C-280

Device for Automated Capture of *E. coli* O157:H7 from Feces : Feasibility for Point of Care Detection.

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ABSTRACT

Detection of pathogens such as *E. coli* O157:H7 in clinical fecal specimens by PCR can be compromised by the specimen matrix, which might contain amplification inhibitors. One interface strategy is to capture pathogens with specific antibodies conjugated to magnetic beads. We are developing an instrument and disposable enteric card (DEC) platform consisting of a microfluidics-enabled 'lab-on-a-card' (lab card) with integrated subcircuits. The subcircuits provide specimen interface, pathogen capture, bacterial lysis, purification of released nucleic acid, PCR amplification, and visual result detection. We report our progress incorporating magnetic beads within a unique lab card that permits efficient capture and enrichment of *E. coli* O157:H7 from feces.

Antibodies specific to O157 LPS were conjugated to 1.08 μM Dynabeads MyOne tosylactivated magnetic beads per the manufacturers protocol. Lab cards, designed and manufactured using Micronics, Inc laminate construction, interface with Micronics' commercial fluidic workstation (microFlow™ system), which controls fluid movement, valving, and mixing on the card.

For initial validation, wild type commensal *E. coli* or *E. coli* O157:H7 were captured from culture media at various colony forming unit (CFU) concentrations, and introduced into the lab cards. Detection was confirmed following lysis of the magnetic bead captured cells using a guanidium buffer, purification on spin columns, and real time PCR. *E. coli* O157:H7 was specifically detected at 10,000 CFU limit of detection. *E. coli* O157:H7 spiked into 1 gram normal feces were diluted 100 fold, then introduced directly into the lab card. We confirmed detection for these spiked feces. Finally, we used the magnetic bead lab card to detect *E. coli* O157:H7 from 6 culture positive patient feces. Six control feces were negative.

INTRODUCTION

Shiga toxin-producing *E. coli* (STEC) belong to a variety of serotypes, but *E. coli* O157:H7 has the most durable link to human disease, and the greatest epidemic potential[1]. The specific diagnosis of enteric bacterial pathogens is complicated because pathogens are defined by a variety of virulence factors, each of which can be encoded on transmissible elements. Because of their transmissible nature, virulence loci are not limited to a specific bacterial genus or serotype, and can be found in commensals. One consequence of this interchangeability is that genetic targets that are highly specific to etiologic agents are difficult to find. Neither individual specific antigen nor individual nucleic acid (NA) detection completely defines a pathogen. One strategy to obtain necessary specificity would be to enrich or capture O157 expressing bacteria directly from analyte (i.e., feces), and then genes specific for pathogenicity. An additional advantage of this approach is that the method separates target bacteria from the specimen, and from PCR inhibitors.

A disposable enteric card (DEC) developed by our team[2] provides an automated, rapid, easy to-use, point-of-care platform to enrich O157 bacteria via capture from feces by magnetic beads conjugated with antibodies to the O157 LPS. The captured cells are then lysed to release NA that is then amplified by PCR to confirm the presence of pathogenic O157:H7 using specific gene targets. This "binary" technology is integrated fluidically on lab cards, which are laminated microfluidic devices produced by Micronics, Inc. For development, each functional step, or subcircuit, is initially optimized independently. Here we report progress for the design and optimization of the subcircuit lab card for magnetic bead capture of pathogenic *E. coli* O157 from feces.

METHODS

A. Reagents

Mouse antibody specific to *E. coli* O157 LPS (US Biological catalog No. E3500-02) wasconjugated to magnetic MyOne™ Tosylactivated Dynabeads® (Dynal®) per to the manufacturer's instructions. Bacteria captured by the beads were introduced into a lysis buffer (4.5 M guanidinium isothiocyanate, 50 mM MES, pH 5.5, 20 mM EDTA, 1% N-lauryl sarcosine, and 5% Triton X-100), and released NA was captured and purified using Qiagen DNeasy columns, eluted, and analyzed by real time PCR on the LightCycler® using the Roche *E. coli* O157:H7 detection kit (catalog no. 03671119001). 100 volumes (wt/vol) of PBS was added to stool (40-60 mg), and the mixture was pipetted to disperse clumps.

B. Magnetic bead microfluidic mixer and capture circuit

Micronics' disposable credit card-sized lab cards have structural elements ranging from 100 to 1000 µm in size. Fluids move in laminae cut by lasers or stamped into the desired shape. Interacting on- and off-card pumps and valves direct fluid flow from sample introduction, reagent mixing, separation and waste storage on card using the microFlow™ System, an integrated microfluidics workstation that uses ultra low-pulse twist syringe pumps to control precisely flow on the card. The microFlow workstation includes electronic hardware to control the individual stepper motors that operate the pumps and valves, air and vacuum control unit to activate card microvalves, or mixing chambers, a serial port for communication between the instrument, a microprocessor, and the system power supply. The flexible system can accommodate different fluidic circuits and functions. Software with script-based as well as real-time programmable user interfaces controls fluid motion. Once the series or sequence of steps on a lab card has been developed, simple commands runs the integrated operations using graphical interfaces.

Figure 1 : microFlow™ workstation provides fluid movement and air control of the mixing lab card.

The specific lab card (figure 2) characteristics that enable efficient magnetic bead capture include:

- single dumbbell chamber to reduce bead loss
- active bellows pumping to quickly disperse and mix beads with sample
- Magnetic capture from the top of the card, allowing debris to settle and then washed out
- repeated volume additions of sample to accommodate large volume
- sequestered waste

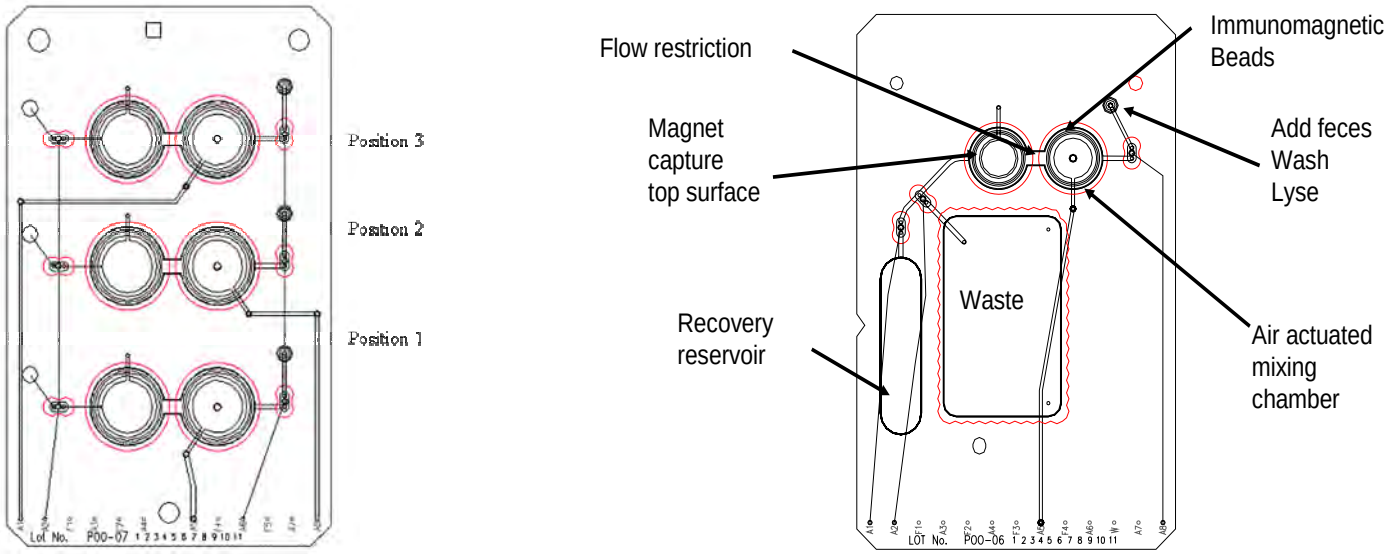

Figure 2 : Micronics' magnetic bead capture and mixing lab card. The dumbbell shaped mixing chamber enables highly efficient mixing through air-actuated to and fro fluid movement through a flow restriction channel. The card on the right processes a single specimen while the design on the left has three positions to process three specimens simultaneously. Magnetic beads conjugated with antibodies to O157 LPS are introduced into the mixing chamber containing 400 μL of diluted feces. The mixture is agitated using the MicroFlow apparatus. A magnet applied from above collects the beads, and feces is shunted to waste. The beads are washed, lysis buffer is added, and the lysate is introduced to Qiagen spin columns for NA purification.

RESULTS

I. Antibody binding specificity

Initially, we assessed the specificity of binding to the antibody coated beads, by enumerating CFU of captured organisms (Table 1).

TABLE I. Measure of binding by CFU method

E. coli strain	% bound to magnetic bead
ATCC 25922	**12**
O157: H7	**57**
0121:H19	**0**
011:HN	**1**

II. EIA specificity

Saturated overnight culture or feces were diluted 1:100 in PBS. Anti-O157 beads are added and vortexed. After incubation (15 min), 200 µL of suspension were transferred in duplicate to a Millipore Multiscreen Plate, and an EIA utilizing E3500-28 (anti-E. coli O157 conjugated to HRP) was performed. Reactions were stopped (100 µL 1N H_2SO_4) and read at 450 nm with a 630 nm reference filter.

TABLE II. ELISA specificity results

isolate	Virulence	Number tested	result
O157:H7	Pathogens	8	+++
O157:H-	Pathogens	2	+++
O157:HNM	Pathogen	1	+++
O157	Non-Pathogens	6	++
Rough:H11; 026:H11; 055:H7	Pathogens	2 each	-
0165:H2	Pathogen	1	+/-*
	Salmonella	1	-
	Shigella	1	-

* indeterminate result

The antibody specifically recognized eight O157:H7 clinical isolates and six non-pathogenic O157 strains, as expected, and borderline reacted with 0165:H2. These strains would require genetic or other confirmatory testing for correct diagnosis. *Salmonella* and *Shigella* were non-reactive. We performed EIAs using MacConkey both cultures of 300 normal stools with no detectable positives. This suggests non-pathogenic O157 strains are infrequent.

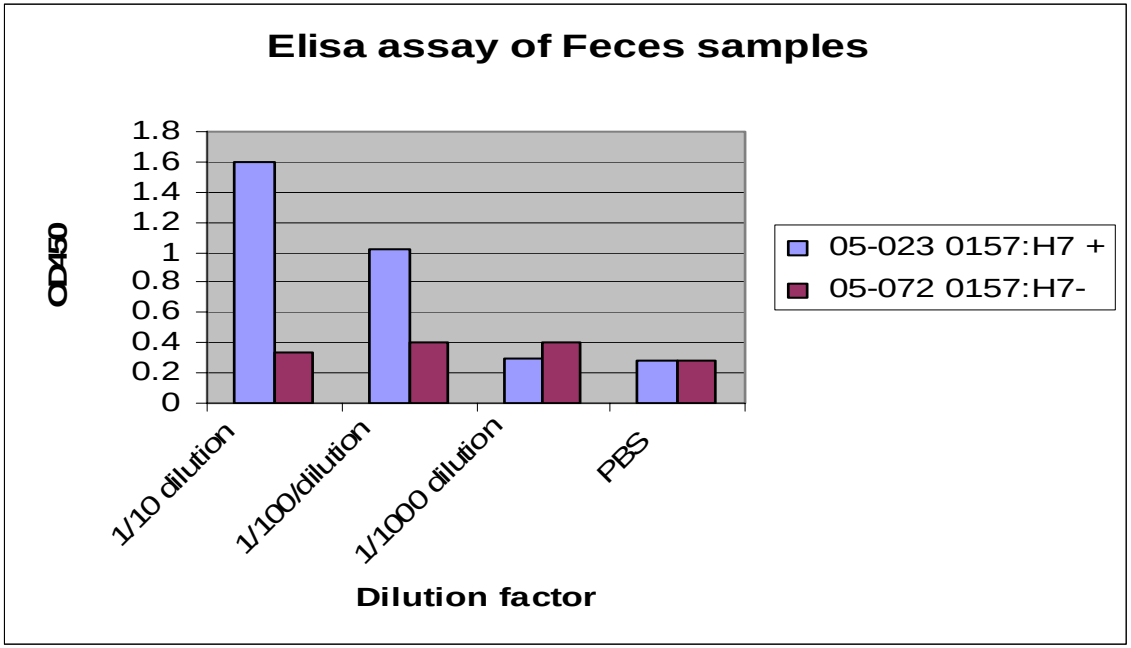

Figure 3 : EIA used to confirm positive clinical specimens.

III. Magnetic bead capture using the microfluidic bead mixing and capture circuit

Initial experiments confirmed that magnetic beads on a DEC card captures 10^4 to 10^8 CFU of *E. coli* (JG, check for itals throughout)O157:H7 from broth cultures (Figure 4). Following NA preparation and elution, the 10^4 CFU number corresponds to only 250 CFU introduced into the PCR reaction. O157:H7 was then spiked into both positive and negative stool specimens to determine if the specimen matrix inhibits bead binding and recovery, or if contact with feces inhibits PCR (TABLE III). For these experiments, normal and O157:H7 positive stools diluted 1:100 were spiked with 10 μL of overnight cultures of O157:H7 to verify the protocols for magnetic bead capture and real time PCR as a measure of recovery efficiency. No inhibition was observed. Finally, six O157:H7 positive clinical specimens and six negative feces were processed using the magnetic bead capture lab card (Figures 5,6); all positives and negatives were confirmed. The lab card displayed a two to three CT value shift compared to off-card capture, indicating its higher efficiency.

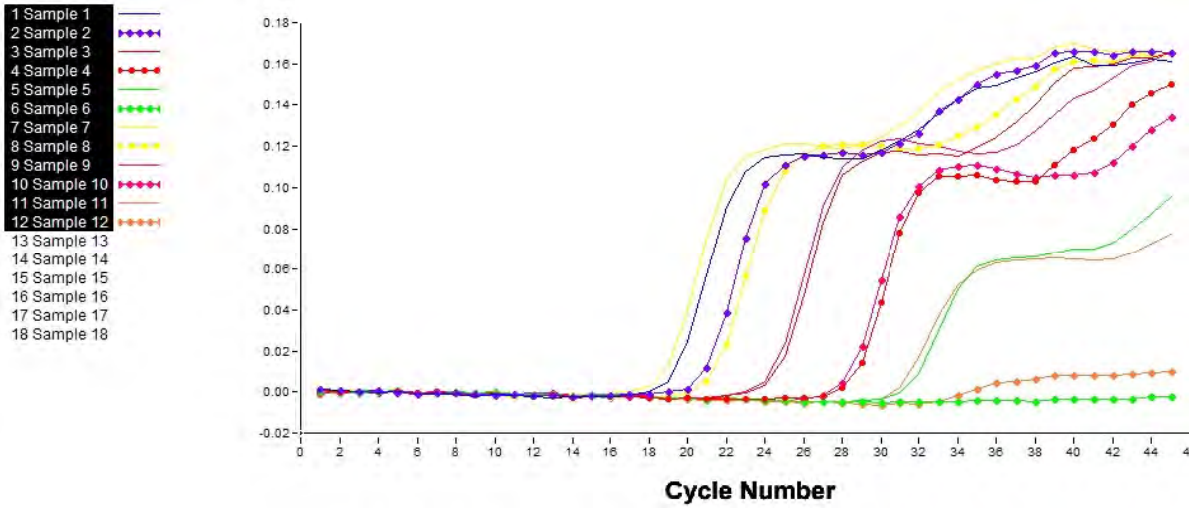

Figure 4 : Limit of Detection for lab card following lab card capture as detected by real time PCR. Duplicate curve sets from left to right represent O157:H7 CFUs of 10^8, 10^7, 10^6, 10^5, 10^4, and negative control.

TABLE III : O157:H7 recovery following CFU spike into feces.

Experiment	Crossing value (ct)
Positive control (direct extractions of CFUs)	20.5
Negative control (PBS)	neg
PBS plus CFU spike	18.52
PBS plus CFU spike	20.62
Normal stool 05-025	neg
Normal stool 05-025	neg
Normal stool 05-025 plus CFU spike	18.74
Normal stool 05-025 plus CFU spike	18.49
Positive stool 05-023	26.04
Positive stool 05-023	25.99
Positive stool 05-023 plus CFU spike	18.51
Positive stool 05-023 plus CFU spike	19.60

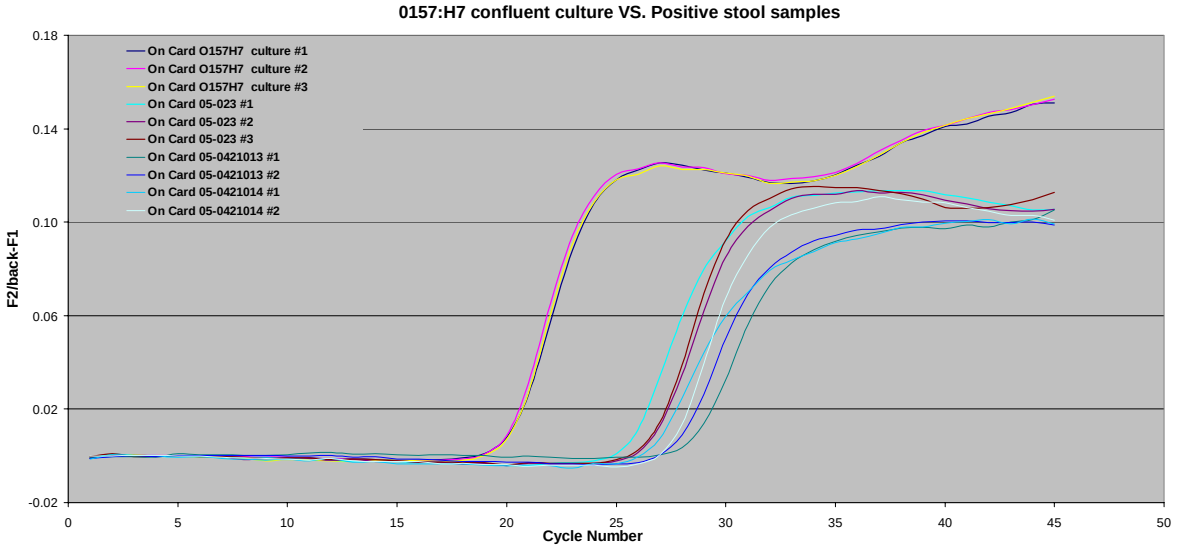

Figure 5 : Real time PCR results for O157:H7-containing feces after magnetic bead capture and mixing on lab card.

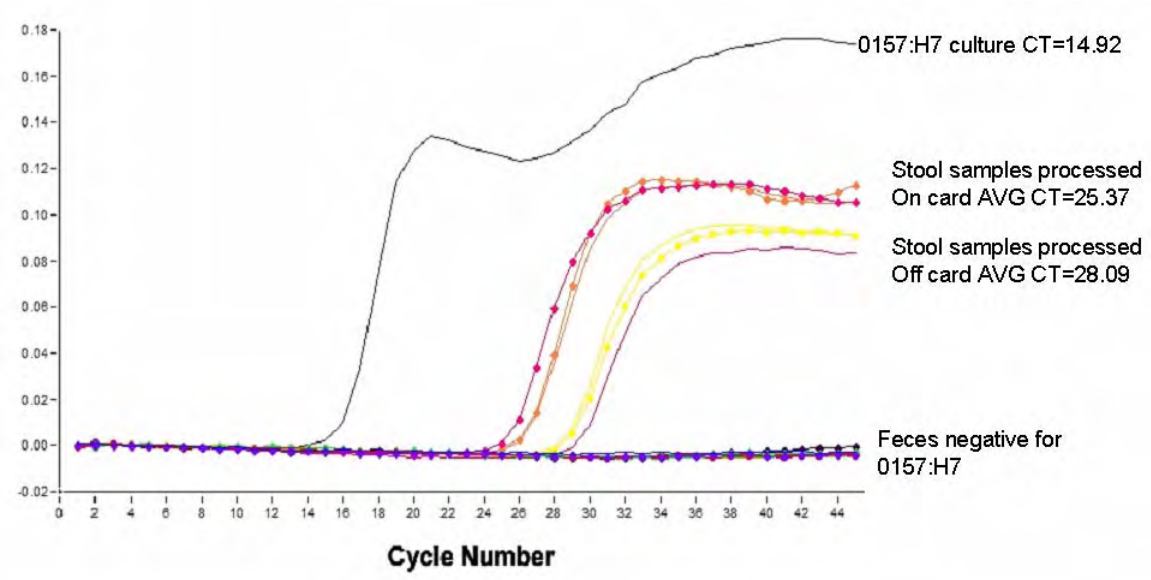

Figure 6 : Direct comparison of manual in tube magnetic bead capture method to microfluidic magnetic capture and mixing on card.

CONCLUSION

- **Anti-O157 distinguishes *E. coli* expressing this LPS from others, but does not differentiate pathogenic from non pathogenic *E. coli* O157; complementary analysis using NA testing must be employed**
- **Anti *E. coli* O157 antibody coated magnetic beads efficiently capture as few as 10^4 CFU O157:H7 from culture broth**
- **Anti *E. coli* O157:H7 antibody coated magnetic beads efficiently capture O157:H7 from naturally infected human feces.**
- **There is no signal from a limited panel of negative specimens.**
- **Infected fecal matter is more efficiently analyzed by the Micronics Microflow apparatus and DEC card than by off-card processes.**

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