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Enrichment and Detection of *Escherichia coli* O157:H7 from Water Samples Using an Antibody Modified Microfluidic Chip

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Abstract

Low abundant (<100 cells mL⁻¹) *E. coli* O157:H7 cells were isolated and enriched from environmental water samples using a microfluidic chip. The poly(methylmethacrylate), PMMA, chip contained 8 devices each equipped with 16 curvilinear high aspect ratio channels that were covalently decorated with polyclonal anti-O157 antibodies (pAb) and could search for rare cells through a pAb mediated process. The chip could process independently 8 different samples or one sample using 8 different parallel inputs to increase volume processing throughput. After cell enrichment, cells were released and enumerated using bench top real-time quantitative PCR, targeting genes which effectively discriminated the O157:H7 serotype from other non-pathogenic bacteria. The recovery of target cells from water samples was determined to be ~72%, and the limit-of-detection was found to be 6 colony forming units (cfu) using the *slt1* gene as a reporter. We subsequently performed analysis of lake and waste water samples. The simplicity in manufacturing and ease of operation makes this device attractive for the selection of pathogenic species from a variety of water supplies suspected of containing bacterial pathogens at extremely low frequencies.

1. Introduction

Threats to human health from *Escherichia coli* contamination of aquatic environments are becoming highly prevalent. Several major water-borne outbreaks have been reported in aquatic systems ranging from freshwater to marine, where *E. coli* accumulates in the water column and sediments.^{1, 2} To better understand *E. coli* ecology in aquatic environments, to

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Supporting Information Available Supporting information is available free of charge via the Internet at <http://pubs.acs.org>.

provide monitoring over time scales appropriate for the study of *E. coli* outbreaks,³⁻⁵ and to protect human populations, monitoring techniques for *E. coli* are needed that have low detection thresholds even for extremely rare cells and rapid processing times.

The US-EPA allowable levels of *E. coli* are 0, 200, and 1,000 cfu per 100 mL of drinking, swimming, and recreational (boating) waters, respectively, and the minimum infectious dose is very low, ~10 cells.⁶ To detect *E. coli* corresponding to doses this low, pre-enrichment of cells is required, especially in drinking and recreational waters, by processing large input volumes to accommodate the readout phases of the measurement assay. There are different technologies for isolating rare cells from heterogeneous samples to aid in their analysis such as fluorescence assisted cell sorting,⁷ flow-through filtrations,⁸ enzyme linked immunosorbent assays (ELISA),⁹ and immunomagnetic assisted cell sorting.¹⁰ These methods, however, rely on time-consuming culturing protocols.

The US-EPA has approved an *E. coli* detection test for the examination of drinking water, which is based on β -D glucuronidase, an enzyme associated with *E. coli* colonies.¹¹ This method (EPA Method 1603) detects the presence of all coliforms, but not specifically the *E. coli* O157:H7 strain due to a two-base frame shift insertion within its genome that yields an inactive *uidA* gene and lack of β -D glucuronidase production.¹² Detection of pathogenic *E. coli* O157:H7 is usually performed with EPA Method 9260F,¹³ which employs a series of incubations at low temperatures for extended periods of time (72 h).

The presence of naturally and anthropogenically-derived dissolved substances in aquatic systems, such as humic materials and residual pharmaceuticals,¹⁴ along with other dominant native bacterial species, can act as interferents that may alter the accuracy of the aforementioned colorimetric tests. In addition, microbiological studies have indicated that stressed *E. coli* O157:H7 become non-culturable even though they may be viable and still capable of producing Shiga-like toxin.¹⁵ More recently, nucleic acid-based methods for pathogen detection, such as PCR, have been developed to target unique bacterial genes. PCR itself, although very sensitive, detects the presence of bacteria but does not allow isolation of rare bacterial cells, which is required to determine the etiological agent responsible for an outbreak.¹⁶

Recent work has shown that cells can be accumulated from biological samples using a microfluidic platform.¹⁷⁻¹⁹ These microfluidic devices utilized the surface of a microchannel or beads trapped within a microchannel for positive cell selection. Liu *et al.* generated a device for processing *E. coli* cells from input volumes of 1 mL with the cell LOD of ~1 cfu μ L⁻¹.²⁰ Beyor and co-workers reported a microfluidic system that could process ~50 μ L of input and search for target cells, such as K-12 or O157:H7 *E. coli*.²¹ The limit-of-detection (LOD) reported was 0.2 cfu μ L⁻¹. With an *E. coli* O157:H7 concentration level of <200 cells per 100 mL, the probability of securing a target cell in 50 μ L would only be 0.01. Therefore, sampling statistics would require processing larger input volumes to provide higher confidence that a target could be processed.

We present a method for the isolation, identification, and quantification of *E. coli* O157:H7 cells from recreational waters. Once the cells were enriched, their quantification was accomplished using off-chip real-time quantitative PCR (qPCR). We used an engineered chip consisting of high-aspect ratio capture beds decorated with polyclonal antibodies (pAbs) specific for antigenic membrane proteins expressed in *E. coli* O157:H7. The method was further demonstrated to be capable of detecting *E. coli* O157:H7 cells in lake and waste water samples. The benefits of this methodology include: (i) pre-selectivity, which is extremely important because the detection is often difficult due to the combination of the high number of non-pathogenic bacteria and the low number of pathogens of interest;¹⁶ (ii)

the microfluidic chip provides a cell purification platform (cells are washed while attached to the surface) and prepares the selected cells for immediate qPCR analysis free from potential interferents; and (iii) rapid analysis as compared to conventional methods relying on culturing.

Materials and Methods

Reagents and buffers

Heat-inactivated *E. coli* O157:H7 cells and goat polyclonal anti-*E. coli* O157 antibodies were purchased from K&P Laboratories Inc. (Gaithersburg, MD). Other chemicals used in these studies are listed in the Supporting Information (SI).

Microfluidic chip fabrication and assembly

Fabrication of microstructures has been reported previously and further details can be found in the SI.^{17, 18, 22} Each of the 8 devices contained 9.5 mm long, 16 curvilinear channels that were 15 μm in width and 80 μm in depth with a radius of curvature of 120 μm (see Figure S1 in SI). The surface area of the 16 channels, which defined the cell selection bed, was 40 mm^2 with a volume of 250 nL. The chip output following selection and release was directed into a PCR microtube. A pre-cell selection rinse of the chip was performed with 100 μL of 150 mM PBS at 50 mm s^{-1} . Then, a cell suspension or water sample was infused into the chip using a syringe pump at 0.5, 1.0, 5, 10, 40, 80 and 100 mm s^{-1} linear velocities. A post-capture rinse with 100 μL of 150 mM PBS at 50 mm/s (55.6 $\mu\text{L min}^{-1}$) was used to remove any non-specifically adsorbed material. Cells were then stripped from the channel walls using a CellstripperTM solution and eluted from the chip in 5 μL of PCR buffer.

Antibody immobilization

Antibody immobilization onto the UV photo-activated chip surface was carried out in a process reported previously.^{17, 18} The procedural details can be found in the SI.

Water sample collection

Lake water samples were secured from two different sites: (i) Baton Rouge University Lake, LA (USA), which is a small man-made lake on the LSU campus created in the early 1920s with the damming of Bayou Duplantier, and (ii) Lake Granbury, TX (USA), which is a reservoir on the Brazos River constructed in 1969. Extensive lake descriptions can be found in the SI. The sampling depth was about 6" below the water surface.

Water sample filtration

Water samples from recreational lakes (100 mL), and waste water samples (1 mL) were filtered using hydrophilic (PVP-coated) polycarbonate track etched membranes (PCTE, Sterlitech, Kent, WA) membrane filters. The pore size of the membranes used was 10 μm for the pre-filtration to remove large particulates and 0.1 μm for further filtration and volume reduction prior to input into the microfluidic device. Water samples were filtered through the 10 μm pore size membranes at 10 mL min^{-1} and the effluent was collected and filtered through the 0.1 μm pore size membranes at 2.5 mL min^{-1} . The membrane was rinsed with ~1 mL of pure water to exhaustively remove material from the surface and used as the input to the microfluidic chip. The 10 μm pore size membrane filter did not change the total volume of the sample however, it did remove solid particulates and debris.

E. coli detection via culturing

E. coli were detected in water by membrane filtration using a modified membrane-thermotolerant *E. coli* agar (Modified mTEC EPA Method 1603). Details of these procedures can be found in the SI.

Polymerase chain reaction (PCR) and real-time qPCR

All PCRs were performed under a positive flow hood (AirClean Systems 600 Workstation, Raleigh, NC) and carried out with *E. coli* cells thermally lysed (10 min. at 96°C). PCRs were performed using a GeneAmp® PCR reagent kit with AmpliTaq® DNA polymerase (Applied Biosystems, Foster City, CA). Real-time qPCRs were performed on a Stratagene Mx4000 real-time PCR machine with Brilliant® II SYBR® Green QPCR Master Mix (Stratagene, La Jolla, CA). Further details are given in the SI.

Fluorescence microscopy

Observations of fluorescently-labeled cells in the chip's selection channels were accomplished using a Zeiss Axiovert 200M Inverted Microscope. Further details are given in the SI.

Results and Discussion

Low-abundant *E. coli* O157:H7 cell processing

Scheme 1 presents an overview of the processing strategy used for the selection and identification of *E. coli* O157:H7 cells. The processing steps involved: (i) chip preparation; (ii) sample filtration using PCTE membranes to remove large particulates from the sample and to provide pre-enrichment; (iii) sample processing on the chip and cell release; and (iv) bench-top real-time qPCR quantification of the selected cells. For the recreational water, the time required to filter a 100 mL input using both the 10 and 0.1 μm filters to reduce the total volume to 1 mL was 50 min, producing a volume reduction of 100-fold and thus, a 10^2 pre-enrichment assuming 100% recovery. Processing a total input volume of 1 mL using the 8 devices poised on the microfluidic chip (see Figure S1 in the SI) at a linear flow velocity of 5 mm s^{-1} ($5.3 \mu\text{L min}^{-1}$) required 24 min and produced an enrichment factor of 2×10^2 . Therefore, the 0.1 μm filter and microfluidic chip generated a total enrichment factor of 2×10^4 with a processing time of 74 min. Membranes before and after filtration were inspected under a microscope and showed that the majority if not all of the solid material had been removed from the sample prior to chip processing (see Figure S2 in the SI). To achieve maximum recovery of bacterial cells from the 0.1 μm filter, the filter was washed with 1 mL of water and control experiments with known amounts of cells were performed. Previously, Wang *et al.* evaluated the performance of these types of membrane filters with pore sizes ranging from 0.1 to 0.45 μm to determine the effect of cell shape and size on bacterial filterability and determined that bacterial shape, rather than their absolute size, was a key factor in determining cell recovery.²³ We recovered $92 \pm 5\%$ ($n = 4$) of *E. coli* O157:H7 microbial material using the 0.1 μm . Bacterial cells were not lysed while on the membrane because this process would not guarantee the high quality quantitative analysis of DNA. A DNA extraction step should be performed to free the sample from any potential inhibitors present on the membrane surface.²⁴ In our approach, the immunocapture of the pathogens of interest using microfluidic channels followed by an extensive wash and subsequent release from the capture surface produced high quality qPCR results that were not seen in the case of using *E. coli* washed directly from the 0.1 μm filter (data not shown). In addition, the use of the microfluidic device provided high pre-concentration factors due to the small selection bed volume, improving the limit-of-detection for the qPCR.

Non-specific cell selection

Even though the affinity purified pAb were directed against *E. coli* O157:H7, other types of *E. coli* serotypes such as O157:H12, O157:H42, O157:H29, O157:H19, O157:H45, could potentially be selected using this reagent as well as other species, such as *S. sonnei* or *C. freundii* (see www.kpl.com). However, even if these bacterial types were selected during microchip enrichment, they would not be transduced during the enumeration process using real-time qPCR because of a lack of the specific marker genes used for reporting specifically on *E. coli* O157:H7.²⁵

We tested the non-specific selection of *E. coli* K12. Cells were infused into the microfluidic channels at 5 mm/s, the same conditions used for *E. coli* O157:H7 selection. After a post-capture rinse, fluorescence micrographs indicated that no *E. coli* K12 cells were found in the selection beds when decorated with the *E. coli* O157 pAbs. Figures 1A and B show fluorescence micrographs taken after introducing both types of cells. In Figure 1A, one can clearly see multiple events of anchored *E. coli* O157:H7 cells (indicated with arrows), while in Figure 1B, there was a lack of any such events for the K12 serotype. Also, minimal non-specific cell interactions with the PMMA channel surface was also found (data not shown) due to the weak adhesion forces and the presence of hydrodynamic shear that could remove any non-specifically bound cells.¹⁷

E. coli O157:H7 cell release from the channel surface

Following a post-cell capture rinse performed with 150 mM PBS, a Cellstripper™ solution was infused into the channels to dislodge antibody-induced adhesion of cells to the channel wall. The captured cells were observed under a microscope until they were removed from the chip surface (see SI, Figure S4). Twenty-five cells were monitored and the average time required for their release was determined to be 3.4 ± 0.3 min. Following incubation with the stripper solution, the released cells were flushed from the selection bed using a total volume of 5 μ L, which was transferred into a PCR micro-tube for amplification. We determined that a concentration of Cellstripper™ up to 10% in the real-time qPCR cocktail did not affect these results (see SI, Figure S5A-D).

Cell recovery

In order to maximize the recovery of *E. coli* O157:H7 cells from water samples and maintain high sampling throughput, the processing linear flow velocity thru the selection microchannels was optimized. *E. coli* O157:H7 capture efficiency at different flow rates and different cell densities ($3 - 30 \times 10^3$ cfu mL⁻¹) were determined using real-time qPCR. Linear flow velocities were varied between 0.5 and 100 mm s⁻¹. The results of these experiments are depicted in Figure 2A as a plot of *E. coli* O157:H7 capture efficiency (%) vs. linear velocity (mm/s). We observed that a linear flow rate of 5 mm s⁻¹ produced the highest recovery; $71.6 \pm 1.4\%$ ($n = 5$). The general trends observed in the plot shown in Figure 2A can be explained based on the Chang/Hammer model for mobile cell interactions with immobilized association elements.^{17, 26} Basically, the two processes that primarily dictate a successful antibody:cell interaction involve: (i) the encounter rate, which describes the rate of delivery of cells to the channel surface and is flow rate dependent with this rate increasing with linear velocity; and (ii) the reaction probability between the surface tethered antibody and the mobile cell antigenic target, which is dependent on the reaction kinetics between the antigen/antibody complex with this probability decreasing at high linear velocities.¹⁷ Therefore, the observed optimal linear velocity (~ 5 mm s⁻¹) results from a balance between the reaction kinetics and the encounter rate.

Capture efficiency decreased with increasing channel width as well^{17, 18} (see Figure 2B). We evaluated the recovery of *E. coli* cells using channels with variable widths, but the same

depth (30 μm) and length (3 cm) and cells flowing at 5 mm s⁻¹ linear velocity. We observed much higher recoveries for narrower channels as opposed to wider channels (68% $\pm$ 2%, 49% $\pm$ 2%, and 18% $\pm$ 5% for channel widths of 10, 20, and 30 μm , respectively). We previously demonstrated that cell recoveries are maximized when the channel width approaches the average cell diameter.¹⁷ Unfortunately, we were limited by the micro-machining technique employed here to a channel width of 10 μm , but the use of narrower channels will most likely increase the recovery by increasing the number of cell:wall contacts. Narrower channels can be fabricated using optical lithography to make the molding tool as opposed to high precision micro-milling.²⁷

We also evaluated the flow dynamics of *E. coli* transport through sinusoidally configured microchannels with hydrodynamic flow (see Figure S3 in the SI). The results indicated that the cells did not cross flow lines even through the turns and thus, the radial distribution of these cells was more randomly distributed at high flow rates.

Cell enumeration in water samples

The selective enumeration of *E. coli* O157:H7 used real-time qPCR and two sets of primers targeting the *slt1* and *uidA* genes. Primers for these genes were reported by Cebula *et al.*²⁸ and were extensively tested for specificity in comparison to other serotypes of *E. coli*. Additionally, we tested these primers with other microbes, some potentially present in the sample. No PCR products were observed for *S. aureus*, *B. subtilis*, *E. aerogenes*, *M. luteus*, or *E. coli* K12 using the *uidA* and *slt1* specific primers. PCR products with the size appropriate for the particular gene being interrogated for *E. coli* O157:H7 were observed (see Figure 2D).

Calibration curves for *E. coli* O157:H7 were generated using serial dilutions of standard *E. coli* O157:H7 cell suspensions between 1 cfu to $\sim 3 \times 10^6$ cfu (see Figure 2C) in samples containing 100 mL of water. The linearity between the Ct values and the number of target cells was observed over 5 orders of magnitude ($r^2 \geq 0.996$). The slope of the calibration curve, which is directly related to the average amplification efficiency throughout the cycling reaction (efficiency = $10^{(-1/\text{slope})} - 1$), was found to be -3.593 ± 0.231 , yielding a 98.5% $\pm$ 1.7% efficiency ($n = 5$) for *slt1*, and -3.616 ± 0.372 for an 89.2% $\pm$ 2.4% efficiency ($n = 5$) for the *uidA* gene. The limit-of-detections (expressed as the amount of cells detected in the 100 mL sample at least 95% of the time) were calculated to be 6 cfu for the *slt1* gene and 10 cfu for the *uidA* gene. Similar or higher real-time qPCR limits-of-detections were reported.²⁹ The analysis of dissociation curves displayed one population of amplicons with a transition temperature around 80 to 82°C indicating the presence of a specific PCR product (see the SI, Figure S6B).

Control samples (single blinded studies) were also evaluated in which *E. coli* O157:H7 samples were spiked at levels of 30 – 800 cfu per 100 mL input. This data was used to construct a calibration plot of Ct versus log (cell density, see Figure 2C). For example, the number ($n = 3$) of *E. coli* O157:H7 cells determined from the calibration plot was 34 ± 4 (RSD of 12%) at the 30 cfu spike level. At a 400 cfu spike level, the average cell count for the assay was 405 ± 5 with an RSD of 1.2%. A blank sample was also analyzed and no *E. coli* O157:H7 was detected.

We next evaluated the microchip/real-time qPCR assay to assess the quality of different water samples. These samples consisted of samples from two lakes and sewer water from a purification plant in Baton Rouge. We should also note that *E. coli* obtained directly from the 0.1 μm filter and tested via real-time qPCR without processing in the microchip sometimes produced failed PCR results due most likely to the presence of potential PCR inhibitors that were removed in the fluidic chip following cell selection and rinsing.

In the Lake Granbury sample, the total *E. coli* levels were detected at 5 cfu 100 mL⁻¹ and *E. coli* O157:H7 serotype at 4 cfu 100 mL⁻¹. Water from University Lake in Baton Rouge was evaluated only for the total *E. coli* level using EPA Method 1603 and was determined to be 15 cfu 100 mL⁻¹ (see Figure S7A). After performing the assay using the microfluidic chip, we determined that for both of these water samples, the level of *E. coli* O157:H7 was below the limit-of-detection. This is understandable given the fact that the recovery is 72% and therefore, the approximate numbers of *E. coli* O157:H7 from Lake Granbury would be ~3 cells, which is below the limit-of-detection for both the *slt1* and *uidA* genes.

Next we evaluated waste water samples using EPA Method 1603 and found *E. coli* levels of 2.6×10^6 cfu 100 mL⁻¹ (see Figure S7B). In waste water samples, we detected $0.96 \times 10^6 \pm 0.2$ cfu 100 mL⁻¹ of *E. coli* with the serotype O157:H7. Also, we calculated the cell capacity of the microchip selection beds possessing a surface area of 40 mm² and this value was determined to be 260×10^6 cells. Thus, the level of cells collected from the waste water sample is well below the saturation limit.

Conclusions

We demonstrated the ability to select and quantitatively enumerate the *E. coli* O157:H7 serotype free from other serotype interferences, which is important due to the large number of *E. coli* types and the O157:H7 serotype health-related issues. The strategy developed offered the ability to monitor water quality without the need for a time consuming cell culture step. We also showed the ability to recover cells with ~72% efficiency from 100 mL input volumes with the real-time qPCR step providing a limit-of-detection of 6 to 10 cfu. The entire processing steps can be implemented in under 5 h and this could significantly be reduced by transitioning more of the processing steps to the chip. For example, increasing the throughput of the fluidic chip through the use of more fluidic vials that are deeper can provide the opportunity to process 100 mL of input directly without the need for the intermediate 0.1 μ m filtering step. In addition, moving the real-time qPCR step to the chip can also reduce processing time significantly due to the more efficient thermal management properties of micro-scale PCR.³⁰

The microchip enrichment procedure provided the following advantages: (i) the enrichment step can recognize O157 types in intact and virulent cells and in stressed and non-culturable cells; (ii) cells enriched on the chip can be isolated from potential contaminants that can interfere with nucleic acid-based analysis; and (iii) the chip can pre-concentrate cells to a detectable level with an enrichment of 2×10^2 in its current rendition. We envision the potential to probe for a large number of different strains of enterohemorrhagic *E. coli*, which includes more than 100 different non-O157 strains.^{31, 32} By proper choice of pAbs and specific probes for the real-time qPCR, one could monitor a large panel of suspected *E. coli* strains from a single sample.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Fluorescence images of a microchannel with (A) *E. coli* O157:H7 cells and (B) *E. coli* K12 cells captured on an antibody-modified PMMA surface. The dashed lines designate the edges of the channel.

Figure 2.

(A) Capture efficiency data of *E. coli* O157:H7 as a function of the cells' translational velocity using the microfluidic chip shown in Figure S1. The number of captured cells was determined via real-time qPCR. (B) Cell capture efficiency as a function of the channel width using a translational velocity of 5 mm/s. Channels used contained a variable width (10, 20, and 30 μm), but the same depth (30 μm) and length (3 cm). (C) Standard curves for the real-time, qPCR analysis of *E. coli* O157:H7 using *slt1* (filled squares) and *uidA* (filled circles) genes. Ct values of known samples were plotted against the corresponding cfu of the bacteria. The linear regression analysis for *slt1*: $y = -3.456 \log(X) + 37.12$ ($r^2=0.997$) and *uidA*: $y = -3.373 \log(X) + 40.99$ ($r^2=0.996$). (D) A fluorescence agarose gel image of the 252 bp and 348 bp PCR products for *uidA* and *slt1* genes, respectively. The amplicons were generated with 10×10^3 cfu/reaction of the *E. coli* O157:H7 serotype.

Scheme 1.

An overview for the processing strategy adopted for analysis of extremely low abundance *E. coli* O157:H7 and other serotypes using positive selection and enrichment via a microfluidic chip with subsequent quantification through real-time qPCR.