Research Article

Evaluation of the Ability of Anti-Lipopolysaccharide Aptamers to Discriminate Various Pathogenic *E. coli* Bacteria

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Abstract | Lengthy 200 base DNA aptamers were developed against extracted lipopolysaccharides (LPS) of *E. coli* O157 and the so called "Big six" non-O157 Shiga-toxin producing *E. coli* (STEC) serovars (O26, O45, O103, O111, O121 and O145). These aptamers were assessed by enzyme-linked aptamer sorbent assay (ELASA) to rank their relative affinities and discriminatory ability for distinguishing between each of the STEC serovars. The most specific biotinylated aptamers from the ELASA screening were then attached to streptavidin-coated magnetic beads (SAv-MBs) and used to capture their cognate and related *E. coli* serovars. Capture was assessed by fluorescence microplate assessment following acridine orange staining. While there was significant cross-reactivity between the various captured serotypes, each of the anti-LPS aptamer candidates showed a preference for its cognate *E. coli* serotype. Results suggest that these aptamers may be useful for enriching specific *E. coli* pathogens during aptamer-magnetic separation in food samples, leading to a greater ability to detect these serotypes if coupled with genetic identification techniques such as PCR or gene probe hybridization.

Editor | Dr. Tarun Kumar Sharma, Centre for Biodesign and Diagnostics, NCR Biotech Science Cluster, 3rd Milestone, Faridabad-Gurgaon Expressway, Faridabad, India.
Received | November 29, 2016; **Accepted** | January 09, 2017; **Published** | January 15, 2017
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Citation | Bruno, J.G. 2017. Evaluation of the ability of anti-lipopolysaccharide aptamers to discriminate various pathogenic *E. coli* bacteria. *Aptamers and Synthetic Antibodies,* 3(1): 55-63.
Keywords | Acridine Orange, Aptamer, Big 6 *E. coli*, Lipopolysaccharide, Magnetic separation, O157, STEC

Introduction

Although originating in the 1930's with the seminal work of Rebecca Lancefield, to this day serotyping is important for identification and classification of pathogenic microbes. In the case of *Escherichia coli* and other Gram negative bacteria, the outer O antigens of lipopolysaccharide (LPS) are the main targets for serotyping, because these repeating oligosaccharides trigger strong immune responses and lead to discriminatory antibodies (DebRoy et al., 2011). While the numerous types of O antigens (at least 186 among *E. coli* serovars to date, Fratamico et al., 2016) have traditionally led to specific serotype discrimination, their complex and sometimes branching sugar structures (Stenutz et al., 2006) are daunting targets for specific antibody or aptamer development. In addition, some O antigens have been shown to be identical or very similar between *E. coli* and related species (Kenyon et al., 2011; Samuel et al., 2004) making aptamer-based discrimination even more challenging.

The United States Department of Agriculture (USDA) has traditionally recognized *E. coli* O157:H7 as the primary threat for testing in various foods, because it appeared to be the most prevalent serotype

which expressed hemorrhagic Shiga toxins or Vero toxins leading to bloody diarrhea and hemolytic uremic syndrome with possible kidney failure. However, in recent years, the USDA has acknowledged and now tests for six more *E. coli* serotypes which often appear to be correlated with Shiga toxin expression and severe disease induction in humans. These "Big 6" non-O157 Shiga-toxin producing *E. coli* or "STEC" are serovars O26, O45, O103, O111, O121 and O145 (Beier et al., 2016; Bosilevac and Koohmaraie, 2012; Kalchayanand et al., 2012). Although, other *E. coli* serotypes such as O104 from the 2011 Hamburg Germany outbreak have been documented to cause severe disease as well (Tahden et al., 2016) and may be added to the foodborne pathogen "watch list" in the future. For now, the USDA tests for the Big 7 (Big 6 plus O157:H7) and the presence of Shiga toxin genes or Shiga toxin production to identify foodborne threats from *E. coli*. Hence, the present report focuses on aptamer development against these Big 7 serotypes and the ability of the lead aptamer candidates to actually discriminate the complex LPS O antigens of these various serovars.

Traditionally, most aptamers have been of 100 bases or less in length, largely due to the difficulty in obtaining good yields for oligonucleotides greater than 100 bases long (Bruno, 2013). However, with the advent of better chemical synthesis techniques leading to routine synthesis of longer oligonucleotides, aptamers of 200 base lengths were developed with the intention of better emulating the very specific 110 amino acid complementarity determining region (CDR) of antibody heavy and light chains (Bruno, 2013). CDRs contain three hypervariable binding regions each which enable multi-epitope (multivalent) binding and enhanced specificity due to the low probability of binding three different epitopes with the same chain. Likewise, longer 200 base multivalent aptamers are likely to be able to bind multiple different epitopes on complex targets such as LPS O antigens, thus enhancing aptamer specificity for *E. coli* serotyping versus shorter aptamers which might only bind one or two different epitopes on LPS (Bruno, 2013). Thus, with the exception of the pre-existing 72-base O157 aptamer (Bruno and Richarte, 2015), longer 200 base aptamers were developed. These longer aptamers were developed to aid the stated objectives of this work which are to determine and enhance the level of discrimination between the Big 7 STEC serovars by the candidate aptamers as assessed by ELASA (ELISA-like plate assay) and a fluorescence microplate cell capture assay using the quantitative nucleic acid-specific fluorescent dye acridine orange (AO).

In addition to antibody and aptamer diagnostic utility against the outer O antigens, since lipid A of LPS is a potentially lethal endotoxin, the ability to specifically bind up or scavenge LPS or its endotoxin component could be an effective therapeutic approach. Several groups have shown that experimental LPS vaccines (Cross, 2014; Poolman and Wacker, 2016; Szu and Ahmed, 2014) and passive anti-LPS therapy with antibodies (Skinner et al., 2015) or aptamers (Ding et al., 2009; Wen et al., 2009) are promisingly effective treatments for endotoxins emanating from Gram negative sepsis. Thus, while the primary objective of the work presented herein is of a diagnostic nature, the same aptamers may also have therapeutic value in cases of life-threatening Gram negative septic shock in the future.

Materials and Methods

Bacteria, Culture Conditions and LPS Extraction

All bacteria were obtained from American Type Culture Collection (ATCC, Manassas, VA). In particular, the bacterial species were: *Escherichia coli* serotypes O26, O45, O103, O111, O121, O145, O157:H7 and *Salmonella enterica* ATCC 13311. All bacterial species were cultured on blood agar plates at 35°C overnight. Bacteria were washed from the blood agar plates in 2 ml of sterile phosphate buffered saline (PBS) and equalized to an absorbance reading of approximately 1.0 at 600 nm in a spectrophotometer. Bacteria were then pelleted by centrifugation at 14,000 x G for 5 min and the supernatant fluid was removed by gentle suction. Bacterial pellets were then extracted to liberate their LPS using an Intron Biotechnology kit (Boca Scientific, Inc., Boca Raton, FL) according the manufacturer's instructions. Crude LPS extracts were washed in 1 ml of 70% ethanol, pelleted at 14,000 X G for 5 min, followed by siphoning of the 70% ethanol and complete drying. The dried LPS pellets (~ 100 µg each) were then dissolved in 1 ml of sterile PBS and stored at 4°C.

Polyacrylamide Gel Electrophoresis (PAGE) of LPS and Silver Staining

The various *E. coli* LPS samples (10 µl or ~ 1 µg) were mixed with 30 µl or 4X Bolt® SDS-PAGE loading buffer (Thermo-Fisher Scientific, Pittsburg, PA). The

40 µL samples were loaded into the wells of 12% Bolt® Bis-Tris Plus polyacrylamide gels and electrophoresed at 125V for approximately 1 h until the dye front reached the bottom of the gel. The gel was then removed, washed briefly in deionized water and silver stained using a SilverQuest™ staining kit (Invitrogen, Thermo-Fisher Scientific) according to the manufacturer's instructions.

LPS-Magnetic Bead Conjugation and Aptamer Development

Extracted LPS was conjugated to amine-coated 2.7 µm diameter magnetic beads (Dynal M-270 amine-MBs, Thermo-Fisher Scientific) by a reductive amination process (Bruno et al., 2008). Ten µg of $NaIO_4$ was added to 100 µl (10 µg) of each LPS solution for 30 min at room temperature (RT). Two hundred µl of amine-MBs (~ 2×10^7 MBs) were washed twice in PBS and added to 100 µl of the $NaIO^4$-treated LPS. Next, 10 µL of a 5M sodium cyanoborohydride in 1M NaOH was added for 2 h at RT. The LPS–MBs were collected using a Dynal MPC® magnetic rack and 1 ml of sterile 0.1M ethanolamine was added in PBS for 15 min at RT with gentle mixing. Thereafter, the LPS-conjugated MBs were washed 4 times in sterile PBS by magnetic separation, resuspended in 1 ml of sterile PBS (~ 10^7 MBs/ml) and stored at 4°C until needed as targets for SELEX aptamer development.

DNA oligonucleotides were obtained from Integrated DNA Technologies (IDT; Coralville, IA). The SELEX template sequence was 5´-ATC CGT CAC ACC TGC TCT-$N_{36/164}$-TGG TGT TGG CTC CCG TAT-3´

Where; $N_{36/164}$ represents the randomized 36 or 164 base region of the DNA library in which dNTPs are mixed in equal molar ratios at each position to yield a 25% probability of each occurring at any position along the randomized stretch. In most cases, the randomized region was 164 bases, thus yielding a 200 base total length for the aptamers, while in one older case for O157 LPS, the randomized 36 base region led to a total length of 72 bases for the lead aptamer candidate. Primer sequences were 5´-ATA CGG GAG CCA ACA CCA-3´ (forward) and 5´-ATC CGT CAC ACC TGC TCT-3´ (reverse) to prime the template and nascent strands, respectively. Ex Taq™ DNA polymerase for SELEX was from Takara Bio (Japan).

The MB-based SELEX method of Bruno and Kiel (2002) was used with LPS-coated MBs except that PCR conditions were: initial denaturation at 96°C for 5 min, 20 cycles of 96°C for 1 min, 53°C for 1 min, and 72°C for 1 min, and a final extension at 72°C for 7 min. Ten rounds of SELEX were accomplished for each of the different *E.coli* LPS targets with ethidium bromide stained gel electrophoresis of the correct 72 or 200 base DNA aptamer products to validate each round of SELEX. Round 10 aptamers were cloned into chemically competent *E. coli* using a Lucigen (Middleton, WI) GC cloning kit followed by delivery of white colonies to SequeTech, Inc. (Mountain View, CA) for proprietary high GC content DNA sequencing.

ELASA Screening for Cross-Reactivity Analysis

One hundred µl or ~ 10^5 of each of the live Big 7 *E. coli* or *Salmonella enterica* cells in 0.1 M $NaHCO_3$ (pH 8.5) were added to the wells of flat-bottom polystyrene 96-well plates (Greiner Bio-One GmbH, Frickenhausen, Germany). The plates were covered and left to stand overnight at 4°C. The following day, plates were decanted and washed 3 times with gentle mixing for 5 min per wash using 200 µl of PBS per well. Wells were then blocked with 150 µl of 2% ethanolamine in 0.1 M $NaHCO_3$ for 1 h at 37°C followed by 3 more washes with 200 µl of PBS as before. The 5'-biotinylated lead candidate Big 7 STEC aptamers shown in Figure 1 were applied to the microplate wells at 1 nanomole per well for 1 h at room temperature (RT ~ 25°C) with gentle mixing. The plates were decanted and washed 3 times in 200 µl of PBS for at least 5 min per wash with gentle mixing. One hundred µl of a 1:5,000 dilution of streptavidin-peroxidase from a 1 mg/ml stock solution (Thermo-Fisher Scientific, Product No. 21126) in PBS was added per well for 30 min at RT with gentle mixing. The plates were decanted and washed 3 more times with 200 µl of PBS per well as before. One hundred µl of One-Component® ABTS substrate (Kirkegaard Perry Laboratories, Inc., Gaithersburg, MD) which had been equilibrated to RT was added to each well and incubated for 15 min at RT. Reactions were halted by addition of 100 µl of 1% SDS as the strongest reactions approached an absorbance of 1.2 at 405 nm using a Thermo Electron MultiSkan™ microplate reader (Thermo-Fisher Scientific; Waltham, MA).

Secondary Structure Analyses

Lead candidate aptamer DNA sequences were subjected to free energy minimization secondary stem-loop

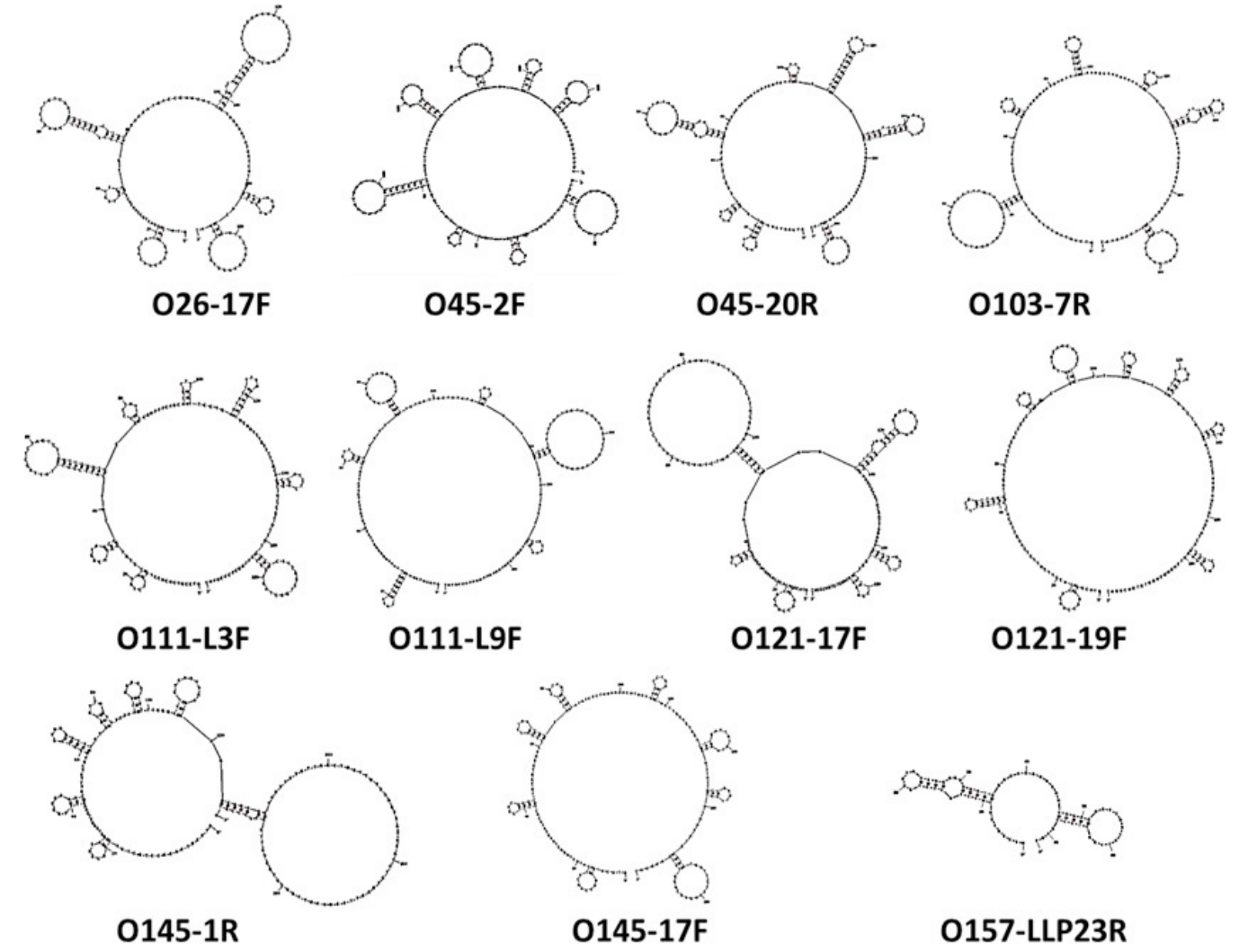

Figure 1: *Secondary stem-loop structures of the leading candidate 200 base DNA aptamers against each of the "Big 6" STEC and the structure for the best 72 base O157 aptamer using UNAFold software*

analyses to determine if any structural commonalities could be recognized. Aptamer DNA sequences were entered into the free web-based UNAFold software program (http://www.idtdna.com/Unafold/) using DNA parameters with 145 mM sodium concentration at 25°C.

Aptamer-Coated Magnetic Separation, Microscopy and Fluorescence Microplate Assessment

Lead DNA aptamers against the Big 7 STEC were synthesized by IDT with 5'-biotin linkers. Each of these aptamers was bound to streptavidin (SAv)-coated M-270 Dynal MBs from Thermo-Fisher Scientific and washed 3 times on an MPC® magnetic rack in sterile PBS. To qualitatively determine if STEC bacteria were being captured, the washed aptamer-MBs (~ 10^5 MBs) were resuspended in 100 µl of PBS and added to 100 µl of each Big 7 *E. coli* bacterial serotypes at ~ 10^4 cells per ml in a microfuge tube. One µl of 2% acridine orange (AO, Sigma-Aldrich Co., St. Louis, MO) was added to each microfuge tube to stain the bacteria and the tubes were mixed gently at RT for 20 min followed by 3 x 1 ml washes in PBS on the MPC® magnetic rack as before. The captured bacteria were resuspended in 200 µl of PBS. An aptamer deletion control consisting of the SAv-MBs without any aptamer but with *E. coli* O157:H7 was also conducted and stained with AO to assess the level of non-specific binding of bacteria to the SAv-MBs. The contents of each tube (200 µl) were added to separate wells of black microtiter plates for fluorometric analysis. A Cary-Varian Eclipse spectrofluorometer equipped with a plate reader was excited at 490 nm and set to read emissions at 530 nm ± 5 nm for AO with a 0.1 sec read time and photomultiplier tube (PMT) setting of 1,000V. Following fluorometric analyses, the contents of several capture wells were microscopically examined using an Olympus BH-2 fluorescence microscope under brightfield illumination and with an FITC (fluorescein/green) filter cube.

Results

Figure 1 gives the secondary stem-loop structures of each of the 200 base lead candidate aptamers for each or the Big 6 non-O157 STEC and the one older 72 base lead aptamer for detecting *E. coli* O157 LPS. While no clear secondary structural similarities are evident among these lead aptamers, the structural data reflect highly complex stem-loop structures, especially among the 200 base aptamers, which probably attest to the complexity of specific binding to the highly variable and branching Big 7 LPS O antigens.

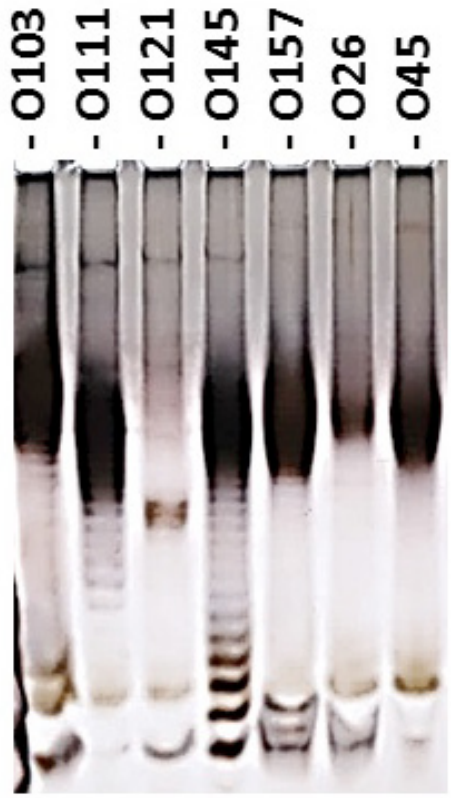

Figure 2: *Silver-stained 12% polyacrylamide electrophoretic gel showing the different banding patterns of extracted LPS from the "Big 7" Shiga toxin-producing E. coli (STEC) pathogens*

Table 1 summarizes the average absorbance values at 405 nm (A_{405nm}) of 3 independent experiments for assessment of specificity (cross-reactivity) of each lead aptamer versus the other Big 7 *E. coli* and *Salmonella enterica* 13311. Many more preliminary ELASA analyses were performed (data not shown for brevity) to narrow the field of candidate aptamers to these few best lead candidates. It is clear from Table 1 that each of these lead aptamers shows some degree of preference for binding its cognate LPS target (bolded values in the table) versus binding and detection of the related *E. coli* serovars or *Salmonella enterica*.

Figure 2 presents results of a silver-stained 12% polyacrylamide electrophoresis gel containing each of the Big 7 STEC LPS extracts. The stained gel clearly shows banding patterns consistent with extracted LPS as reported frequently in the literature, thereby proving that high quality LPS was available for attachment to MBs and use as targets for SELEX aptamer development.

The general ability Big 7 aptamers to capture live STEC cells on the surface of aptamer-coated MBs is demonstrated in Figure 3. Panel A shows the typical appearance of possible *E. coli* bacteria adhering to the surface of the aptamer-MBs when using brightfield microscopy (600X total magnification), but some of these adherent particles could be debris as well. Figure 3B, however, strongly suggests that these adherent particles are in fact live *E. coli* cells because they fluoresce with the characteristic green emission associated with AO staining.

Table 1: *Summary of ELASA Cross-Reactivity Studies*

	Leading Candidate Aptamer										
***E. coli* seorvar**	**O26-17F**	**O45-2F**	**O45-20R**	**O103-7R**	**O111-L3F**	**O111-L9F**	**O121-17F**	**O121-18F**	**O145-1R**	**O145-17F**	**O157-LL-P23R**
O26	**0.910**	0.813	0.860	0.728	0.614	0.724	0.617	0.927	0.965	0.570	0.209
O45	0.884	**1.168**	**1.020**	0.753	0.877	0.773	0.637	0.955	1.086	0.653	0.251
O103	0.544	0.929	0.593	**0.908**	0.771	0.742	0.578	0.641	0.663	0.554	0.368
O111	0.518	0.890	0.578	0.795	**1.200**	**1.202**	0.656	0.564	0.994	0.550	0.372
O121	0.325	0.843	0.463	0.514	0.682	0.688	**1.091**	**1.230**	0.701	0.434	0.399
O145	0.403	0.856	0.582	0.733	0.915	0.697	0.778	0.546	**1.281**	**0.999**	0.581
O157	0.472	0.985	0.659	0.766	0.737	0.801	0.538	0.674	1.048	0.630	**1.370**
Salmon. enterica	0.563	0.685	0.653	0.731	0.693	0.733	0.359	0.831	0.806	0.406	0.818

Note: *Absorbance values represent the means of 3 independent experiments*

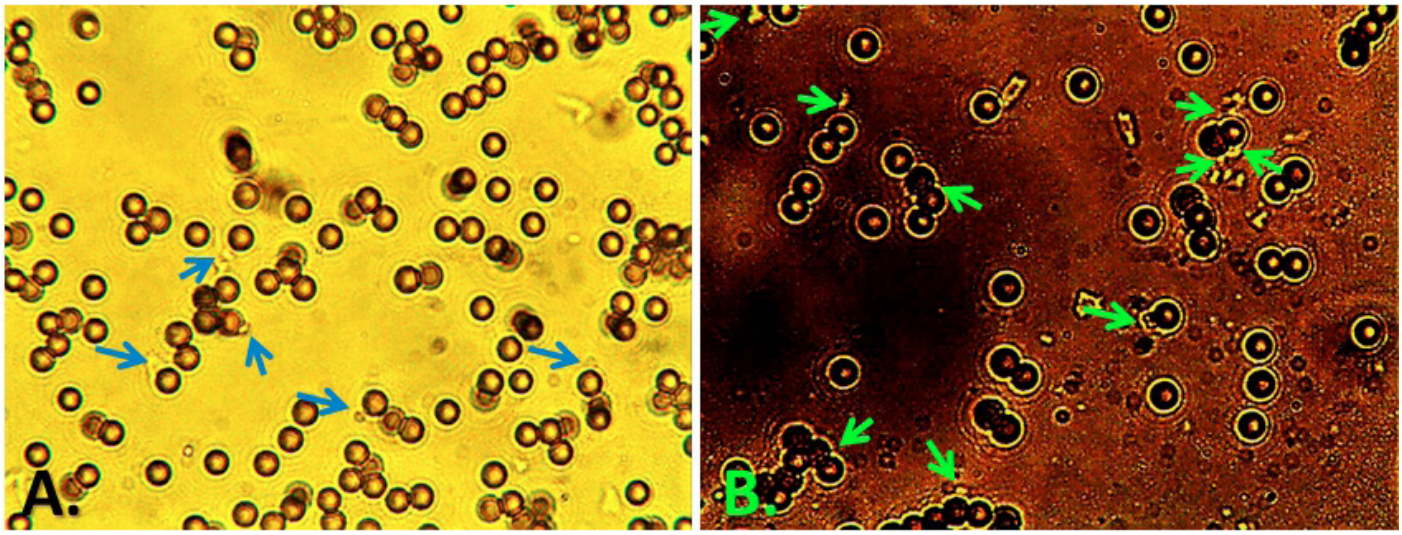

Figure 3: *Typical 600X microscopic images of captured acridine orange (AO)-stained Big 7 E. coli bacteria on aptamer-coated magnetic beads (MBs) seen under* **A)** *brightfield and* **B)** *fluorescence illumination using an FITC (fluorescein) optical cube. Arrows point to captured E. coli bacteria on the MB surfaces*

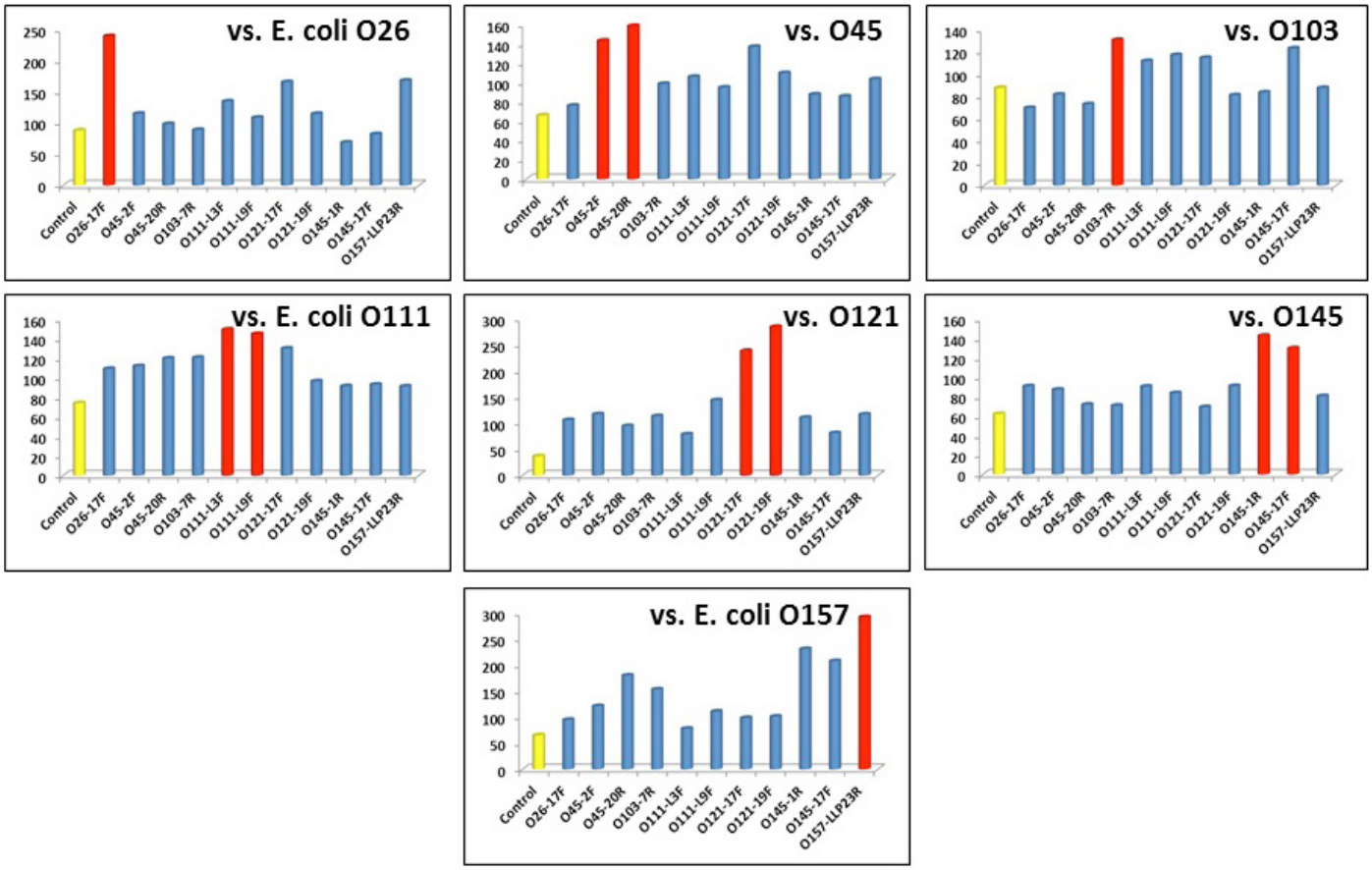

Figure 4: *Results of fluorescence microplate-based aptamer-MB capture cross-reactivity experiments. X-axes list the type of aptamer-coated MBs used to capture the added E. coli serovar cells listed in the upper right corner of each panel (e.g., vs. E. coli O26, etc.). Y-axes give the relative fluorescence intensity of each microwell measurement. Yellow bars represent the negative control (evaluation of AO-stained E. coli O157 non-specific adherence to SAv-MBs) fluorescence intensity values in each graph. Note that despite significant cross-reactivity between serotypes, the aptamers captured their cognate serotypes best (red bars), suggesting some utility for enrichment of specific serotypes from food samples followed by specific PCR or other genetic detection techniques*

The quantitative AO fluorescence results of each of the Big 7 STEC cells per each panel versus each type of aptamer-coated-MB capture (along each of the x-axes) in black microwell plates are given in Figure 4. While there is significant cross-reactivity based on the high AO fluorescence signals from interaction and binding of some aptamer-MBs with particular Big 7 serotypes, it is clear that the strongest fluorescence

signals for detection of each serotype (red bars in Figure 4) resulted from the aptamer-MBs binding their cognate serotype targets. Therefore, one would expect that the aptamer-MBs might be useful for enriching food samples for the targeted Big 7 serotypes and if so, genetic tests such as PCR or gene probe hybridization could confirm the presence of one or more Big 7 serotypes in food samples following aptamer-MB capture and enrichment.

Discussion

In summary, the present work illustrates that, while not perfect, aptamer-coated SAv-MBs may be useful for enrichment of the various pathogenic "Big 7" *E. coli* serotypes. Table 1 and Figure 4 suggest that while significant cross-reactivity exists, each aptamer demonstrated a noteworthy preference for the type of LPS against which it was developed. These results should perhaps be expected, because LPS is such a complex polymer composed of a core region bound to lipids and complex repeating and branched oligosaccharide areas (O antigens). In addition, it has been shown that some different *E. coli* serotypes express the exact same O antigens (Samuel et al., 2004), making highly specific LPS discrimination by antibodies or aptamers extremely difficult.

Given that whole LPS was immobilized onto amine-derivatized MBs for aptamer selection in this project, it is possible that some of the candidate aptamer sequences were directed against the LPS core or fatty acids instead of the O antigen saccharides. Many of the O antigen sugars were covalently bonded to the amino-MBs via reductive amination (Gildersleeve et al., 2008) and were therefore perhaps sterically hindered and less available for interaction with the random aptamer library. In addition, reductive amination has other drawbacks for immobilization of LPS such as opening of the sugar rings (Gildersleeve et al., 2008) and the formation of insoluble fatty aggregates (Cox et al., 2010). Alternative LPS immobilization strategies including maleimide-thiol linkage (Cox et al., 2010) and Mannich formaldehyde condensation (Ünlü et al., 2015) from any abstractable hydrogen in the LPS structure promise to be more efficient and facile while leaving the oligosaccharide sugar rings intact for presentation of a more native and true LPS structure on amine-conjugated MBs. In the future, it would be optimal to select aptamers against the Big 7 O antigens themselves bound to amine-MBs by the Mannich reaction. Ding et al. (2009) used hydrophilic and hydrophobic microtiter wells for oriented immobilization of LPS to develop aptamers against the accessible O antigens or lipid ends of LPS. Still, despite the molecular "handicap" of developing aptamers against relatively unoriented whole LPS immobilized on amine-MBs by reductive amination, the present aptamers were screened against whole Big 7 *E. coli* cells by ELASA in which the outward facing and accessible O antigens of LPS were presented to the candidate aptamers, thus any LPS core- or fatty acid-binding aptamer candidates were probably screened out, leading to the vetted final set of O antigen binding aptamers shown in Table 1 and Figure 1. It is also interesting to note that while longer 200 base aptamers were originally thought to perhaps perform better for discrimination of the complex *E. coli* O antigens, the shorter 72 base aptamer previously developed against *E. coli* O157 (designated O157-LLP23R in Figure 1) and reported by Bruno and Richarte (2015) performed equally well for discrimination of its target bacteria (Table 1 and Figure 4). This fact may attest to the notion that as long as an aptamer has a unique or specific epitope to bind in a complex structure, it will be highly specific in its discriminatory performance regardless of length of the aptamer or the lack of "multivalence".

In the final analysis, a small family of Big 7 LPS O antigen-binding aptamers were selected (Figure 1) which appear to prefer their cognate serotypes (Table 1 and Figure 4). While these aptamers may not be ideal for single step assays requiring high specificity such as lateral flow test strips (Bruno and Richarte, 2015), they may be better suited for capture, concentration and enrichment roles in multi-step assays which culminate in genetic analysis for confirmation of captured bacteria. Other researchers (Fischer et al., 2015; Hünninger et al., 2015; Ozalp et al., 2014; Suh and Jaykus, 2013; Suh et al., 2014) have already shown that aptamer-coated MBs are very useful for enrichment of target pathogenic bacteria in food samples followed by confirmatory PCR of genes contained in the captured bacteria.

Conclusions

While not entirely specific for their intended Big 7 STEC targets, the present work describes aptamers which prefer binding to their cognate *E. coli* serotypes. Thus, these aptamers may have utility when coupled

to MBs and allowed to capture and concentrate (enrich for) specific STEC serovars in food samples. If coupled to confirmatory genetic analysis techniques involving PCR primers or other genetic probes, the aptamers may play a key "immunomagnetic-like" enrichment role to enhance the efficacy of PCR and other genetic assays for the food safety testing industry.

Acknowledgments

Funding was provided by a USDA Phase 2 Small Business Innovative Research (SBIR) Grant No. 2015-33610-23787. The author expresses thanks to Ms. Alicia Richarte for the LPS extractions and conducting the SELEX aptamer development against various types of LPS.

Author Contributions

JGB conceived and conducted all reported experiments and wrote the manuscript.

Conflicts of Interest

The author owns 8% of OTC Biotechnologies, Llc. and Pronucleotein Biotechnologies, Inc. which intend to compete in the food safety testing industry using aptamer-based technologies in the future.

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