

Specific detection of *Shigella sonnei* by enzyme-linked aptamer sedimentation assay

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Development of potent new anti-*Shigella* agents for rapid and specific detection and treatment is of great importance. Aptamers, nucleic acid oligomers capable of specific binding to a wide range of non-nucleic acid targets, may be of value for this purpose. In the present study, we used a Systematic Evolution of Ligands by Exponential enrichment (SELEX) process to select DNA aptamers that bind to whole *S. sonnei* cells. The resulting aptamers exhibited specificity in binding only to *S. sonnei* cells. Five unique DNA sequences were isolated from the aptamer cocktail by cloning, among which ASA4 showed the highest affinity. © 2011 Progress in Biological Sciences, Vol. 1, No.1, 11-15.

KEY WORDS: DNA aptamer; *Shigella sonnei*; SELEX; ELASA.

INTRODUCTION

The ingestion of food contaminated with infectious or toxicogenic microorganisms is a major cause of morbidity and a significant cause of death throughout the world. Many of the cases of acute diarrheal disease in both developing and industrialized countries are due to infection by *Shigella* species (Niyogi, 2005; Sansonetti, 2006). The estimated incidence of *Shigella* infection is 164.7 million per year, of which 163.2 million occur in developing countries and 1.1 million result in death. Furthermore, 69% of all occurrences of shigellosis and 61% of *Shigella* caused deaths involve children younger than 5 years (Kotloff et al., 1999); however more recent epidemiological studies suggested that this is an underestimate (Sansonetti, 2006). *Shigella sonnei* is the predominant species in developed countries and also one of the most frequent in the tropics (Preston & Borczyk, 1994; Heier et al., 2009). *Shigella sonnei* is a non-motile, non-spore-forming, facultative anaerobic Gram-negative bacterium (Yang et al., 2005). Transmission is mainly through direct contact with

infected individuals by the fecal-oral route, although outbreaks may be occasionally associated with the consumption of contaminated food or water (De et al., 2006). *Shigella* may be missed by routine microbiological detection methods (Niyogi, 2005), and can be difficult to distinguish from entero-invasive *Escherichia coli*, another major cause of dysentery, via biochemical properties such as O-antigens (Cheasty & Rowe, 1983) and plasmid-associated virulence determinants (Lan et al., 2001). Moreover, discriminating among *Shigella* species is of value in epidemiologic studies (Sur et al., 2004; Niyogi, 2005). Therefore, development of rapid and highly specific approaches for detection of this bacterium in environmental and biological samples is urgently required.

Systematic Evolution of Ligands by Exponential Enrichment (SELEX) as a combinatorial chemical method to produce novel ligands to target molecules has been used extensively to isolate RNA or DNA oligonucleotides that specifically and efficiently bind to target molecules (Abelson, 1990; Tuerk & Gold, 1990). These oligo-

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nucleotide aptamers have several potential advantages over antibodies. For example, being smaller than antibodies, aptamers are better candidates for cell penetration, blood clearance, and a variety of chemical modifications, and they are readily synthesized and non-immunogenic and therefore do not cause side effects resulting from unwanted immune responses in hosts (Pan *et al.*, 2005). Aptamers are beginning to emerge as a class of molecules that rival antibodies in both therapeutic and diagnostic applications including bacterial detection. In this study we applied a SELEX approach to identify aptamer oligonucleotides as ligands that bind to whole cells of *S. sonnei* with high affinity and specificity.

MATERIAL AND METHODS

Bacterial strains and reagents

All bacterial strains, *S. sonnei*, *S. flexneri*, *E. coli* O157, *Salmonella typhi*, and *Staphylococcus epidermis* were obtained from the Pasteur Institute of Iran (Tehran, Iran) and grown in Luria-Bertania (LB) broth for 14-16 h at 37°C or solid medium containing 1.5% (w/v) agar. Glycerol, EDTA, NaCl, KCl, Tween20, triptone, yeast extract, and other reagents were obtained from Merck (Darmstadt, Germany).

Oligonucleotide library and primers

A synthetic random DNA library, biotin labeled primers, and other primers were from Bioneer (Daejeon, Republic of Korea). Reagents for PCR and DNA extraction kit were purchased from Fermentas (Burlington, ON, Canada). Streptavidin-conjugated horseradish peroxidase and OPD were from Bangalore Genie (Bangalore, India). The TA cloning kit and plasmid purification kit were from Intron (Seoul, Republic of Korea). The oligonucleotide template was synthesized as a single stranded 80-mer with the following sequence: 5'-CATCCATGGGAATTCGTCGAC(N)₄₀CTGCCTAGGCTCGAGCTCG-3', where the central N₄₀ represents random oligonucleotides based on equal incorporation of A,G,C and T at each position (Bioneer). The dsDNA molecules were generated by PCR amplification using primer 1: 5'-CATCCA

TGGGAATTCGTCGAC-3' and primer 2: 5'-CGAGCTCGAGCCTAGGCAG-3' (Bioneer)

Whole bacterial cell SELEX procedure

To initiate *in vitro* selection, the random DNA library was heated at 85°C for 15 min, snap-cooled on ice for 5 min and incubated in binding buffer (25 mM Tris-HCl, 50 mM KCl, 200 mM NaCl, 0.2 mM EDTA, 5% (v/v) glycerol, and 0.5 mM DTT) for 30 min at 37°C together with 10⁹ CFU *S. sonnei*. Following washing with at least 10 column volumes of binding buffer, bacterial cells with bound ssDNA was centrifuged at 15000×g for 5 min, and the supernatant was discarded. The precipitant was diluted by adding 100 µl ddH₂O, boiled for 5 min, centrifuged at 15 000×g for 5 min, and the supernatant was used as the template for PCR to obtain ssDNA pool for the next round of selection. To select aptamers specifically recognizing *S. sonnei*, we progressively increased the selective pressure by increasing the number of washings and by decreasing the incubation time.

Cloning and sequencing

After thirteen rounds of selection, PCR products cloned into M13mp9/pUC9. The cloned sequences were transformed into *E. coli* BL21 (DE3). Plasmid DNA was isolated from individual clones, purified, and analyzed by sequencing.

Enzyme-Linked Aptamer Sedimentation Assay (ELASA)

To demonstrate the affinity and specificity of aptamers to *S. sonnei*, biotin-labeled aptamers (30 µg) were incubated in a 1.5 ml tube with 10⁹ CFU of *S. sonnei*, *S. flexneri*, *E. coli* O157, *S. typhi*, or *S. epidermis* at 37°C for 15 min and centrifuged at 2500×g for 5 min. Unbound biotin aptamers were removed and the plates washed three times with 1.5% Tween-20 in binding buffer. Finally, 100 µl of a 1:250 dilution of a solution of Streptavidin conjugated to HRP was added and vortexed. Following 30 min incubation at room temperature on a shaking platform, centrifuged at 2500×g for 5 min, and plates were washed three times with 1.5% Tween-20 in binding buffer and developed using

OPD as substrate (Bangalore Genie) according to the manufacturer's instructions. The sorbent was centrifuged at $2500\times g$ for 5 min and the supernatant transferred to 96-well micro titer plates. Absorbance was read at 492 nm using a standard ELISA reader instrument. Each experiment was performed in triplicate.

Statistical analyses

Data from ELASA are expressed as means \pm SEM (standard error of means). Significance of differences between absorptions (with $P < 0.01$) were analyzed by one-way ANOVA (analysis of variance) using SPSS 16.0 software (SPSS, Chicago, IL, USA).

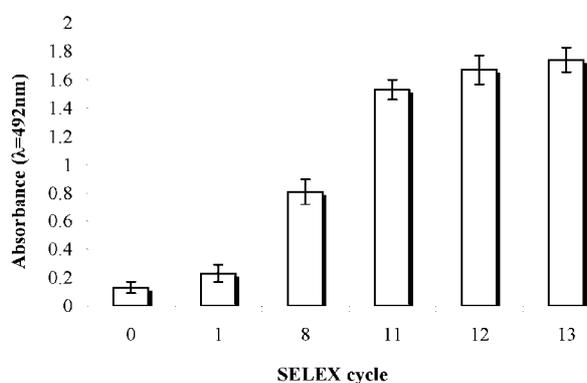


Fig. 1. Maturation of aptamers for *S. sonnei* during SELEX cycles. The ELASA was performed in triplicate and data expressed as means \pm SEM of absorption at 492 nm vs. number of SELEX cycle. Control is the random library.

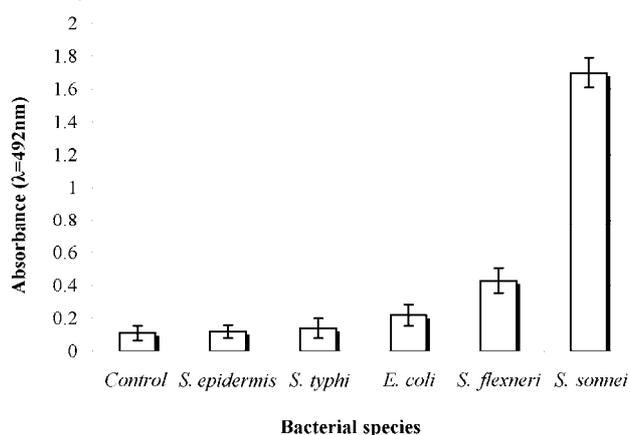


Fig. 2. ELASA to assess specificity of anti-*sonnei* aptamer cocktail. The data are presented as OD at 492 nm vs. bacterial species. In negative control ELASA carried out without aptamer. Means \pm SEM of three replicate assays are given.

RESULTS

Affinity maturation of aptamer cocktail

The biotin-labeled aptamer cocktail obtained from the 8th, 11th, 12th, and 13th round of SELEX was separately assessed by ELASA against an equal value of bacteria (10^9 CFU) compared with a control containing no aptamer. A significant signal was observed above background level. Data for three replicate samples ranged from 0.1 (background) to 1.76 absorbance units. As seen in Fig. 1, in comparing absorbance of aptamers from the 8th round with the 13th round, it is confirmed that during selection, binding affinity of aptamers to *S. sonnei* increased, and absorbance of aptamers from round 11, 12, and 13 did not differ significantly.

S. sonnei-specificity of aptamers

ELASA was used to provide information of specificity of resulting aptamers to *S. sonnei* from those of other species. For this purpose, binding affinities of these aptamers against four other species, *S. flexneri*, *E. coli* O157, *S. typhi*, and *S. epidermis*, were tested by ELASA. As can be seen in Fig. 2, the signal output showed very low affinity, ranging from 0.13 absorbance units for *S. epidermis* to 0.49 for *S. flexneri*, while significantly stronger *S. sonnei*-binding activity indicated the value of obtained aptamers in discrimination of *S. sonnei* from other bacteria.

Cloning and evaluation of individual aptamers

After the 13th round of selection, the SELEX yielded aptamer mixture was cloned into M13mp9/pUC9 vector, and ten randomly selected clones were sequenced, yielding five different sequences (Fig. 3). To evaluate the affinity of unique aptamers to *S. sonnei* in comparison with aptamer mixture obtained with SELEX, the *S. sonnei* cells (10^9 CFU) were exposed to equal amounts of these aptamers (1 μ g) separately, and ELASA assays carried out. As shown in Fig. 3, absorbance of ASA4 is highest in comparison with other individual aptamers and the mixed cocktail, confirming a higher binding affinity of ASA4. After ASA4, ASA2 had the most affinity.

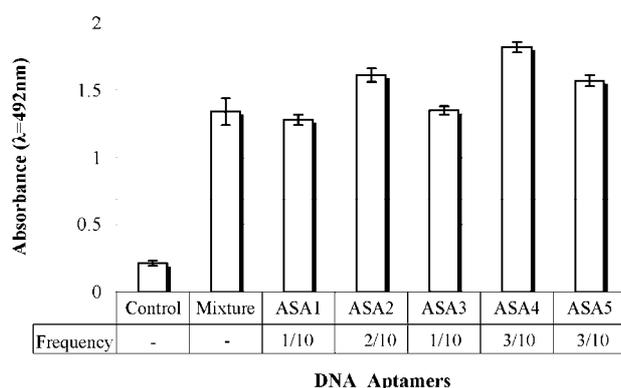


Fig. 3. ELASA to assess the affinity of individual anti-*sonnei* aptamers compared to mixed aptamer cocktail from 13th cycle. The data are presented as OD at 492 nm vs. aptamers. In negative control ELASA carried out without aptamer. Means \pm SEM of three replicate measurements given. Table presents frequency of each aptamer sequence among ten randomly selected clones.

DISCUSSION

To our knowledge this is the first report of successful generation of DNA aptamers that bind specifically to *S. sonnei*, using a whole bacterial cell-SELEX. This approach, relying on using whole cells instead of individual target molecules to create aptamers, exhibits advantages, including simplicity of selection procedure and comprehensive targeting of surface antigens (Chen et al., 2007). Similar approaches have been shown to result in selection of specific aptamers in previous studies (Vivekananda & Kiel, 2006; Chen et al., 2009). Using a similar whole-cell-SELEX approach, Vivekananda & Kiel (2006) developed DNA aptamers against *Francisella tularensis* which had almost 1/3 affinity to an unrelated bacteria *Bartonella henselae*. Our results showed better aptamer specificity, as the selected aptamers exhibited affinity to *S. sonnei* more than threefold that to its related species *S. flexneri* (Fig. 2). In other words, although anti-*S. sonnei* aptamers showed slightly higher affinity to *S. flexneri* compared with other distant bacterial species (*E. coli*, *S. typhi*, *S. epidermis*), they still exhibited a discriminating specificity to *S. sonnei*. Although no counterselection was performed, this high specificity may be largely due to the innate specificity property of aptamer-target interaction. Aptamers have been shown to be able to discriminate closely

related molecules from their targets (Jenison et al., 1994; Huang et al., 2003). This optimal discrimination potential was obtained by maturation of aptamer affinity and specificity during the multiple selection cycles of increasing severity. These specific aptamers may be used for detection purposes, as demonstrated by several previous studies using strategies to apply aptamers for microbial detection (Ulrich et al., 2006; Huang & Liu, 2010; Lautner et al., 2010; Wu et al. 2010). Although the precise target molecules of these aptamers are unknown, they can nevertheless be used as detection and therapeutic agents due to their high specificity. Similarly DNA aptamers have been used in cancer studies without prior knowledge of target molecules (Shangguan et al., 2006).

These whole bacterium-specific aptamers are of value in identification of novel specific surface antigens for *S. sonnei*. In this work, affinity assessment was performed by the use of ELASA described here for the first time. This simple and cost effective method takes advantage of sedimentation of target-bound biotin-labeled aptamers and washing out of unbound aptamers, followed by color generation with enzyme-conjugated Streptoavidin. In addition to its simplicity, the method reassembles the selection procedure and condition, suggesting its valuable account to monitor the selection process. Cloning of anti-*S. sonnei* aptamers revealed five different sequences among ten sequenced clones. This frequency of different sequences is similar to previous experiments, such as a recent study by Chen et al. (2009) in which six different sequences were obtained among eight clones, using a similar whole cell SELEX approach. Furthermore, our approach involves DNA aptamers, which present several advantages over RNA aptamers, such as stability in harsh environments, long shelf-life, and ease of large scale chemical synthesis at a relatively low cost (Ulrich et al., 2006). In summary, we have generated a subpopulation of oligonucleotide anti-ligands that bind with high specificity to *S. sonnei* whole cells. These ssDNA molecules recognize *S. sonnei* from other bacteria. The current work also demonstrates that aptamers can be used in ELASA format for detection of cells.

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