devices are also an ideal platform for AMR detection because paper is easily modifiable, inexpensive, and simple to manufacture in large quantities.[1] Our paper-based device will first confirm the capture of specific bacteria within a sample, and then identify whether these bacteria express β-lactamase, which mediates antibiotic resistance to penicillin and cephalosporin antibiotics. The first part of this assay will be achieved using immunomagnetic separation (IMS) to identify the bacterial infection with antibodies specific to target pathogens, including (but not limited to) *Enterococci coli* (from

the family *Enterobacteriaceae*) and *Staphylococcus aureus*. These bacterial species were selected because of their role in clinical bacterial infections and their CDC classification as a serious level threat.[2] Because IMS simply requires different antibodies for different species, possibilities for different pathogenic bacteria tests are extensive and can be expanded beyond the initial target species. After target pathogens have been separated from the sample matrix and identified, the bacteria will then undergo an assay to detect the presence of β-lactamase as an indicator of ESBL-mediated resistance, which has also been classified as a serious level threat by the CDC.[2]

IMS consists of multiple steps starting with specific to the target pathogen, followed by separation of the beads from the sample matrix using a magnet (Figure 1A).[3] Next, the user adds another biotion-conjugated antibody also specific to the target pathogen, followed by the addition of an enzyme conjugated to streptavidin, which will react with biotin to form a stable conjugate. There are washing steps between each step to ensure no enzyme is present if the targeted analyte is not present in the sample. The pathogens present in the patient sample are identified by reacting a specific substrate with the enzyme conjugated-antibodies bound to the pathogen through IMS, similar to a traditional enzyme-linked immunosorbent assay (ELISA). The IMS procedure has already been optimized by our lab in unpublished work using a microcentrifuge tube for the detection of *Salmonella typhimurium* using β-galactosidase (β-gal) and chlorophenyl-red-galactopyranoside (CPRG) as the enzyme-substrate pair. The reaction results in a detectable color change from yellow to red (Figure 1B and 1C) to indicate *Salmonella* is present. This assay has been demonstrated for the specific detection of *Salmonella* with a LOD of 100 CFU/mL in bird feces (See Appendix sections 1 and 2). Because β-gal and CPRG assays are a user-friendly and sensitive system, this enzyme-substrate pair will be used throughout this project.

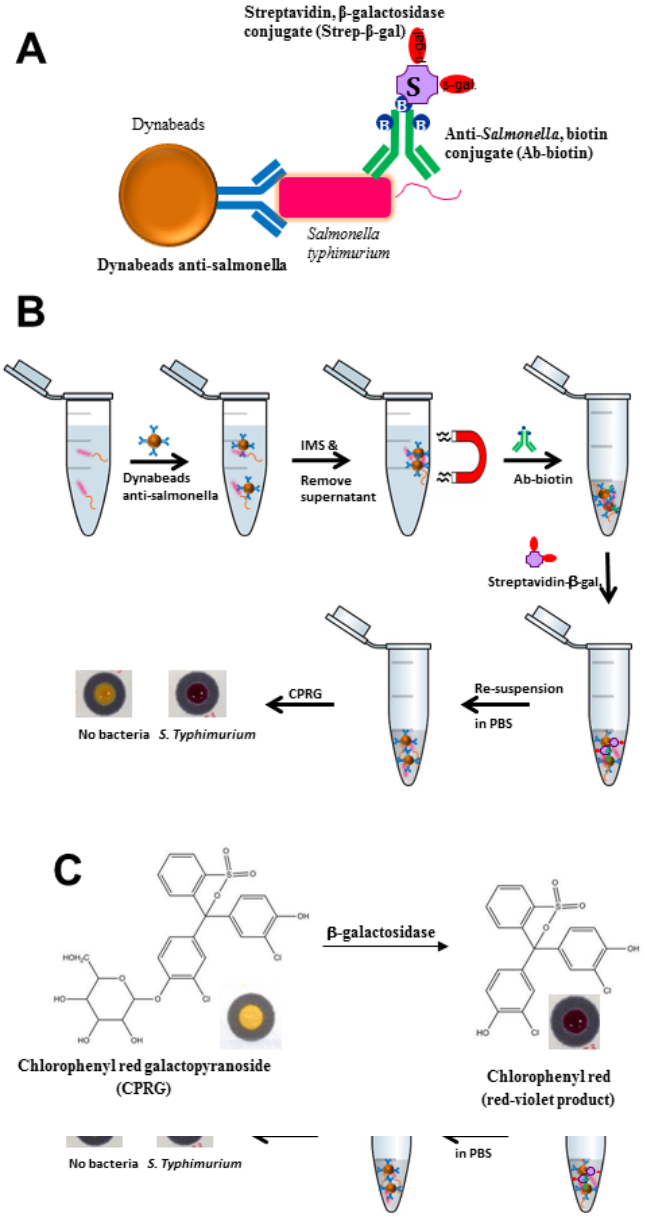
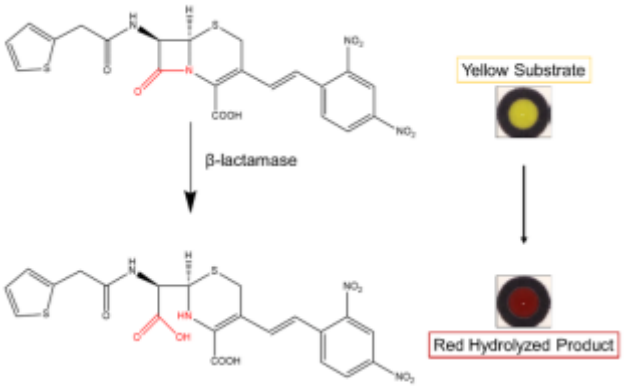


Figure 1 | Immunomagnetic separation (IMS) for *Salmonella* detection. (A) The detection motif for *Salmonella*. **(B)** The procedure for IMS. **(C)** The reaction scheme for β-galactosidase and chlorophenyl-red-galatopyranoside (CPRG), which was used to detect the presence of *Salmonella*.



Scheme 1 | Reaction scheme for detection of β-lactamase. When β-lactamase hydrolyzes the β-lactam ring in nitrocefin, there is a distinguishable color change from yellow to red.

After the first step of IMS (Figure 1A), where bacteria have been separated and concentrated from the sample, we can perform two different tests. The first is to identify that bacteria are present based on the antibody test as previously described, and the second is to identify the bacteria's resistance to penicillin and cephalosporin antibiotics based on their expression of β-lactamase. β-Lactamase expression is one of the most common (but certainly not the only) motifs for antimicrobial resistance in bacteria. Detection of β-lactamase activity has been optimized using nitrocefin, a chromogenic cephalosporin that undergoes a distinguishable color change from yellow to red when the enzyme hydrolyzes the β-lactam ring (Scheme 1).[4] This method has been optimized (Appendix 3) and demonstrated for the detection of β-lactam resistance in sewage samples (see Appendix 4), clinically significant bacterial isolates (Appendix 5), and inoculated urine and plasma samples (Appendix 6). During optimization, it was determined that cell lysis was not necessary (Appendix 3). Studies show that β-lactamase is translocated into the periplasm, but not outside the cell entirely.[5,6] This translocation indicates that nitrocefin is likely cell permeable, as opposed to β-lactamase secretion from the cell. This will be ideal for detection of β-lactamase activity in samples where bacteria will be separated from the supernatant in a sample, because minimal detectable enzyme should be lost. Although cell lysis is not necessary for detection, we will investigate whether a permeabilization step will improve assay LOD. The biggest challenge for this assay is the bacteria LOD of 10^6-10^7 CFU/mL, a fairly high concentration that would not be present in patient samples. By implementing IMS as the first step of the assay, this should lower our LOD as seen with *Salmonella* detection. Using IMS as a pre-concentration step should also eliminate the need for sample culturing, saving hours from sample-to-result.

The first phase of this project is to optimize the IMS assay for *E. coli* and *S. aureus* detection in plasma and urine samples and to identify whether the captured bacteria express β-lactamase. The second part of this project is to integrate this system into a semi-automated paper-based device that requires minimal instrumentation and training for the device user. This will be done using an inexpensive, reusable 3D-printed manifold that contains interchangeable and disposable paper-based tests. The 3D-printed manifold will consist of three separate pieces. The bottom piece houses the sample and waste layer of the device, which is secured directly into the manifold (Figure 2-3, 2-7, and 2-19, respectively). The center piece allows insertion of the reagent layer of the device, which contains eight unique paper channels that are modified with reagents for each step of the assay (Figure 2-2, 2-6, and 2-10, respectively). The top piece secures a magnet at the sample zone of the device for the IMS, and contains a buffer reservoir for fluid delivery to the reagent layer of the device (Figure 2-1, 2-5, and 2-4, respectively). To assemble the manifold in preparation for the assay, the sample layer, preloaded with the patient's sample, is placed in the bottom piece of the manifold. The center and upper pieces of the manifold are then assembled, and the entire manifold is secured with screws, springs, and fasteners (Figure 2-8). The springs maintain constant pressure between the layers of the device during the assay. Buffer is loaded into the reservoir on the manifold, providing reagent transport down the first channel of the device to the sample (Figure 2-4). For sequential reagent delivery, the center layer of the manifold (2-2), along with the reagent layer of the device, is rotated by

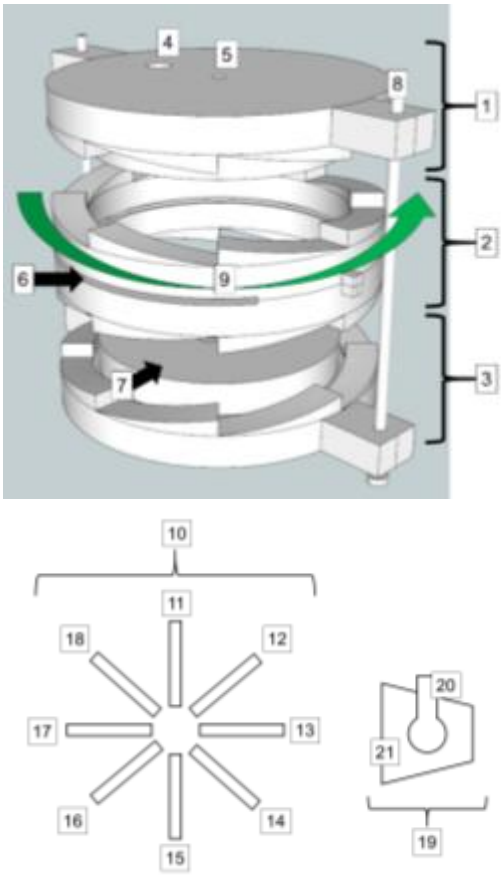


Figure 2 | CAD representative of the 3D printed manifold and paper-based devices design. (1) Top piece. (2) Center piece. (3) Bottom piece. (4) Buffer deposit. (5) Magnet placement. (6) Insert disposable and interchangeable reagent layer of the device. (7) Place sample and waste layer of the device. (8) Screws and wingnuts to hold entire manifold together. (9) Rotate center piece counter-clockwise between each step of assay. (10) Reagent layer of the device where each section is modified with a different reagent necessary for the assay. (11) Immobilized biotinylated antibody. (12) Immobilized Tween-20 for washing. (13) Immobilized streptavidin-beta-gal. (14) Immobilized Tween-20. (15) CPRG. (16) Nitrocefin. (17 and 18) Extra channels. (19) Sample and waste layer of the device. (20) Sample deposit with Dynabeads®. (21) Waste layer and reservoir.

45º to connect the buffer reservoir to the next channel of the device (Figure 2-9). Interlocking wedges on the edges of each piece of the manifold snap the device into place using a mechanism similar to that used in a retractable pen. The wedges also cause the upper and lower layers of the manifold to be lifted away from the center, preventing leakage between paper channels during the different assay steps upon device rotation. Our current design for the interchangeable and disposable paper-based devices uses Whatman #4 filter paper and lamination to direct sample flow and to help preserve reagents in the reagent layer of the device (Figure 2-10). Images of this 3D-printed manifold are displayed in its assembled form (Figure 3A) and separated into its component pieces (Figure 3B-D).

To perform the assay, the reagent layer of the device is inserted into a slot located in the center piece of the manifold (2-6). The reagent layer contains individual reagent steps of the assay immobilized on paper that will be rehydrated with buffer by the user and flowed through the paper to the sample layer (Figure 2-20) and waste reservoir (Figure 2-21) located below the reagent layer. Each step of the assay has the appropriate reagent immobilized in its own channel as displayed in Figure 2-11 through 2-18. Two of the channels currently do not contain reagents in case more steps are required for the final assay. Before the assay is started, the sample will undergo one of two processes, which will be determined through optimization. The first option will be to premix the patient sample with Dynabeads® before adding the mixture to the sample layer of the device. The second approach is to immobilize Dynabeads® to the paper in the sample layer for rehydration similar to the rest of the reagents. With the latter approach, the technician running the test will simply need to add the patient sample to the sample layer of the device before proceeding with the rest of the assay. Once this initial step is completed, the magnet in the device will ensure that the Dynabeads® with bacteria remain in the sample layer instead of being washed into the waste reservoir during washing steps. The device user will then add buffer through the buffer deposit of the 3D manifold (Figure 2-4) to undergo the next step of the assay, transporting the biotinylated antibody to the sample to adhere to the bacteria (or wash through the sample layer to the waste reservoir if no bacteria is present in the sample). After this step is completed, the user will rotate the device manifold 45° to the next channel in the reagent layer, and add more buffer to wash the sample with Tween 20, a surfactant often

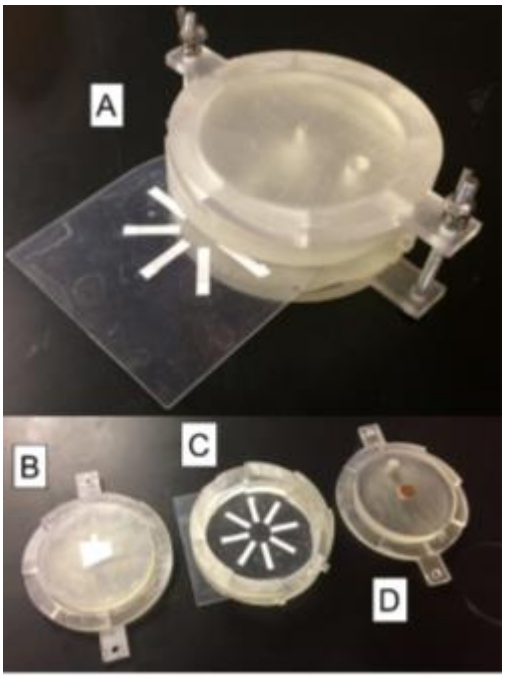


Figure 3 | 3D printed manifold. (A) Entire manifold screwed together with reagent layer insert. (B) Bottom piece of manifold with sample and waste device layer. (C) Center piece with reagent device layer. (D) Top piece with magnet.

used for washing steps in immunoassays. These steps of device rotation and buffer deposition will be executed until the assay is complete and CPRG is deposited into the sample region. The final goal will be to generate a system where the device user does not need to continue depositing buffer with each step, but where all of the buffer will be added to the manifold at the beginning of the assay and buffer flow will be controlled throughout the process. At the end of the assay, if the sample spot turns red, then the targeted pathogens are present within the patient sample and are likely the cause of infection. If the sample stays yellow, then target pathogens are not present in the patient sample. Although not included in this preliminary design, determination of β-lactam resistance will be completed by having a second sample spot in the sample layer of the device that will be connected to the channel containing nitrocefin (Figure 2-16). Therefore, the device user will simply have to move the device another 45°, deposit more buffer, and observe whether the second sample spot turns red to determine whether the bacteria express ESBL.

Development Approach

The first step in the development of this device will be to optimize the detection of *E. coli* and *S. aureus* using IMS in a microcentrifuge tube format as was completed for optimization of *Salmonella* detection. β-lactamase detection will also be optimized following the initial IMS step to confirm that the enzyme can be detected when bacteria are purified and concentrated from a sample. Detecting bacteria and β-lactamase activity will be demonstrated in plasma and urine within microcentrifuge tubes before moving to the paper-based devices and the 3D-printed manifold (Figure 3). Although IMS has been demonstrated in the successful detection of *Salmonella* in bird feces, almost the entire process was completed in a microfuge tube, with only the final reaction between CPRG and β-gal from the bacteria-Dynabead® complex taking place on a paper device. Because the IMS procedure is extensive and requires training, we are developing a method that integrates all steps into a single device with minimal user operation. With the completed *in vitro* diagnostic, there will be minimal user-training required to operate the device and execute diagnostic tests. We have designed and printed an initial design for the 3D-printed manifold, and IMS will soon be optimized for use within the manifold so that modifications can be made. Once the paper-based device and manifold have been optimized for one IMS test, we will work to incorporate three tests into one device. The final diagnostic platform will perform at least three tests at once, to detect the presence of *E. coli* and *S. aureus*, and to test for the bacteria's expression of β-lactamase. We will also demonstrate the in-field feasibility of this device by determining how long reagents are stable in paper. The device will focus on detecting *E. coli* and *S. aureus*, while contining to use the β-lactamase detection motif to obtain a profile of ESBL-expressing bacterial isolates that are detected.

Challenges and Risks

The primary challenge of this method will be the implementation of IMS into a paper-based device for a final detection motif that has minimal hands-on time by the user. For example, antibodies and Dynabeads® may be difficult to flow through paper due to their large size. Most immunoassays hold antibodies in one location for detection, but there has been published research on selecting the correct paper substrate for optimal antibody release and flow.[7] Antibodies are typically around 10 nm, vs. Dynabeads® which range from 1-5 µm, approximately 100-500x larger in diameter. Per Abe et al., ADVANTEC filter paper no. 5C with 1 µm pores is ideal for antibody release and flow. Although this substrate was selected as the ideal paper for antibody flow, this might not necessarily be the case for Dynabeads®. Initial studies have shown flow of Dynabeads occurs in Whatman 4 chromatography paper with good efficiency. Consequently, selecting the appropriate paper for the device will need optimization with possible paper modification to enable completion of the assay steps. There is also the possibility that there will be background due to nonspecific adsorption to the paper substrate that will be difficult to remove during the washing steps. Not only will the paper substrate have to be optimized, but washing and addition steps will be modified to best suit the paper-based devices compared to microcentrifuge tubes.

There is also the possibility that the reagents associated with IMS will not be stable in urine and plasma solutions, resulting in decreased sensitivity and/or selectivity. While our β-lactamase assay has been tested in plasma and urine samples, IMS has not (it has been tested with feces samples). There are components of the IMS assay that might not be stable at the variable pHs that have been measured in urine (pH 4.5-8).[8] Previous studies of β-gal demonstrate the enzyme works optimally around pH 7 and loses activity around pH 5.5.[9] Because β-gal will not be implemented into the assay until after the bacteria have been separated from the original sample matrix, this should not be an issue with proper washing. If antibodies are affected by the sample matrix and conditions, this could pose problems and will have to be addressed in the procedure with a possible pH neutralization step. Another issue that could be problematic includes nitrocefin's instability in plasma samples and in acidic and basic pH solutions. We have demonstrated that it is possible to detect resistance in inoculated urine and plasma samples when cultured in media. However, reacting nitrocefin directly with urine and plasma results in false positives and negatives, respectively. It has been previously published, and our studies confirm, that nitrocefin is inherently unstable in plasma due to serum proteins.[10] Our results also indicated that nitrocefin did not react efficiently in urine samples, which was likely due to solution pH. Similar to the risk of β-gal not reacting in acidic solutions, both of these problems should not be an issue when bacteria are separated from the original sample matrix. In the case that IMS does not fully purify the bacteria from the sample matrices, ultrafiltration will also be considered.

State of the art statement

The World Health Organization (WHO) notes that AMR bacteria is an emerging water, sanitation and hygiene issue worsened by a lack of reliable, well documented and validated human health risk assessments.[11] Surveillance for AMR bacteria and resistance genes from environmental exposures merits interdisciplinary attention. Improved surveillance and monitoring of AMR in a cost effective and efficient manner across environments, animals, and people is critical for increasing infectious disease control and prevention globally.

There are diverse screening approaches for phenotypic and genotypic characterization of bacterial isolates with AMR for both environmental and clinical applications. The conventional approaches in use by research and diagnostic laboratories for screening of AMR bacteria

involves plating samples on non-selective or antibiotic-selective agar plates, purifying bacterial colonies, and using disc diffusion, broth dilution and other similar methods to determine the minimum inhibitory concentration (MIC) for resistance to antibiotics. These methods are accurate and informative, but are also slow, often taking several days to perform the full panel of MIC on purified isolates. Automated bacterial characterization systems that are relatively rapid still take approximately 12 hours following the initial isolation of bacteria.

Our assay will be much faster, taking <60 minutes from sample to result and will be sensitive enough to eliminate the need for initial culturing. Traditional laboratory methods also require laboratory personnel who have been trained to execute these tests. Our test will require minimal hands on time beyond loading the sample and clicking the system through a few steps, and will have results that are easy to interpret with colorimetric readout. The test will also be much cheaper costing on the order of $1-2USD per test as opposed to ~$20 per test for antibiotic susceptibility testing. It will similarly not require the need for expensive equipment as the tests utilize paper and an inexpensive and reusable 3D-printed manifold. To decrease the spread of AMR bacteria, not only is it of critical importance to diagnose AMR bacterial infections, but it is also important to monitor AMR at other sources of infection such as contaminated water. Because of the device's ease of use and cost, not only could this device be used in outpatient services and in bedside diagnostics, but also in the field to monitor AMR bacteria from environmental sources. This is a device that is lacking in the field of AMR bacteria, and could be of significant impact. Preliminary data using the paper-based devices on bacterial isolates also indicates that these devices could have quantitative properties. With some bacterial isolates, the test would turn a weaker color of red compared to other isolates. These weakly positive tests corresponded to weakly resistant species as confirmed by traditional culture and plating methods, but presented the results in a user-friendly format.

How Solvers Will Complete Step 2

One of the many advantages of using a paper-based device is the simplicity of its fabrication. With the collaboration of our three labs, we will have all the technologies and resources needed to create and optimize this detection method. The Henry laboratory has been involved with paper-based diagnostic technologies since close to their introduction, as they were the first lab to integrate electrochemical detection into paper-based devices.[12] They have since published over 30 journal articles in the field since 2009 that include innovative detection motifs and review articles, many of which are in high impact journals. Because of their experience with paper-based methods, the Henry lab is equipped with all the materials necessary to develop this device including a 3D printer and software necessary for designing and making the 3D manifold. The Geiss laboratory has collaborated with the Henry laboratory for the soon-to-be published article using IMS and paper-based devices to detect *Salmonella* in bird feces samples. Therefore, the Geiss lab is already equipped with all the materials necessary for IMS and the biosafety cabinets required for sterile handling of biological samples and pathogenic bacteria. The Ryan laboratory has extensive experience in the field of environmental monitoring of AMR bacteria as well as animal and human samples. Therefore, the Ryan laboratory has the resources necessary for obtaining environmental samples along with urine and plasma samples for this project. The Ryan laboratory also has extensive experience addressing the NIH Human Subjects Protections and Inclusion of Women, Children, and Minorities, for their previous projects, and will not be an issue for this development. All three of our labs have been collaborating for several months to write and submit a manuscript to *Angwendte Chemie* on paper-based devices for monitoring AMR bacteria in environmental samples. The combined research experience, knowledge, and resources from all three laboratories spanning analytical chemistry, engineering, microbiology, and environmental science will make developing the

proposed device achievable. Because IMS and nitrocefin have already been optimized for other projects, along with the 3D manifold having been designed and printed, this project should take an estimated 12-16 months to complete.

Appendices

1. Immunomagnetic Separation (IMS) Assay

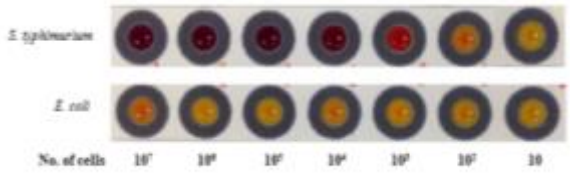


Figure 4 | The *Salmonella* limit-of-detection utilizing IMS was around 100 cells.

Each step of the IMS procedure was optimized for a short, but accurate test at 45 min from start to finish. Using the optimized incubation times, the limit of detection for this approach was determined and *Salmonella* was detected as low as 100 CFU/mL. In addition, *E*. *coli* was also applied to investigate the selectivity of the proposed detection motif. As seen in Figure 4, *E*. *coli* only showed a positive result with a concentration of 10^7 CFU/mL with a color intensity equal to the intensity of 100 CFU/mL of *Salmonella*. Normally, *E*. *coli* can produce β-gal within the cell, but the enzyme is not often secreted and is not detectable with CPRG in intact organisms. Accordingly, the slightly positive result from 10^7 CFU/mL of *E*. *coli* could be due to non-specific binding of the anti-*Salmonella* antibody. However, non-specific signals only occurred at high concentrations of *E*. *coli*. Therefore, this is not a major concern because it is not expected to observe the presence of bacteria at high concentrations in real samples. Detection of *Salmonella* using the proposed system was achieved at as low as 100 CFU/mL, with the analysis time of 75 min (45 min for the IMS assay and 30 min for reaction with CPRG) and an additional time of 10-15 min for washing steps. Accordingly, the total analysis time should be within 90 min without any requirement for complex instruments and enrichment steps for bacteria incubation. Therefore, the proposed approach has shown its ability to be a sensitive, easy, rapid, instrument-free, reliable, and portable method for detection of *Salmonella* and could be an ideal platform for on-site analysis.

2. Specificity of IMS

In real-world samples, *Salmonella* is found mixed with many different species of bacteria, so a key question is if the assay is able to specifically detect *Salmonella* in the presence of other bacteria. Therefore, the specificity of the proposed system was evaluated by mixing *Salmonella* and *E. coli* at different concentration ratios. Three different numbers of *Salmonella* (10^2, 10^3 and 10^4 CFU/mL) were used as representatives of low, medium and high levels, respectively. Each *Salmonella* suspension was inoculated with different amounts of *E. coli* to observe the effect of *E. coli* on the signal intensity from the assay. Results in Figure 5 show that no difference was observed from the color developed from the assays obtained from the mixtures and control, even though the number of *E*. *coli* was 1,000 times higher than that of *Salmonella*. These results show high specificity of the proposed approach for detection of *Salmonella* without an interfering effect from *E. coli.*

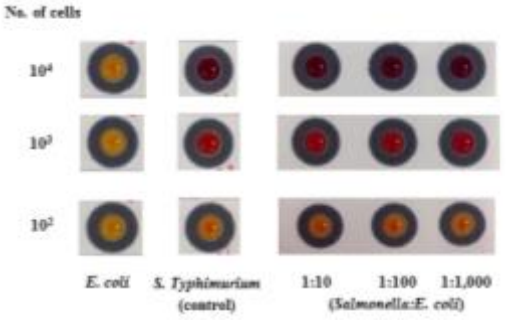


Figure 5 | The specificity of the IMS assay was demonstrated by mixing *Salmonella* with *E. coli* at different ratios.

3. Laboratory-Based AMR Bacteria Detection

To detect β-lactamase in live bacteria, we used optimized reaction conditions (between nitrocefin and β-lactamase) for *E. coli* as a model species. Serial dilutions of β-lactamase-expressing *E. coli* and control *E. coli* were incubated with 0.5 mM nitrocefin at room temperature. We observed no color change unless the bacteria expressed β-lactamase (Figure 1A). We observed color change in the assay with more than $3.8x10^6$ CFU/mL bacteria, but were not able to detect β-lactamase from lower concentrations of bacteria. To determine whether non-β-lactamase producing bacteria in co-culture 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 compared using the same mixing ratios for β-lactamase-expressing bacteria to media without any bacteria present (Figure 1B). *E. coli* not expressing β-lactamase do not appear to interfere with the reaction as similar color intensities were observed in pure or mixed cultures. β-lactamase is produced within bacteria, so to attempt increasing sensitivity, we tested whether lysing the cells would decrease the limit-of-detection. For DH5α *E. coli* cells expressing β-lactamase, lysing the cells using probe sonication helped obtain a faster and more intense signal, but only marginally compared to no lysing (Figure 1C). These results indicate that these cells either secrete β-lactamase or nitrocefin is cell permeable.

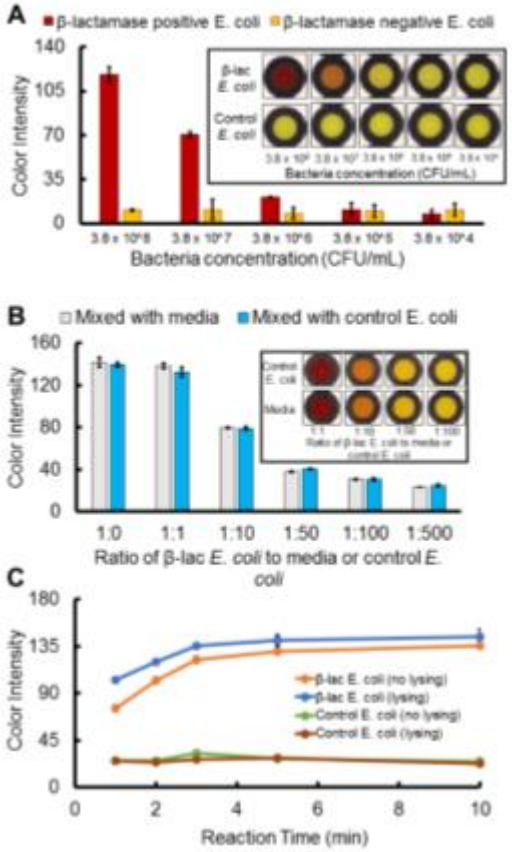


Fig. 1| **Optimizing the bacterial reaction**. **(A)** Specificity was demonstrated by using the test on E. coli that do and do not express β-lactamase. **(B)** β-lactamase expressing bacteria was mixed with either non-β-lactamase expressing bacteria or pure media demonstrate that non-ESBL bacteria do not interfere with the assay. **(C)** The reaction rates of cells that were lysed vs. intact cells were compared.

4. AMR Detection in Urban Sewage Water

In order to confirm that this method would work with real-world samples, influent and effluent water was obtained from the Drake Water Reclamation Facility located in Fort Collins, CO. 1 mL of influent or effluent was incubated with 3 mL of media. Samples of the enrichment process were taken every 2 hrs for testing up to 12 hr. 20 µL of sample was reacted with 20 µL of 0.5 mM nitrocefin for 2 hr in order to obtain a confident and detectable signal. In the influent, β-lactamase was detected after only 2 hr of incubation and the signal continued increasing until it reached its maximum signal at ~10 hr of incubation. The effluent, which should contain less bacteria, did not show a signal until 8 hr of incubation (Figure 2). Results were confirmed by a separate party using dilution and plating methods, which showed a concentration of $4.50x10^6$ CFU/mL of total bacteria in the influent, and $5.08x10^3$ CFU/mL of total bacteria in the effluent. AMR bacteria were confirmed using commercially available extended-spectrum-β-lactamase (ESBL) plates [CHROMagar™]. On these plates, there were $4.96x10^4$ CFU/mL of total ESBL-containing bacteria in the influent and $1.30x10^1$ CFU/mL in the effluent. This comes to 1.1% and 0.257% ESBL bacteria in the influent and effluent respectively. Why such a high signal was obtained in the influent sample, considering a 1:99 ratio of β-lactam-resistant bacteria to non-

resistant bacteria, could be due to several factors. First, bacteria resistant to β-lactam antibiotics could be growing at a faster rate compared to non-resistant bacteria, therefore occupying more of the sample once it was concentrated enough to detect resistance. 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 entirely resistant bacteria.

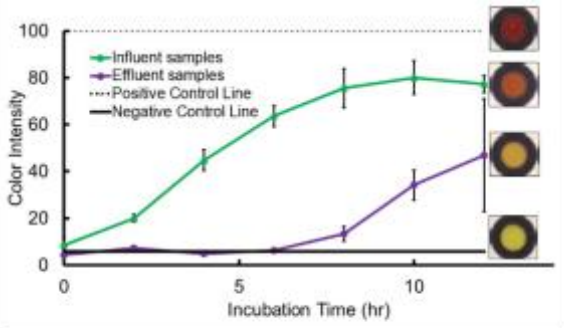


Fig. 2│ Samples of influent and effluent water were tested for ESBL resistance, and both the influent and effluent tested positive after concentration.

5. Detecting AMR in Clinically Significant Bacteria Species

In order to determine how many different bacterial species were detected in the sewage samples, several bacteria species were isolated and cultured from the original sewage and other environmental samples after being plated on agar and ESBL-selecting plates. The bacteria cultures were then given to the tester blind to ensure no biases when using the paper-based tests to confirm β-lactam resistance. Of 12 different bacteria isolates tested from a variety of different species and environmental sources, there were no false positives and one false negative (Table 1). Bacteria solutions were not lysed to have consistent data. Based on the results displayed, when using bacteria that has not been lysed with this device, the assay could also be an indicator of resistivity for different bacteria species. The "slightly positive" paper tests corresponded to "weak positives" that were confirmed via CHROMagar™ ESBL plates by someone other than the tester. "Slightly positive" was defined as having a weaker color change than the positive control *E. coli*, and "weak positive" was defined as less or slower growth on ESBL plates compared to other resistant species. There was one bacteria isolate, *Chromobacterium violaceum,* which tested negative using the paper-based test, but tested positive using a commercial 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 plate was correct, the isolate was sent to a separate diagnostic lab to determine the minimum inhibitory concentration (MIC) to different β-lactam antibiotics. *C. violaceum* was resistant to cephalosporins like cefazolin, cefpodoxime, and cephalothin, but was susceptible to penicillins like amoxicillin and ticarcillin. The bacteria were

Nitrocefin Test	Bacteria Species	ESBL Status	Isolation Source	Nitrocefin Test	Bacteria Species	ESBL Status	Isolation Source
	Positive Control *Escherichia coli*	(+)	Lab		Negative Control *Escherichia coli*	(-)	Lab
	Enterobacter (cloacae/ absurdum)	(+)	Effluent		*Cirtobacter wermanii*	(+)	City Sewage
	Chryseobacterium gleum	(+)	Effluent		*Serratia liquefaciens*	(+) (weak)	Effluent
	Pseudomonas alcaligenes	(+) (weak)	Effluent		*Pseudomonas putida*	(+)	City Sewage
	Enterococcus faecalis	(-)	Colon Tissue from Human		*Escherichia coli*	(+) (weak)	River Water
	Escherichia coli	(+)	Influent		*Serratia fonticola*	(-)	River Water
	Pseudomonas putida	(+)	Influent		*Chromobacterium violaceum*	(+)	Influent

Table 1│ Different bacteria 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).

also susceptible to imipenem, a carbapenem β-lactam antibiotic that is used as a last resort in clinical cases. Interestingly, this isolate was also considered to have "intermediate" resistance against ampicillin with an MIC of 9.5 µg/mL, despite its lack of growth on an agar plate containing ampicillin. This is likely due to the ampicillin plate containing 50 µg/mL of ampicillin, higher than the calculated MIC. Overall, this resistance profile is inconclusive on which test was accurate, but is similar to a previously published profile on resistance to cephalosporins, but sensitive to penicillins.[13]

6. Detecting ESBL in Urine and Plasma Samples

To determine whether this assay would work with clinical samples, several plasma and urine samples were also tested. Each sample was inoculated with ESBL-expressing or non-ESBL-expressing *E. coli* isolated from environmental samples. Three urine samples and five plasma samples were used. They were either not inoculated, inoculated with ESBL-negative bacteria, or inoculated with ESBL-positive bacteria. Samples were incubated overnight to allow bacteria to enumerate to a detectable concentration. To determine whether the actual sample matrix would interfere with the nitrocefin assay, 10 µL of concentrated bacteria was diluted with 90 µL of urine or plasma sample. This was effectively a 1:10 dilution of bacteria, which is a concentration that should still be detectable by nitrocefin. The same samples were inoculated or not inoculated for accurate comparisons between concentrating samples in media vs. detecting bacteria directly in the sample matrix. 20 µL of sample was reacted with 20 µL of nitrocefin. As seen in Table 2, concentrating inoculated urine in media yielded accurate results as confirmed by traditional culturing on ESBL-selecting plates [CHROMagar™]. However, using the assay on cultured plasma results in weakly positive tests that were confirmed negative by culture. When comparing these results to using the paper-based tests on the original sample matrices, plasma resulted in nitrocefin turning dark red regardless of whether the sample contained ESBL-positive bacteria. This is consistent with previous publications reporting nitrocefin's instability in plasma due to serum proteins. Unlike plasma, inoculated urine showed very little color change in the presence of nitrocefin compared to cultured urine samples. This is likely due to pH as urine pH varies drastically between 4.5 and 8, and β-lactamase and nitrocefin react best at pH 7.

Cultured Sample	PAD Test	Confirmation by Culture Methods	Original Sample Matrix	PAD Test
Positive Control		Positive	Positive Control	
Negative Control		Negative	Negative Control	
Original Urine (No Inoculation)		Negative	Original Urine	
Original Plasma (No Inoculation)		Negative	Original Plasma	
Positive Inoculated Urine		Positive	Positive Inoculated Urine	
Negative Inoculated Urine		Negative	Negative Inoculated Urine	
Positive Inoculated Plasma		Positive	Positive Inoculated Plasma	
Negative Inoculated Plasma		Negative	Negative Inoculated Plasma	

Table 2 | Several plasma and urine samples were either inoculated with enough bacteria to immediately detect with nitrocefin, or cultured in media for bacteria concentration. Nitrocefin is unstable directly in plasma, and does not react efficiently in urine (likely due to pH).

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