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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

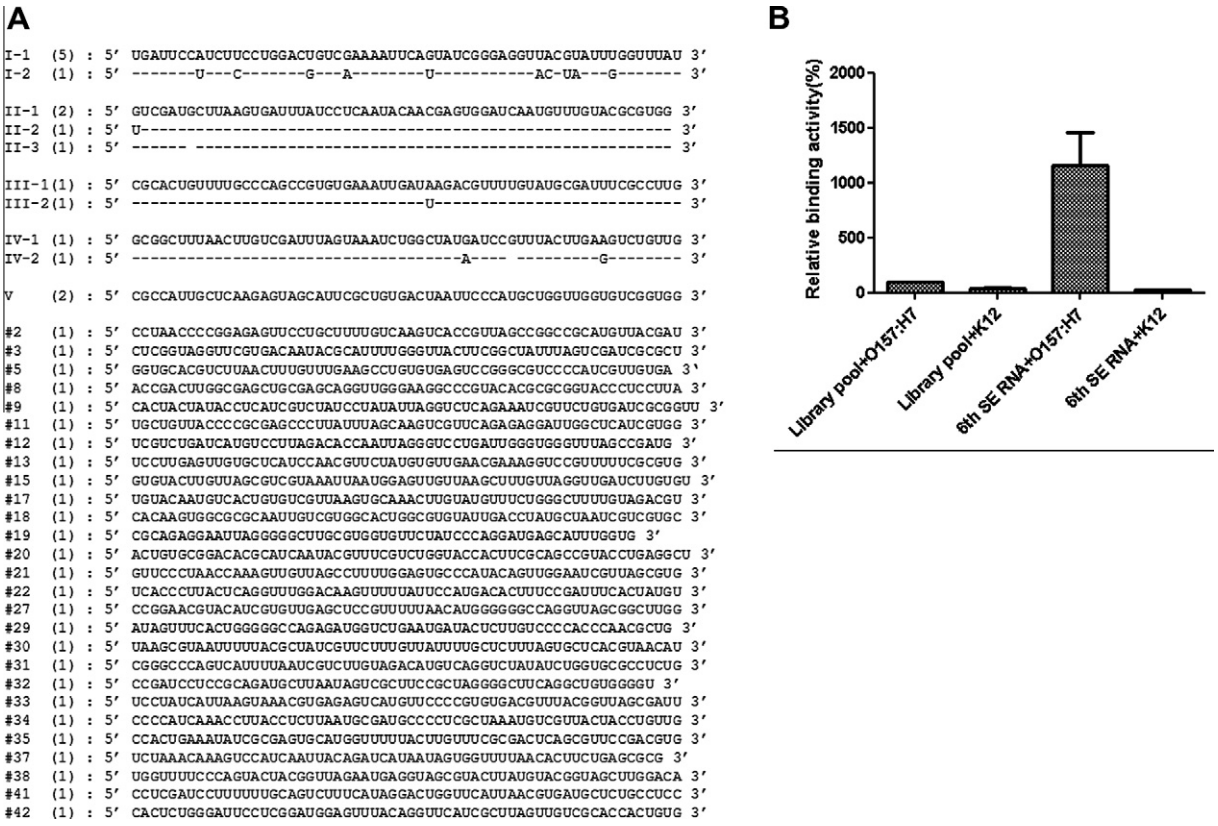

Fig. 1. Sequences and specificity of selected RNA aptamers against the O157:H7 strain. (A) Selected RNAs were found to encode 35 different RNA sequences. Several of the RNA sequences were present multiple times (numbers in parentheses). The lines drawn indicate that nucleotides found at these positions were identical. C and U in this figure correspond to 2′-fluoro C and 2′-fluoro U, respectively. (B) Library RNA or the RNA pool after the 6th round SELEX was incubated with *E. coli* K12 or O157:H7 strain. RNAs bound to each cell were then purified and amplified. The amount of real time RT-PCR products from the bound RNAs was quantified relative to the amount of library RNA pool bound to O157:H7. Averages of 3 independent measurements are presented with bars indicating standard deviations.

O157:H7 strain or K12 strain. Briefly, *E. coli* O157:H7 or K12 strain (1×10^9 cells each) was resuspended in 1 ml of lysis buffer, 200 μl of chloroform was added, and the mixture was vortexed vigorously for 10–20 s. The lysed cells were then centrifuged at 13,000 rpm for 10 min at 4 °C, and 400 μl of supernatant was transferred to a new tube. Purification buffer (800 μl) was added, incubated for 10 min at −20 °C, and centrifuged at 13,000 rpm for 15 min at 4 °C. The LPS pellet was washed with 1 ml of 70% EtOH, dried completely, and dissolved with 10 mM Tris–HCl buffer (pH 8.0).

2.9. LPS binding assay of aptamer

Test wells in a 96-well flat-bottom microtiter plate (Techno Plastic Products AG, Zollstrasse, Switzerland) were coated with 100 μl of purified LPS from O157:H7 or K12 strain (3 mg/ml in PBS) by incubation at 37 °C for 1 h and overnight at 4 °C. The plates were rewarmed at 37 °C for 1 h before use. The wells were treated with blocking buffer (PBS with 5% BSA and 10 μg of yeast tRNA) for 1 h at 37 °C, and a series of truncated RNA aptamer with biotin-conjugated oligodT were incubated in the plate coated with LPS at RT for 30 min. Streptavidin-HRP solution (Thermo Scientific, 1:1000) and 100 μl of QuantaBlu peroxidase substrate solution (Thermo Scientific) were then added to each well. The binding efficacies of RNA aptamers to the LPS were measured using a fluorometer (Thermo Scientific, excitation/emission; 325/420 nm).

3. Results

3.1. Selection of RNA aptamers

For the selection of RNA aptamers targeting a specific bacterial strain, we incubated an RNA library with the specific cells, followed by the extraction and amplification of specific RNAs bound to the cells using RT-PCR and *in vitro* transcription. Therefore, inherent cellular RNAs from naïve cells targeted in this study should not be amplified with primer sets used for the amplification of RNA aptamers. In contrast to cDNA detection from library RNA, no cDNA products were amplified with the primers from any bacterial cells (Supplementary Fig. 1).

To identify RNA aptamers specific to *E. coli* O157:H7, but not to other stains, we established a cell-based subtractive SELEX procedure, as described in Section 2. After 6 iterative cycles of the subtractive-SELEX, the amplified cDNAs were cloned, and 43 different clones were sequenced (Fig. 1A). RNAs with very diverse sequences were then identified. However, several RNAs had the same or very similar sequences and were found in multiple clones. Enrichment of RNA aptamers specific to the *E. coli* O157:H7 strain was confirmed using semi-quantitative real-time RT-PCR (Fig. 1B). The amount of RNAs that bound to the nonpathogenic K12 strain in the selected RNA pools decreased by up to 37%. In sharp contrast, the amount of RNAs that bound to the pathogenic *E. coli* O157:H7 strain was markedly increased by more than 11-fold in the selected RNA pools, compared with that of library RNA bound to the strain.

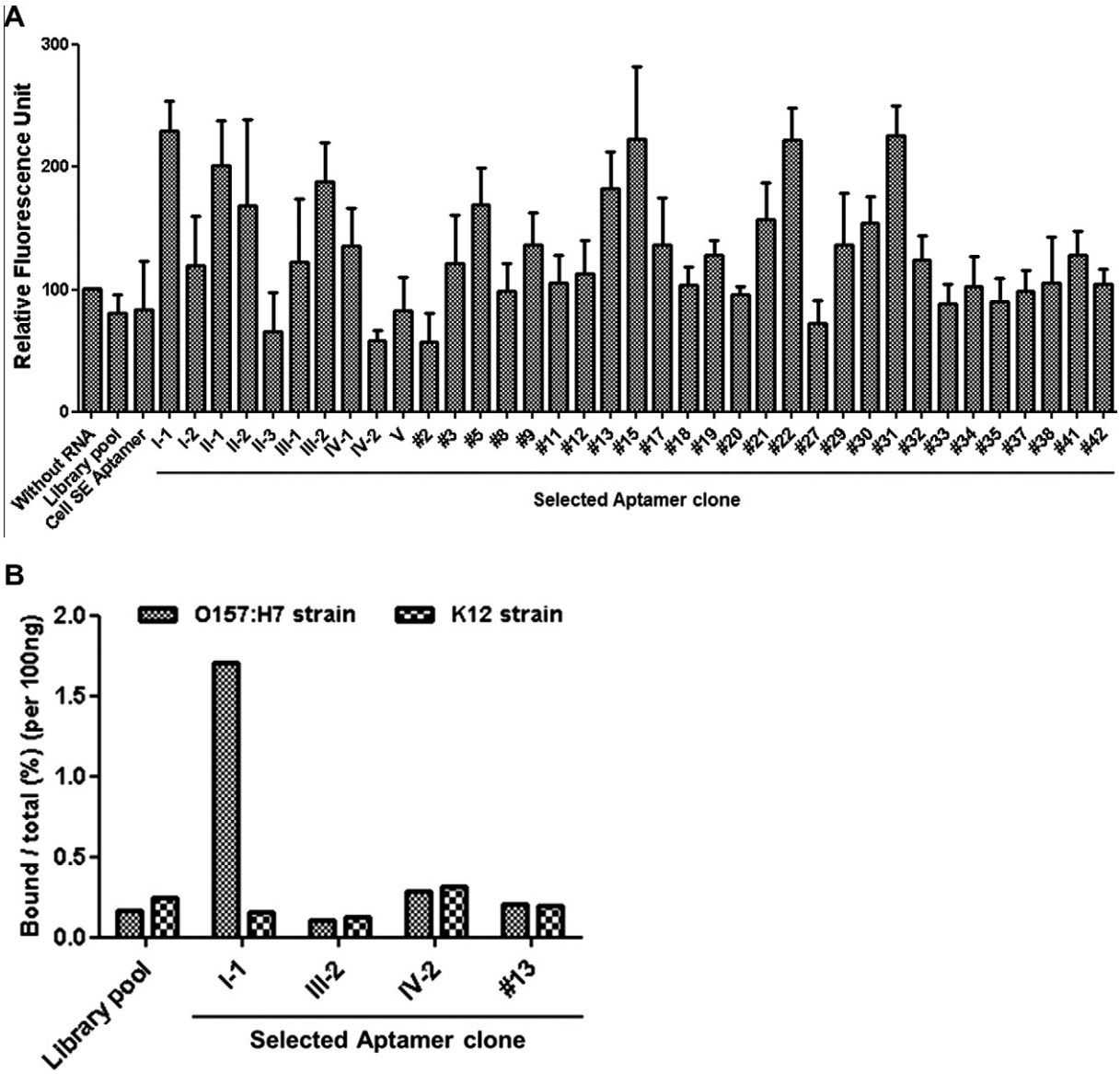

Fig. 2. Validation of binding specificity of selected RNA aptamer clones. (A) Screening of selected RNA aptamers which can bind to O157:H7 strain. Binding ability of each aptamer clone to a specific strain is presented as the relative fluorescence unit (%) to the sample without any RNA. Each value represents the average of 3 independent experiments with standard deviation. (B) Specific binding activity of RNA aptamer clones. Library pool RNA or RNA aptamers I-1, III-2, IV-2, #13 bound to each cell were eluted and amplified. The amount of real RT-PCR products from the bound RNAs was quantitated and expressed as bound RNA/total input RNA (%).

3.2. Screening of the selected RNA clones which can specifically bind O157:H7 strain

The aptamer-immobilized ELISA method described in Section 2 was used to screen and identify RNA aptamer clones that could bind to the pathogenic target strain. (Fig. 2A). The library RNA pool or RNA aptamer against Jurkat T cells (Cell SE aptamer) [19] was used as negative controls. As a result, aptamer clones I-1 and III-2 bound to the *E. coli* O157:H7 strain, 2.8- and 2.3-fold better, respectively, than the negative control RNAs. In addition, the binding capacities of several selected clones including #15, #22, and #31 were almost 2-fold higher than capacities of the negative controls.

After extraction of the RNAs bound to K12 or O157:H7 strain, quantitative real-time RT-PCR was employed to validate the binding specificity of the selected aptamer clones to the target strain (Fig. 2B). RNA aptamer clone I-1 specifically and efficiently bound to the *E. coli* O157:H7 strain, but not to the K12 strain, while no binding specificity was shown by other selected RNA clones (Fig. 2B).

3.3. Mapping the minimal binding domain of RNA aptamer I-1

On the basis of preliminary screening and binding confirmation, RNA aptamer I-1 was selected for further characterization. To determine the minimal region in the aptamer I-1 needed for binding to the *E coli* O157:H7 strain, full-length aptamer I-1 was truncated to generate 3 different aptamer derivatives: 'Two arm', 'Long arm', and 'Short arm' aptamers (Fig. 3A). In the secondary configuration of aptamer I-1 predicted using the Mulfold program [23], the region selected from the randomized sequence of the library RNA was anticipated to form a relatively short stem-loop and a longer stem-loop structure (Fig. 3B). Truncated 'Two arm' aptamer harbors both stem-loop conformations.

3.4. Specific binding against O157:H7 strain by minimized aptamer and target characterization

We performed a direct binding assay through precipitation of aptamer–cell complexes to determine the minimal binding domain

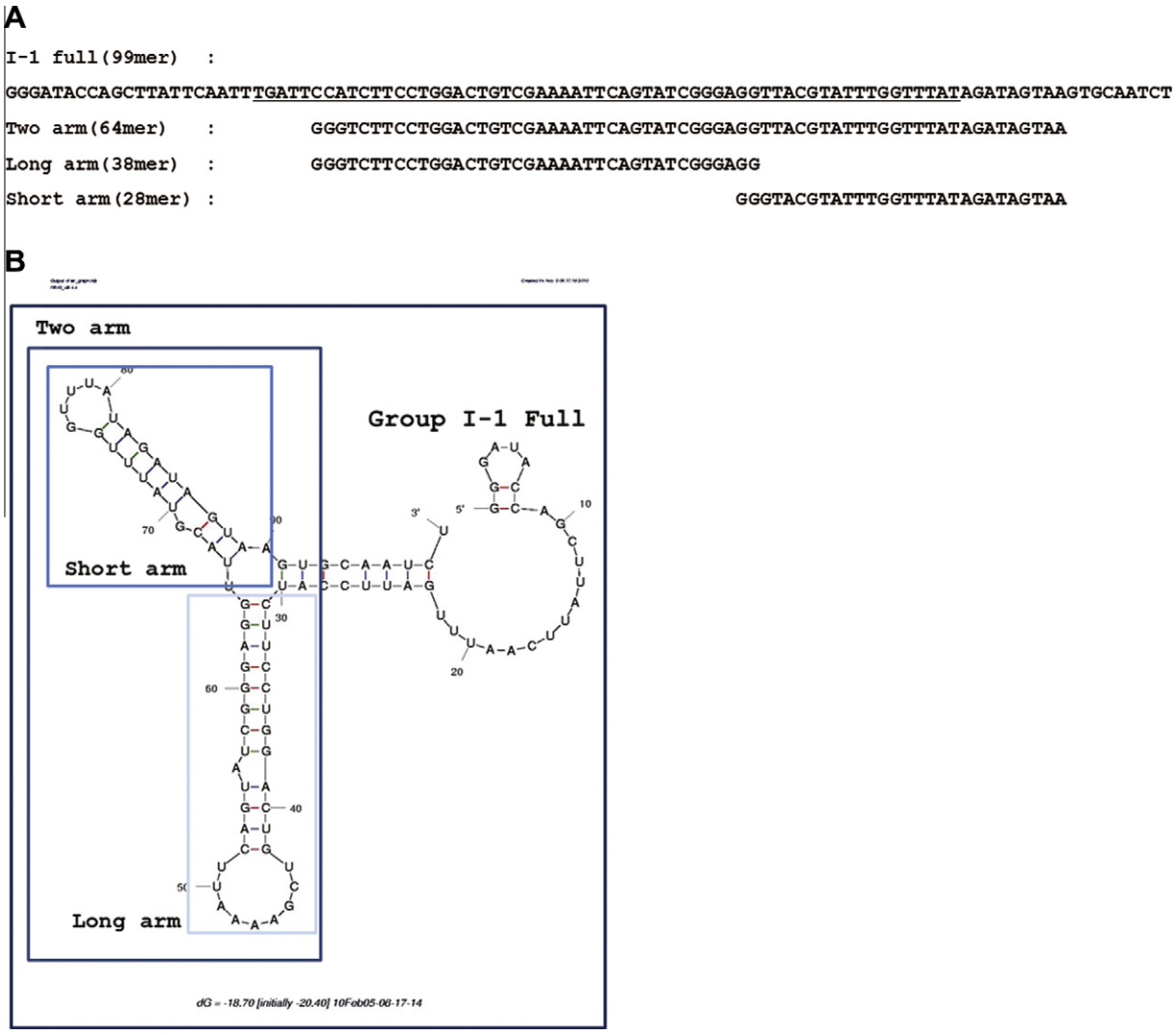

Fig. 3. Mapping minimal region of RNA aptamer I-1. (A) Sequence of full length RNA aptamer I-1 and its truncated aptamer series. (B) Predicted secondary structure of full length aptamer clone I-1 and truncated aptamer series.

of RNA aptamer I-1 and the binding specificity of the minimized RNA (Fig. 4A). One of the aptamer I-1 variations, 'Two arm' aptamer, specifically bound to the O157:H7 strain with a binding specificity and efficacy comparable to those associated with the full-length aptamer I-1. In contrast, additional truncation found in another aptamer series ('Long arm' and 'Short arm' aptamers) abolished binding to the O157:H7 strain.

We next determined the binding affinity between the 'Two arm' aptamer and the O157:H7 strain by quantifying the amount of aptamer bound to the strain after incubating a constant number of the cells with varying concentrations of the RNA aptamer (Fig. 4B). *E. coli* K12 strain was shown to have little affinity for the 'Two arm' aptamer, even at the highest concentration of the aptamer. By contrast, the *E. coli* O157:H7 strain exhibited high affinity with an apparent equilibrium dissociation constant (K_d) of ~110 nM with the 'Two arm' aptamer, demonstrating that the selected RNA binds tightly to the pathogenic strain.

Once the specificity and avidity of the selected aptamer for the O157:H7 strain were evidenced, we next investigated which molecule on the O157:H7 strain surface was the target of the aptamer (Fig. 4C). To this end, we isolated LPS from the O157:H7 or K12 strain and then tested the binding activity of aptamer I-1 and its derivatives with each LPS. In accordance with results from the binding studies with *E. coli* strains, the 'Two arm' aptamer specifically bound to the LPS from the O157:H7 strain, but not to the LPS from the K12 strain. The binding specificity and efficacy of the 'Two arm' aptamer were comparable to those associated with full-length aptamer I-1.

4. Discussion

In this study, we designed subtractive SELEX strategy to isolate a nuclease resistant RNA aptamer specifically binding to the *E. coli* strain O157:H7. Moreover, we truncated and minimized the aptamer sequence without compromising its binding specificity and efficacy. This minimized aptamer, called 'Two arm' aptamer, could specifically discriminate the pathogenic *E. coli* O157:H7 strain from the nonpathogenic K12 strain with high affinity, hence being a potential diagnostic and/or capturing ligand for the pathogenic strain.

Cell-targeting SELEX was employed for the selection of an aptamer specific to the O157:H7 strain surface, rather than using a conventional SELEX approach with crude or purified extracellular target molecules. An obvious advantage of the cell-based SELEX method is that a specific cell type can be targeted without prior knowledge of the target molecule [24], and aptamers can be

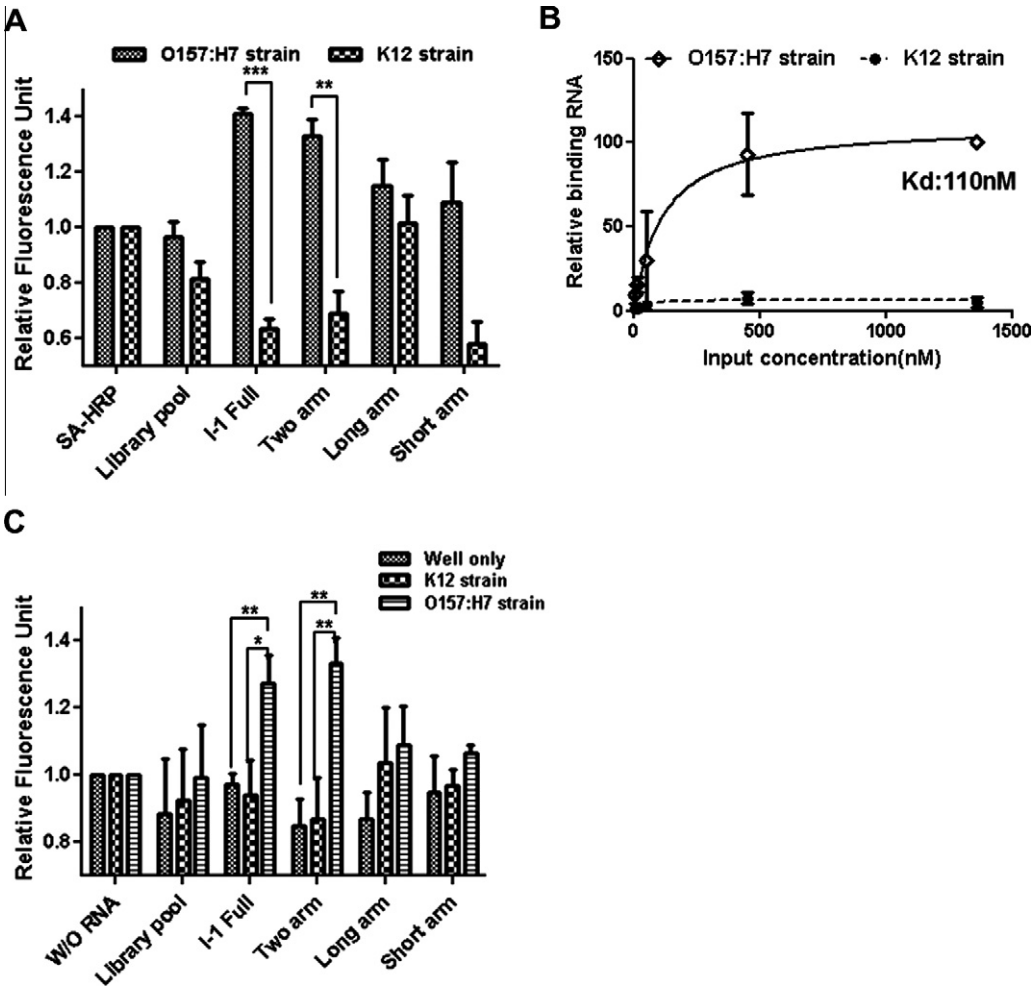

Fig. 4. Binding specificity and efficacy of truncated and minimized RNA aptamer. (A) Comparison of binding ability of RNA aptamer I-1 and its truncated aptamers to O157:H7 strain versus K12 strain. Binding capacity of each aptamer or library RNA to a specific strain is presented as relative fluorescence units to the sample without any RNA (SA-HRP). (B) Determination of the dissociation constant (K_d) for 'Two arm' aptamer to the O157:H7 strain. The percentage of aptamer bound to the cell was calculated by determining the fraction of RNA amount present in the RNA–cell complexes. A maximum binding percentage of the aptamer to the O157:H7 strain was seen at a 1.36 μM concentration of RNA. The plotted numbers have been normalized to that amount. (C) Comparison of binding ability of aptamer I-1 and its truncated aptamers to O157:H7 LPS versus K12 LPS. Binding capacity of each aptamer or library RNA to the LPS from a specific strain is presented as relative fluorescence units to the sample without RNA. The values of aptamer binding and K_d represent the average of 3 independent experiments with standard deviation. $^{***}p < 0.0001$, $^{**}p < 0.005$, $^{*}p < 0.05$.

isolated against targets in their native configuration and physiological circumstances [25]. Moreover, characterization of molecular targets against aptamers selected by cell-SELEX would be useful for the identification of molecular markers specific to the targeted cell type. Importantly, the RNA aptamer isolated in this study specifically and efficiently bound to LPS from the O157:H7 strain, but not to LPS from the K12 strain, indicating that LPS of the O157:H7 strain is the specific target molecule for the aptamer. Accordingly, aptamer interaction with the O157:H7 strain remained unaltered by proteinase treatment of the cell surface (data not shown). This result coincides with the finding that the presence of LPS O antigen is one of the major surface differences between the *E. coli* O157:H7 strain and the normal K12 strain [26].

Current methods used for detection of *E. coli* O157:H7 include broth cell culture and colony counting, PCR based assay, and ELISA using antibodies. However, most of these methods have several shortcomings as follows. Although the traditional culture method using a special biochemical media provides selective isolation and specific detection of EHEC O157:H7, it requires 4–5 days to complete analysis, which would be long after an outbreak of disease [27]. The PCR based method has a high sensitivity and can detect at least 1–10 microbial pathogens per sample. However, the methods frequently generate a high rate of false-positives. Traditional immunoassays (ELISA) have advantages such as simplicity, speed, and high reproducibility. The major limitation of the immunological techniques resides in the quality and stability of the antibody, and the sensitivity required for direct measurement of pathogenic bacteria.

Aptamers have significant benefits over antibodies with regard to their use as diagnostic ligands. One advantage is that there are no batch-to-batch variations because they are generated entirely by *in vitro* chemical synthesis [16,28]. In addition to their target specificity, affinity, and high sensitivity, which equal or surpass those of antibodies, aptamers can be easily modified, optimized, or fixed with variable reporters, depending on the type of diagnostic platform. Recently, various types of biosensor systems, including immunosensors, have been developed for real-time detection of pathogens with high sensitivity and speed [29,30]. The RNA aptamer isolated in this study could potentially be mounted on a biosensor system as a ligand to directly detect *E. coli* O157:H7.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.130.

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