Appendices

1. Immunomagnetic Separation (IMS) Assay

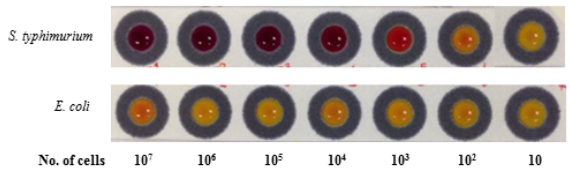


Figure 4 | The *Salmonella* limit-of-detection utilizing IMS was around 100 cells.

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.*

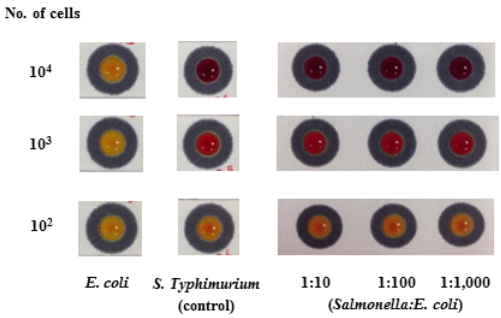


Figure 5 | The specificity of the IMS assay was demonstrated by mixing *Salmonella* with *E. coli* at different ratios.

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.

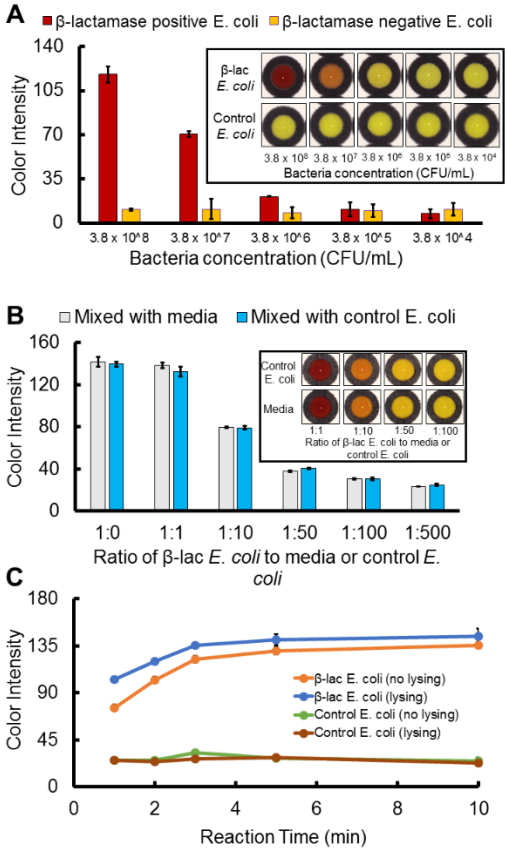


Fig. 1 | Optimizing the bacterial reaction. (A) Specificity was demonstrated by using the test on E. coli that do and do not express β-lactamase. **(B)** β-lactamase expressing bacteria was mixed with either non-β-lactamase expressing bacteria or pure media demonstrate that non-ESBL bacteria do not interfere 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.

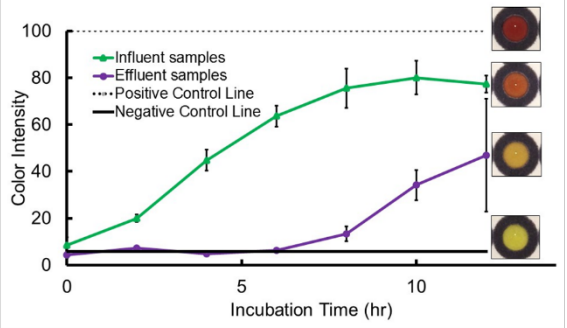


Fig. 2| Samples of influent and effluent water were tested for ESBL resistance, and both the influent and effluent tested positive after concentration.

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/ absurdium)	(+)	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.

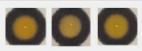
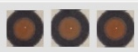
Cultured Sample	PAD Test	Confirmation by Culture Methods	Original Sample Matrix	PAD Test
Positive Control		Positive	Positive Control	
Negative Control		Negative	Negative Control	
Original Urine (No Inoculation)		Negative	Original Urine	
Original Plasma (No Inoculation)		Negative	Original Plasma	
Positive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