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In vitro selection of *Escherichia coli* O157:H7-specific RNA aptamer

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ABSTRACT

Escherichia coli (*E. coli*) O157:H7 is a major foodborne pathogen that causes life-threatening symptoms in humans worldwide. To rapidly and properly identify the pathogen and avoid its toxic effects, ligands which can directly and specifically bind to the virulent *E. coli* O157:H7 serotype should be identified. In this study, a RNA aptamer-based ligand which can specifically distinguish the pathogen *E. coli* O157:H7 from others was developed by a subtractive cell-SELEX method. To this end, an RNA library was first incubated with the *E. coli* K12 strain, and the RNAs binding to the strain were discarded. The precluded RNAs were then used for the selection of O157:H7-specific aptamers. After 6 rounds of the subtractive cell-SELEX process, the selected aptamer was found to specifically bind to the O157:H7 serotype, but not to the K12 strain. This was evidenced by aptamer-immobilized ELISA, real-time PCR analysis, or an aptamer-linked precipitation experiment. Importantly, the isolated RNA aptamer that distinguishes between the virulent serotype and the nonpathogenic strain specifically bound to an O157:H7-specific lipopolysaccharide which includes the O antigen. This novel O157:H7-specific aptamer could be of potential application as a diagnostic ligand against the pathogen-related food borne illness.

1. Introduction

Escherichia coli (*E. coli*) is a common and usually harmless microorganism found in the intestinal tract. It causes pathogenic effects in a weakened or immunosuppressed host. Especially, the *E. coli* O157:H7 strain, a type of the most frequently found enterohemorrhagic *E. coli* (EHEC), is one of the most dangerous food borne pathogens [1], and produces exotoxins known as verotoxins (also termed Shiga-like toxins). Verotoxins play a major role in pathogenesis through cytopathic effects on vascular endothelial cells, kidneys, intestines, the central nervous system, and other organs [2]. These cytopathic effects of EHEC have been associated with several diseases, such as hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura [2,3]. This pathogen is abundant in undercooked or raw ground beef, milk, fruits and vegetables, and it can be easily spread through food and the use of common facilities. At least 20,000 cases of food poisoning caused by the *E. coli* O157:H7 strain occur in the US each year [4].

The traditional detection methods for a bacterial pathogen in foods depend on the enrichment of liquid samples at 35–37 °C for 18–24 h to increase the *E. coli* population to a detectable level [5,6]. However, the methods have drawbacks such as labor-intensiveness and a long analysis time. Noticeably, only a low infectious dose of the pathogen is needed to cause disease. Therefore, a method for sensitive, rapid on-site screening, as well as accurate identification of pathogens, is required for food safety and quality control. A number of new techniques have recently been developed to assay for bacterial pathogens; for example, DNA hybridization tests [7], PCR based methods [8], surface plasmon resonance measurements [9], electrochemilluminescent and cytometric bead array biosensors [10], and solid-phase enzyme-linked immunosorbent assays (ELISA) [11]. To increase sensitivity, selectivity, efficacy, speed, and accuracy for detection of pathogens, high affinity ligands which can directly and specifically recognize the *E. coli* pathogenic strain should be identified.

Aptamers are single-stranded DNA, RNA, or modified nucleic acids which can recognize target molecules such as small chemicals, proteins, and cells through folding into specific three-dimensional structures [12,13]. Aptamers with randomized oligonucleotide sequences can be obtained from a combinatorial library via an *in vitro* selection process known as the systematic evolution of ligands by exponential enrichment (SELEX) method [14,15]. Due to their high specificity and affinity, broader target range, chemical manufacturability, stability, easy discovery and modification, and lack of immunogenicity, aptamers have been considered as emerging ligands which can rival antibodies for diagnostics as well as therapeutics [16–18]. In this study, to isolate an *E. coli* O157:H7 strain-specific ligand, we established and employed a cell-based

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subtractive SELEX technology and identified an RNase-resistant RNA aptamer that specifically binds to the cell surface of O157:H7, and thus selectively distinguishes the pathogenic strain from other strains. Moreover, we characterized a target molecule on the surface of the *E. coli* O157:H7 strain that specifically binds to the RNA aptamer.

2. Materials and methods

2.1. Microorganisms and preparation of cells

E. coli O157:H7 (ATCC 43895) and K12 strain (ATCC 29425) were purchased from ATCC. For aptamer selection and characterization, *E. coli* O157:H7 or K12 strain (1×10^9 mL^{-1}) was incubated at 37 °C for 24 h in Brucella broth with 0.04% formaldehyde. Bacteria were then washed, resuspended in sterile phosphate buffered saline, and stored at 4 °C until use.

2.2. Selection procedure

An RNA library of $\sim 10^{14}$ different molecules was generated by *in vitro* transcription of synthetic DNA templates with 2′-deoxy-2′-fluoro CTP and UTP (Epicentre Technologies) and normal GTP, ATP, and T7 RNA polymerase, as described [19]. This modification of the 2′ position of RNA increased its stability in mammalian serum by >10,000-fold, when compared with unmodified RNA with the 2′-hydroxyl group [20,21]. The sequence of the resulting RNA library was 5′-GGGAUACCAGCUUAUUCAAUUN$_{60}$AGAUAGU AAGUGCAAUCU-3′, where N_{60} represents 60 nucleotides (nts) with equimolar incorporation of each base at each position.

Cell-based subtractive SELEX was designed to select nuclease-resistant RNA aptamers specific to the cell surface of the *E. coli* O157:H7 strain. To eliminate RNAs that bound nonspecifically to the cell surface, the RNA library (30 μg) was first incubated with 3×10^6 *E. coli* K12 cells at 25 °C for 10 min in 200 μl of a binding buffer (30 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM dithiothreitol, and 1% BSA) in the presence of 750 pmol of tRNA prior to each round of selection. The cell–RNA complexes were separated by centrifugation, and any RNAs bound to molecules on the *E. coli* K12 cell surface were discarded. The precleared supernatant RNA pools were incubated with the 1.0×10^6 target *E. coli* O157:H7 cells at 25 °C for 10 min using the binding buffer with tRNA. O157:H7–RNA complexes were separated by centrifugation, washed 5–7 times with 0.4 ml of the binding buffer, and resuspended in 100 μl TE buffer with 10 mM EDTA. The bound RNAs were recovered by heating at 80 °C for 5 min, followed by phenol/chloroform extraction. The RNAs were then amplified by RT-PCR with reverse (5′-AGATTGCACTTACTATCT-3′) and forward primer (5′-GGGTA ATACGACTCACTATAGGGATACCAGCTTATTCAATT-3′), transcribed, and utilized for the following selection rounds as previously described [22]. After 6 rounds of selection, the amplified cDNA was cloned and sequenced.

2.3. Aptamer-immobilized ELISA

The A(16) sequence was extended on the 3′ terminal of each RNA aptamer clone. Then, the A16-tailed RNA clones were hybridized with biotin-conjugated oligodT at RT for 30 min. Each biotin–RNA hybrid was incubated on a streptavidin-coated 96 well plate (Techno Plastic Products AG, Zollstrasse, Switzerland) at RT for 30 min. The wells were blocked with blocking buffer (PBS with 5% BSA/10 μg yeast tRNA). The *E. coli* O157:H7 strain (5×10^6 cells) was resuspended in binding buffer (0.05% Tween 20, 1.5 mM $MgCl_2$ in 1× PBS), and then incubated in each RNA clone-coated well at RT for 20 min. After incubation of each well with *E. coli* O157:H7 primary antibody (100 ng/well) (Abcam) at RT for 1 h, HRP-conjugated secondary antibody (100 ng/well) (Santa Cruz Biotechnology Inc.) was added at RT for 1 h, followed by addition of 100 μl of QuantaBlu peroxidase substrate solution (Thermo Scientific). Bound *E. coli* O157:H7 cells were measured using a fluorometer (Thermo Scientific, excitation/emission; 325/420 nm).

2.4. Real-time PCR analysis

After binding of *E. coli* O157:H7 or K12 strain cells (5×10^6 cells each) with aptamer clone RNA or library RNA (6 pmol each), bound RNA was extracted by heating at 80 °C and phenol/chloroform extraction, and reverse-transcribed with MMLV reverse transcriptase (Finnzymes Oy, Vantaa, Finland) using the reverse primer used for SELEX. Real time PCR was performed using the Rotor-Gene (Roter-gene 6000, Qiagen, Hilden, Germany) and EvaGreen™ PCR Core Reagents (SolGent Co., Ltd., Daejeon, South Korea), according to the manufacturer's protocol. Conditions for PCR were 95 °C for 30 s, 48 °C for 30 s, and 72 °C for 30 s, for 40 cycles with the reverse and forward primer utilized for SELEX. For standardization, a known concentration of RNA aptamers was amplified by the same method.

2.5. Aptamer truncation

We constructed 3 types of truncated aptamer I-1 by PCR and *in vitro* transcription; 'Two arm' (64 mer), 'Long arm' (38 mer), and 'Short arm' aptamer (28 mer). Primers for the PCR were as follows. 'Two arm'; forward (5′-GGTAATACGACTCACTATAGGGTC TTCCTGGACTGTCGAAAA-3′), reverse (5′-TTTTTTTTTTTTTTTTTACT ATCTATAAACCAAATACG-3′): 'Long arm'; forward (5′-GGTAATAC-GACTCACTATAGGGTCTTCCTGGACTGTCGAAAA-3′), reverse (5′-TTTTTTTTTTTTTTTTCCTCCCGATACTGAATTTTCGACAGTC-3′): 'Short arm'; forward (5′-GGTAATACGACTCACTATAGGGTACGTATTTGG TT-3′), reverse (5′-TTTTTTTTTTTTTTTTTACTATCTATAAACCAAATAC G-3′).

2.6. Aptamer-linked precipitation experiment

Minimized aptamer series were hybridized with biotin-conjugated oligodT. Each biotin–RNA hybrid was incubated with *E. coli* O157:H7 or K12 strain (5×10^6 cells each) at RT for 30 min, and centrifuged. Pelleted RNA-bacteria complexes were resuspended in streptavidin-HRP solution (Millipore, 1:1000), incubated at RT for 30 min, and then resuspended in 100 μl of QuantaBlu peroxidase substrate solution (Thermo Scientific). Binding levels of minimized aptamer series were measured using a fluorometer (Thermo Scientific, excitation/emission; 325/420 nm).

2.7. K_d analysis

Increasing concentrations of the 'Two arm' aptamer (5.5 nM to 1.36 μM) were incubated with *E. coli* O157:H7 or K12 strain (1×10^6 cells each) in the binding buffer at RT for 30 min. The amount of aptamers bound to the cells was assessed using quantitative RT-PCR analysis by real-time PCR (Roter-gene 6000, Qiagen, Hilden, Germany) with forward (5′-GGGTCTTCCTGGACTGTC-GAAAA-3′) and reverse primer (5′-ACTATCTATAAACCAAATACG-3′). Saturation curves were plotted based on the quantitative RT-PCR data and the dissociation constant of the aptamer was calculated by non-linear regression analysis.

2.8. Lipopolysaccharide (LPS) purification

A LPS extraction kit (Intronbio, Gyeonggi-do, South Korea) was used according to the manufacturer's protocol to purify the LPS of