**Filter-Based Assay for *Escherichia coli* in Aqueous Samples Using Bacteriophage-Based Amplification**

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**Supporting Information**

**ABSTRACT:** This paper describes a method to detect the presence of bacteria in aqueous samples, based on the capture of bacteria on a syringe filter, and the infection of targeted bacterial species with a bacteriophage (phage). The use of phage as a reagent provides two opportunities for signal amplification: (i) the replication of phage inside a live bacterial host and (ii) the delivery and expression of the complementing gene that turns on enzymatic activity and produces a colored or fluorescent product. Here we demonstrate a phage-based amplification scheme with an M13KE phage that delivers a small peptide motif to an F+ strain of *Escherichia coli* K12, which expresses the ω-domain of β-galactosidase (β-gal). The result of this complementation—an active form of β-gal—was detected colorimetrically, and the high level of expression of the ω-domain of β-gal in the model K12 strains allowed us to detect, on average, five colony-forming units (CFUs) of this strain in 1 L of water with an overnight culture-based assay. We also detected 50 CFUs of the model K12 strain in 1 L of water (or 10 mL of orange juice, or 10 mL of skim milk) in less than 4 h with a solution-based assay with visual readout. The solution-based assay does not require specialized equipment or access to a laboratory, and is more rapid than existing tests that are suitable for use at the point of access. This method could potentially be extended to detect different bacteria with bacteriophages that deliver genes encoding a full-length enzyme that is not natively expressed in the target bacteria.

The guidelines for monitoring bacterial contamination in a public supply of water, or in food, are made stringent to mitigate threats to public health. Analytical methods approved by the Environmental Protection Agency (EPA) must ensure that a public water supply maintains fewer than one colony-forming unit (CFU) of *Escherichia coli* in 100 mL of water, and the Food and Drug Administration (FDA) has a “zero tolerance” policy for the presence of bacterial species such as *Escherichia coli* O157:H7, *Salmonella* sp., and *Listeria monocytogenes* in foodstuffs.

The detection of such small numbers of bacteria requires an amplification step: EPA-approved methods rely on microbiological culture, affinity capture (based on antibodies), or the amplification of nucleic acids. Microbiological cultures determine the number of live bacteria in a sample but require incubation periods ranging from several hours to several days at temperatures between 30 and 45 °C.9,9 the “rapid” test for *Escherichia coli* bacteria approved by the EPA requires a 16 h incubation at 35 °C. Nucleic acid-based methods such as multiplexed polymerase chain reaction (PCR) can determine the identity and number of bacteria present in a sample within a period of 2–8 h but are more difficult and costly to run than cultures because they require access to laboratory equipment and reagents. In addition, they cannot distinguish between live and dead bacteria in samples that could have been disinfected with chlorine or other disinfectants. There are a number of lab-on-chip and paper-based prototypes that utilize surface plasmon resonance, amperometric, or impedimetric measurements. None have yet provided a simple analytical method that meets the criteria for approval by the EPA. Several commercial products are also available to detect *Escherichia coli* in a sample of water, at the point of access, but these products are culture-based and require incubation periods of 12 h or longer.

Bacteriophages (phages) are viruses that selectively infect a bacterial host and utilize the cellular machinery of the host to replicate in number. Phages are well-suited as a reagent for...
detecting the presence of bacteria in a sample because they (i) amplify in number naturally, once they have infected the targeted host; (ii) are species- or serotype-specific, and this specificity reduces the probability of a false-positive result; (iii) require a single reagent, the phage of interest; (iv) can be produced in large numbers at a low cost; (v) can be stored for long periods in a dry state; (vi) pose no threats to humans and can be handled without fear of infection or illness; and (vii) can be engineered to coexpress enzymes or peptide sequences that are not natively expressed in the targeted bacteria.

Phage-based detection of bacteria is a field with more than 20 years of history and offers four specific advantages over other detection methods: (i) The rate of replication of phage in its host is always faster than the replication of the host organism. Detecting phage progeny replicated in the host accelerates the detection of host microorganisms, which can be nonreplicating or slow-growing (e.g., *Mycobacterium tuberculosis*). (ii) Phage can be engineered readily with reporter genes such as galactosidase (gal), luciferase (luc), and fluorescent proteins (e.g., gfp). The host bacteria can be detected by fluorescence microscopy or cell sorting when these genes are delivered by the phage. (iii) Most phages evolved receptor-binding proteins (RBP) that allow for potent and specific recognition of unique protein and carbohydrate receptors on a bacterium. Multiple copies of RBPs are displayed on a phage and make them highly specific antibacteria antibodies capable of labeling the surface of the target bacterium. To facilitate such detection, phages could be prelabeled with a fluorophore or affinity tag, or engineered to display a specific peptide sequence. Alternatively, RBP–bacteria interactions allow for the capture and enrichment of specific bacteria in diagnostic devices.

A point-of-access assay for bacteria that combines the simplicity of culture with the short time periods required for nucleic acid-based methods is not currently available. We describe a simple, portable, filter-based assay that can detect fewer than 50 CFUs of *E. coli* in 1 L of liquid in 4 h by exploiting two different types of selective signal amplification: the replication of phage within live bacteria and the production of hundreds (or thousands) of colored or fluorescent molecules per second from an enzymatic reaction (Scheme 1). We use this bacteriophage-based method to identify *E. coli* in samples of drinking water, milk, and orange juice.

**EXPERIMENTAL SECTION**

**Choice of Bacteriophage–Host System.** We chose M13KE phage as a model phage, and *E. coli* K12 (ER2378) as a model host, for the phage-based assay because (i) this

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*Scheme 1. Schematic of the Assay Based on Bacteriophage Amplification*
bacterium is a coliform bacterium, which is a rod-shaped bacterium that is not necessarily pathogenic but may be indicative of pathogenic bacteria associated with fecal matter, and thus a target organism for EPA-approved methods; (ii) a single E. coli infected by M13 phage produces an exponential burst of 1000 plaque-forming units (PFUs) of phage within 40 min of infection; 28,29 (iii) the M13KEd phage is nonlytic, and the host continues to proliferate and produce the M13 progeny at a linear rate; 28,29 (iv) both M13KE and ER2378 are commercially available strains engineered for α complementation assays.30–34 Specifically, ER2378 is a lacZα-complementing strain of E. coli and expresses high levels of the ω-domain of the β-gal (ωGal), which is not functional until it binds to a short peptide denoted as αGal. The αGal peptide is cloned into the intergenic region of the M13KE genome. 32–34 The αGal peptide is expressed quickly after injection of the phage DNA into the host and complements the ωGal to yield functional β-gal.

A β-gal-expressing phage is advantageous because the enzymatic turnover of β-gal (e.g., 620 molecules/s at pH = 7.0 and 20 °C) 35 provides a second stage of amplification and the products of the enzymatic assay can be detected visually, eliminating the need for microbiological culture (or a plaque-forming assay). We note that this model system, which is based on complementation will not work for the bacteria commonly found in environmental samples because they do not express ωGal. Nevertheless, we anticipate this assay can be implemented to detect naturally occurring bacteria with previously published phage, engineered to contain genes for full-length enzymes (e.g., gal or luc). The only anticipated difference is that the rate of expression and maturation—folding and tetramerization—of full-length gal gene will be reduced, when compared with the rate of expression of short complementing peptide αGal.

Choice of Liquid Samples. We detected and quantified the number of CFUs of ER2378 E. coli in drinking water, drinking water contaminated with particulates of soil (5 g/100 mL; i.e., “dirty water”), skim milk, and pulp-free orange juice. We chose to test samples of water because a significant number (15%) of Americans, 6,37 and a larger proportion of people in the developing world, obtain drinking water from private sources (e.g., a well, cistern, or stream) that are not monitored unless an outbreak of water-borne disease has occurred. We selected milk and orange juice because they are commodity foodstuffs and require constant monitoring; there is currently no diagnostic capable of detecting the presence of bacteria in a sample of milk at the site of milk collection and processing. The collection and pooling of milk samples in the developing world, or other locales where pasteurization is not feasible, may result in a single sample of bacteria-containing milk contaminating an entire pool. 38 Milk and orange juice also pose analytical challenges because they are opaque and not compatible with assays based on a visual readout.

Concentration and Amplification of E. coli Using a Syringe Filter. We began every assay by filtering the sample through a 0.2 μm syringe filter (surfactant-free cellulose acetate, Thermo Scientific) to capture the bacteria from the sample (Scheme 1). These filters are available in prepackaged sterile units and represent a self-contained microbiology laboratory in which the captured bacteria can be incubated and handled with low risk of contamination. The filters retain the bacteria throughout multiple washing steps, which are necessary to reduce colored contaminants or excess salts from samples, such as milk or orange juice, that may interfere with detection, but allow for the elution and collection of newly produced phage, or newly assembled molecules of functional β-gal, following incubation.

The filtration of liquid samples through an inline filter is an industry standard to capture bacteria, and a portable version of this method was first described in the 1950s. 39 To date, the bacteria captured on these filters are detected with a culture-based assay, and the assay requires the aseptic transfer of the inline filter to, and culture on, an agar-containing dish. We combined the inline filter approach with secondary phage-based detection into an integrated device that requires no aseptic handling and could detect model bacteria in as few as 4 h.

Indirect Detection of E. coli with Phage- and β-gal-Based Assays. The quantity of phage (or β-gal) collected after incubation correlates with the number of viable bacteria captured on the syringe filter, because bacteriophages can only replicate in a live bacterial host. To validate this correlation, we quantified the newly produced phages with a plaque assay, 40 a standard microbiological assay in which the phage are introduced to solid agar containing E. coli, and plaques (regions of dead bacteria) are counted after incubation. The M13-phage-infected bacteria produce blue-colored plaques in the presence of a colorimetric substrate for β-gal: 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside, Xgal.

For the portable assay, we detected the captured E. coli with a solution-based assay that relies on the enzymatic activity of β-gal to produce a colorimetric or fluorescent product. A readout based on the production of a fluorescent molecule is desirable because fluorescence readings are less susceptible to interference from colored solutions.

**RESULTS AND DISCUSSION**

**Filtration of Liquids through the Syringe Filter Improves the Detection of Colored Products.** The background absorption and/or scattering of light by opaque or colored samples make the detection of a colorimetric product difficult. We prepared samples of drinking water, orange juice, skim milk, and dirty water with increasing concentrations of chlorophenol red—one possible product of the β-gal assay—ranging from 4 to 250 μM (Figure 1a). The red color of chlorophenol red can be observed at a concentration of 8 μM in drinking water, but is more difficult to detect in orange juice (125 μM), milk (63 μM), and dirty water (16 μM).

Figure 1b shows samples of orange juice, milk, and dirty water before and after filtration. The increased transparency of the samples after filtration facilitates the detection of a colored or fluorescent molecule. Figure 1c compares the transmittance (λ = 570 nm, the maximum absorption of chlorophenol red) of the liquid samples in Figure 1b before and after filtration.

Samples of orange juice and dirty water passed easily through the 0.2 μm filter, but less than 1 mL of skim milk clogged the filter. We found that adding solutions of sodium hydroxide (to a final concentration of 0.4% w/v) to the samples of milk greatly reduced their viscosity and allowed them to pass through the filter. Basic solutions are known to be antibacterial, but short exposures are not lethal. 40

**Filtration of Liquids through the Syringe Filter Captures and Retains Bacteria.** To ensure the bacteria contained in a liquid sample were captured (and retained) during filtration and several washing steps, we filtered 5 mL samples of drinking water, orange juice, skim milk, and dirty water containing 5000 CFUs of a β-gal-expressing E. coli
(NCTC 9001) and rinsed each sample with 10 mL of 1× phosphate-buffered saline (1× PBS). We then centrifuged the filtrates at 14,000g for 10 min, removed the supernatant, resuspended the pellet with 1 mL of 1× PBS, and plated it on an agar plate containing Xgal. We included several positive controls (containing 5, 50, and 500 CFUs of E. coli NCTC 9001), which we suspended in 10 mL of 1× PBS, centrifuged, and plated. The positive control samples each contained blue-colored plaques, while the filtrates contained no plaques (n = 3 samples of each liquid); these results show that the syringes effectively capture bacteria from the sample and sterilize the filtrate.

We performed a separate set of controls to determine if the viability of E. coli in a sample of milk decreased when exposed to sodium hydroxide (0.4% w/v); we found that exposures within the time required to add sodium hydroxide to a sample of milk, filter the sample, and rinse it with 1× PBS (~15 min) did not decrease the viability of 5 CFUs of E. coli in the sample (see the Supporting Information).

**Culture-Based Readout Has a Limit of Detection of 5 CFUs of E. coli in 1 L of Drinking Water.** We determined the limit of detection of the phage-based assay with an overnight culture-based readout (i.e., a “plaque assay”) for samples containing between 0 and 5000 CFUs of E. coli K12. Because the plaque assay cannot distinguish between newly produced phage and excess phage remaining on the filter, we inactivated the excess phage by rinsing the filter with a solution of ferrous ammonium sulfate,31 followed by a solution of sodium citrate to chelate and remove excess ferrous ions (Scheme 1, see the Supporting Information for experimental details). We incubated the syringe filters for an additional 60 min to allow the phages to complete their replication cycle prior passing 1 mL of 1× PBS through the filter and applying the solutions to a plate of solid agar containing E. coli and Xgal (detailed procedure in the Supporting Information). We counted the number of plaque-forming units (PFUs) after a 12 h incubation and plotted the number of PFUs as a function of CFUs of E. coli captured on the filter (Figure 2a).

Samples of drinking water containing a low copy number of CFUs of E. coli—estimated to be five or fewer by an independent colony-forming assay—produced 1100 ± 500 PFUs (Figure 2, parts a and b). The distribution of data and its standard deviation were similar to the Poisson distribution with an expectation value of 5 (Figure 2, parts b and c). This observation confirmed that experiments were indeed detecting low copy numbers of bacteria. The signal originating from samples containing zero E. coli was 30 ± 15 PFUs, which was significantly different from 1100 ± 500 PFUs produced by samples with a small number of E. coli. The simulated Poisson distribution predicted that each CFU of bacteria generated 210 PFU of phage. This value was below the expected number of phage produced by an average burst size of an M13 phage (500–1000).28,42 It is possible that the phage was lost in this assay due to nonspecific adsorption to the filter. This loss was exacerbated for higher numbers of E. coli, and resulted in a nonlinear relationship between captured CFUs of bacteria and produced PFUs of phage (Figure 2a).

Both the limit of detection (LOD) of the culture-based readout and the average number of PFUs observed for samples containing ca. 5 CFUs of E. coli were independent of the volume of the liquid sample (Figure 2b). Due to the number of bacteria on the filter, our assay, by design, has increased sensitivity for samples of increased volumes.

**Dirty Water, or the Presence of Other Species of Bacteria, Does Not Affect the Phage-Based Assay.** Dirty water, which is often associated with sources of water that may be contaminated does not interfere with the assay (Figure 3a); the numbers of PFUs detected from samples of drinking water and dirty water containing 50 CFUs of E. coli are statistically indistinguishable (p > 0.05, by Student’s t test). We also found that the number of plaques produced from samples of drinking water containing 50 CFUs of bacteria not targeted by the M13 phage—E. coli BL21 (an F− E. coli), Pseudomonas aeruginosa, and Staphylococcus aureus—are statistically indistinguishable from samples containing zero bacteria (Figure 3b). Samples of drinking water containing a mixture of bacteria (e.g., 50 CFUs of E. coli K12 and 50 CFUs of E. coli BL21) and samples containing only 50 CFUs of E. coli K12 produce numbers of PFUs that are statistically indistinguishable (p > 0.05, by Student’s t test).

The species specificity of a bacteriophage reduces the likelihood of false-positive readings; it is, however, important to note that a false positive is possible if the excess phage on the filter is not properly deactivated with a ferrous ion containing...
solution. The need for a sterile laboratory environment (for plating and culturing the phage-containing samples) and numerous controls (to ensure that the excess phage are inactivated with ferrous ions and excess ferrous ions are inactivated with citrate ions) makes a culture-based assay difficult to implement at the point of access. An ideal assay would require few experimental steps, no access to a laboratory, and produce a visual signal in less time than required for culture.

Portable, Visual Readout-Based Assay Has a Limit of Detection of 50 CFUs of E. coli in 1 L in 4 h. While the overlay-based assay surpasses the requirements set by the EPA for a coliform test—the ability to detect 1 CFU of E. coli in 100 mL of water in less than 24 h—a solution-based assay, with a visual readout, would be attractive because there are no approved point-of-access assays for bacteria that do not rely on culture of the sample.

A readout based on β-gal (rather than the collection of newly produced phage), which is activated in the model E. coli after M13 phage infection eliminates the need for washing the filter with ferrous ammonium sulfate and sodium citrate because the presence of excess phage does not affect the result. Following the incubation of the sample with the phage, we treated the filter with a solution that contained lysozyme, which lysed any bacteria present on the filter and released their contents, and a substrate for β-gal (see the Supporting Information). We collected the filtrate and monitored the enzymatic reaction for changes in color.

There are a number of substrates that are converted to a colored product in the presence of β-gal.43 We compared three substrates that are listed in methods already approved by the EPA:1 2-nitrophenyl β-D-galactopyranoside (ONPG), which yields 2-nitrophenol (ONP, yellow in color); chlorophenol red-β-D-galactopyranoside (CPRG), which yields chlorophenol red (CPR, red in color); and 4-methylumbelliferyl β-D-galactopyranoside (MUG), which yields 4-methylumbelliferone (MU, fluorescent).

Figure 4a shows the visual limits of detection for ONP, CPR, and MU, based on the measurement of a series of standard solutions; we also measured the absorbance and fluorescence of each solution with a spectrometer to ensure that the calibration trends were linear (see the Supporting Information). The visual limit of detection of CPR (∼0.01 mM) is approximately 20-fold lower than that of ONP (0.2 mM), which is a more commonly used substrate for β-gal. MU was the most sensitive of the three substrates, with a visual limit of detection of ∼0.00003 mM when excited with a hand-held UV lamp or an light-emitting diode (LED) emitting in the UV. LEDs are compatible with a

Figure 2. (a) Number of PFUs of M13 phage detected with a plaque assay from 10 mL samples of drinking water containing known CFUs of E. coli. The relationship between the number of E. coli and the number of PFUs of M13 phage detected in the plaque assay is nonlinear as evidenced by the log fit. As a result, a 1000-fold increase in the number of E. coli yielded only a 4-fold increase in the number of PFUs. All data are shown (n = 9). (b) Distribution of PFU values generated by samples with 5 CFUs of E. coli resemble a Poisson distribution with an expectation value of 5. This distribution predicts that each bacteria produce, on average, 210 PFU. (c) Poisson distribution with an expectation value of 5, and an overlay of the box-plot with average, high/low values, and 95% confidence interval. Probability to observe zero bacteria in this distribution is beyond the 95% interval. (d) Number of PFUs of M13 phage detected for 1, 10, 100, and 1000 mL samples of drinking water containing 0 CFUs of E. coli (top row) and 5 CFUs of E. coli (top row). The gray regions demarcate a predicted 95% confidence interval for samples containing five bacteria and a range of signal observed for samples without bacteria. Each point is the average of n = 9 experiments, and the error bars represent one standard deviation from the mean.

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portable assay, are easy to use, inexpensive (<$1 per flashlight), and require little power (~18 h of continuous light, or over 10,000 samples, on a single lithium battery).

To determine the limit of detection of the solution-based assay, we captured and infected between 0 and 5000 CFUs of E. coli on the syringe filter, lysed the bacteria in the presence of MUG or CPRG, and collected the lysate in 1.5 mL centrifuge tubes. The fluorescent signal (of MU) in samples of water, milk, and orange juice containing 50 CFUs of E. coli was observed after 3 h of incubation. To achieve a visible colorimetric result (of CPR) in the same period of time required ∼1×10^6 CFUs of E. coli.

Figure 4b shows a 0.2 μm syringe filter before (left) and after (right) it was used to filter 10 mL of soil-contaminated water. The filtrate from the dirty water did not interfere with the visual detection of CPR or MU, whereas the visual detection of ONP was limited by the discoloration of the filtrate (Figure 4c). The presence of particulates of soil does not interfere with the visualization of MU (Figure 4d).

We note that the rapid β-gal readout in the M13KE-ER2378 model strains is the result of the unnaturally high concentration of galactosidase caused by the presence of lacIq mutation in the ER2378 strain. Nonengineered coliform bacteria (e.g., E. coli NCTC 9001, which was collected from urine) express low, wild-type levels of galactosidase. The time required for 5000 CFUs of NCTC 9001 to produce a visible signal of MU was approximately 8 h. To accelerate detection, such strains could be infected by the phage that delivers the gal gene under the control of strong promoters and enables the wild-type bacterium to express unnaturally high levels of the enzyme. Phages engineered to yield overexpressed levels of galactosidase to E. coli are known; there are also phages which express reporter genes such as lacZ, luc, and gfp to target clinically relevant bacteria.

### CONCLUSION

There is presently no convenient or cost-effective method to test samples of liquid for the presence of bacteria at a point of interest (e.g., a water source, an assembly line in a food processing plant, a container of pooled milk samples, etc.). Commercially available kits rely, as do most laboratory-based
assays, on the overnight culture of the sample. This paper describes an alternative assay, based on the species-specific infection of bacteria in a sample with a self-amplifying system: a bacteriophage. We have detected 50 CFUs of model α-complementing E. coli strain in a 1 L sample of drinking water in less than 4 h with a visually based readout. Phages are an ideal reagent for diagnostics because, in addition to their selectivity, they can be stored dry and can be engineered to coexpress a variety of reporter enzymes that produce colored, fluorescent, or electrochemically active species. There are a number of repositories of already sequenced phages, and an exponential rise of new phage species from metagenomic studies. The procedures for inserting a gene of interest into the phage genome are among the most established techniques in molecular biology because phage genomes are the basis of the cloning and sequencing vectors developed in 1970s and 1980s.

The sensitivity of the assay described in this report benefits from two types of amplification: the replication of phage, which can amplify in number by a factor of up to 1000, and the catalytic activity of a high-turnover-rate enzyme, which is known to amplify 10^6-fold.4,34 The system we described here is based on complementation of a small peptide delivered by the phage is, however, a highly developed technology. The authors declare no competing financial interest.

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