Progress in Biological Sciences
Vol. 1, No.1, 2011,
xx-xx

PBS ISSN: xxxx-xxxx
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Specific detection of *Shigella sonnei* by Enzyme-Linked Aptamer Sedimentation Assay

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Abstract
Shigella species are able to cause Shigellosis – an enteric infection considered as a global human health threat. Development of potent new anti-*Shigella* agents for rapid and specific detection and treatment is therefore of great importance. Aptamers, nucleic acid oligomers capable of specific binding to a wide range of non-nucleic acid targets, seem to helpthis purpose. In this study, we selected DNA aptamers that bind to *S. sonnei* whole cell using a Systematic Evolution of Ligands by Exponential enrichment (SELEX) process. The resulting aptamers exhibited specificity in their ability to bind only to *S. sonnei* cells. Five unique DNA sequences were isolated from the aptamer cocktail by specifically cloning to *S. sonnei*, among them ASA4 showed the highest affinity.

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 very 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 (Sansonetti 2006; Niyogi 2005). 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 percent of all occurrence of shigellosis and 61 percent of *Shigella* caused deaths involve children younger than 5 years old (Kotloff *et al.* 1999), however more recent epidemiological studies suggested that this is an underestimation and there should be a higher incidence of shigellosis (Sansonetti 2006). *S. sonnei* is the predominant species in developed countries and also one of the most frequency ones in the tropics (Preston and Borczyk 1994; Heier *et al.* 2009). *Shigella sonnei* is a non-motile, nonspore-forming, facultative anaerobic Gram-negative bacterium (Yang *et al.* 2005). Transmission is mainly by direct contact with infected individuals by the fecal-oral route although outbreaks may occasionally be associated with the consumption of contaminated food or water (De *et al.* 2006). *Shigella* may be missed in routine microbiological detection methods (Niyogi 2005) and also can hardly be distinguished from enteroinvasive *Escherichia coli* – another major cause of dysentery – via the biochemical properties such as O-antigens (Cheasty and Rowe 1983) and plasmid-associated virulence determinants (Lan *et al.* 2001). Moreover, distinguishing between different *Shigella* species is of valuable worth in epidemiologic studies (Sur *et al.* 2004; Niyogi 2005). Therefore, development of new rapid and highly specific approaches for detection of this bacterium in environmental and biological samples is urgently required.

SELEX (Systematic Evolution of Ligands by Exponential enrichment) as combinatorial

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chemistry method that produce novel ligands to target molecules has been used extensively to isolate RNA or DNA oligonucleotides that specifically and efficiently bind to target molecules (Tuerk and Gold 1990; Abelson 1990). These oligonucleotide aptamers have several potential advantages over antibodies, for instance, being of smaller size than antibodies, aptamers are better candidates for cell penetration, blood clearance, and a variety of chemical modifications, and they are nonimmunogenic, readily synthesized, and therefore do not cause side effects resulting from unwanted immune responses in hosts (Pan *et al.* 2005). Therefore 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 aptamers oligonucleotides as ligands that bind to whole cell of *S. sonnei* with high affinity and specificity.

Material and methods

Bacterial strains and reagents

All bacterial strains, *Shigella sonnei*, *Shigella flexneri, E.coli O157, Salmonella typhi, Staphilococos epidermis* were obtained from Pastour Institute of Iran (Tehran, Iran) and grown in Luria-bertania (LB) broth for 14 to 16 h at 37°C or solid medium contained 1.5% (w/v) agar. Glycerol, EDTA, NACL, KCL, Tween20, Triptone, yeast extract and other reagents obtained from Merck.

Oligonucleotide library and primers

A synthetic random DNA library, biotin labeled primers and other primers were from Bioneer (Daejeon, Korea Rep.). 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, Korea Rep.). The oligonucleotide template was synthesized as a single stranded 80-mer with the following sequence: 5´-CATCCATGGGAATTCGTCGAC$(N)_{40}$CTGC CTAGGCTCGAGCTCG-3´, were the central N_{40} represents random oligonucleotides based on equal incorporation of A,G,C and T at each position (Bioneer, Daejeon, Korea Rep.). The dsDNA molecules were generated by PCR amplification using primer 1: 5´-CATCCATGGGAATTCGTCGAC-3´ and primer 2: 5´-CGAGCTCGAGCCTAGGCAG-3´ (Bioneer, Daejeon, Korea Rep.).

Whole bacterial cell SELEX procedure

To initiate in vitro selection, random DNA library were heated at 85°C for 15 min, snap-cooled on ice for 5 min, 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 10^9 CFU *S. sonnei*. Following washing with at least 10 column volumes of binding buffer, bacterial cells with bounded ssDNA was centrifuged at 13000rpm for 5 min, and supernatant was discarded. The precipitant was diluted by adding 100ul ddH20, boiled for 5 min, centrifuged at 13000rpm for 5min, and the supernatant was used as the template for PCR to obtain ssDNA pool for the next round of selection. In order 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* during, in a 1.5 ml tube, biotin-labeled aptamers (30µg) were incubated with 10^9 CFU of *S. sonnei S. flexneri*, *E. coli O157, S. typhi* or *S. epidermis* at 37°C for 15 min, centrifuged at 5000rpm for 5 min. Unbound biotin aptamers were removed and the

plates wash thrice with 1.5% Tween-20 in binding buffer. Finally 100µl of a 1:250 dilution of a solution of streptavidin conjugated to HRP were added to them and vortex. Following 30-min incubation at room temperature on a shaking platform, centrifuged at 5000rpm for 5 min, and plates were washed thrice with 1.5% Tween-20 in binding buffer again and developed using OPD as substrate (Bangalore Genie) according to the manufacturer's instructions. Then sorbent was centrifuged at 5000rpm for 5 min and supernatant was transfer to 96-well micro titer plates and absorbance was read at 492 nm using a standard ELISA reader instrument. Each experiment is performed in triplicate.

Statistical analyses

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

Results

Affinity maturation of aptamer cocktail

Biotin-labeled aptamer cocktail obtained from 8^{th}, 11^{th}, 12^{th}, 13^{th} round of SELEX was separately assessed by ELASA against equal value of bacteria (10^9 CFU) compared with a control containing no aptamer. A significant signal was observed over background, and data for three replicate samples ranged from 0.1 (background) to 1.76 absorbance units. As seen in Figure 1, in comparing absorbance of aptamers from 8^{th} round with 13^{th} 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 significantly differ.

***S. sonnei*-specificity of aptamers**

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

Cloning and evaluation of individual aptamers

After the 13^{th} round of selection, SELEX yielded aptamer mixture were cloned into M13mp9/pUC9 vector and ten randomly selected clones were sequenced, observing five different sequences (Figure 3). In order to evaluate the affinity of unique aptamers to *S. sonnei* and comparison with aptamers mixture obtained from SELEX, the *S. sonnei* cells (10^9 CFU) were exposed to equally amount of these aptamers (1 µgr) separately and ELASA assays carried out. As shown in Figure 3 absorbance of ASA4 in comparison with other aptamers individually or in mixed cocktail is the highest. It confirms that binding affinity of ASA4 is more than others. After that ASA2 had the most absorbance and affinity among others.

Discussion

As 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 relies on using whole cells instead of individual target molecule to evolve aptamers exhibit some advantages including simplicity of selection procedure and comprehensive targeting of surface antigens (Chen *et al.* 2007). Interestingly similar approaches also have been showed to result in selection of specific aptamers in previous studies (Chen *et al.* 2009; Vivekananda and Kiel 2006). Using similar whole-cell-SELEX approach, Vivekananda and Kiel developed DNA aptamers against *Francisella tularensis* which had almost 1/3 affinity to an unrelated bacteria *Bartonella henselae* (Vivekananda and Kiel 2006). Our results showed even better aptamer specificity as the selected aptamers showed more than threefold higher affinity to *S. sonnei* than its relative

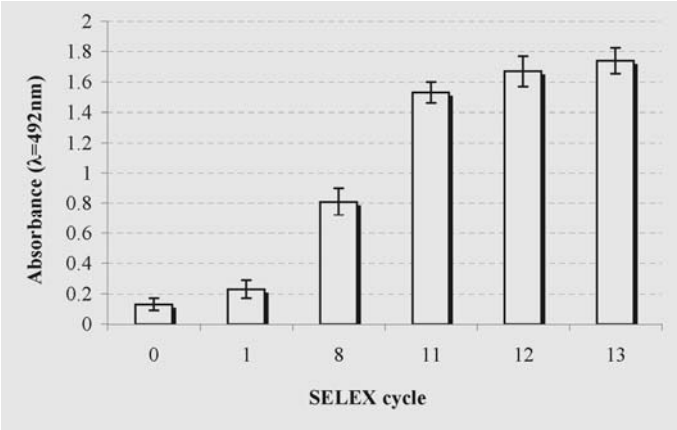

Fig.1. Maturation of aptamers for *S. sonnei* during SELEX cycles. The ELASA were performed in triplicate and data expressed as mean±SEM of absorbtion at 492 nm *vs.* number of SELEX cycle. control is the random library

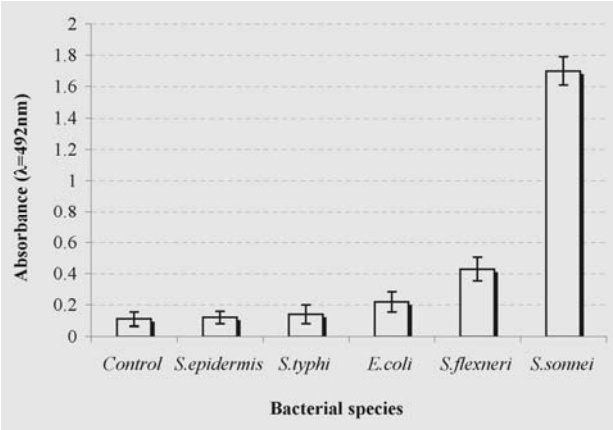

Fig. 2. ELASA to analyze the specificity of anti-*sonnei* aptamer cocktail. The data are presented as OD at 492 nm *vs.* bacterial species. In negative control ELASA carried out without adding aptamer. Means±SEM of three replication assay are shown in the figure.

species *S. flexneri* (Figure 2). In other words, although anti-*S. sonnei* aptamers showed slightly higher affinity to *S. flexneri* compared with other distant bacterial species (*Escherichia coli, Salmonella typhi, Staphylococcus epidermis*), they still exhibited a detectable specificity to *S. sonnei*. Whereas no counterselection was performed, this great specificity may be largely due to innate specificity property of aptamer-target interaction. Indeed, 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 with ascending severity. These specific aptamers may be used for detection purposes as several previous studies used various strategies to apply aptamers for microbial detection (Ulrich *et al.* 2006; Lautner *et al.* 2010; Huang and Liu 2010; Wu *et al.* 2010). Although, the precise target molecule of these aptamers are unknown they can still used as detection and therapeutic agent due to their high specificity. Similarly DNA aptamers have been used in cancer study without prior knowledge of target molecules (Shangguan *et al.* 2006). However these whole bacterium-specific aptamers are of valuable worth for

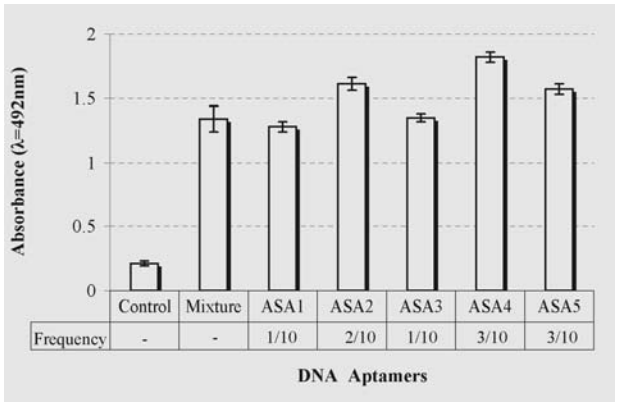

Fig. 3. ELASA to analyze the affinity of anti-*sonnei* aptamers individually compared with mixed aptamer cocktail from 13th cycle. The data are presented as OD at 492 nm *vs.* aptamers. In negative control ELASA carried out without adding aptamer. Means±SEM of three replication measurements are shown. Table at the bottom presents frequency of each aptamer sequence between ten randomly selected colons.

identification of novel specific surface antigens for *S. sonnei*. In this work, affinity assessment was performed by the use of an Enzyme-Linked Aptamer Sedimentation Assay (ELASA) which has been 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 unbound aptamers, followed by color generation with enzyme-conjugated streptoavidin. In addition to its simplicity, the method reassembles greatly 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 not far from pervious experiments such as a recent study by Chen and colleagues where six different sequences were obtained among eighth clones, using a similar whole cell SELEX approach (Chen *et al.* 2009). Furthermore, our approach involves DNA aptamers, which present several advantages over RNA aptamers, such as their 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 cell. 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.

References

Abelson J (1990) Directed evolution of nucleic acids by independent replication and selection. Science 249:488-489

Cheasty T, Rowe B (1983) Antigenic relationships between the enteroinvasive Escherichia coli O antigens O28ac, O112ac, O124, O136, O143, O144 ,O152, and O164 and Shigella O antigens. J Clin Microbiol 17:681-684

Chen F, Hu Y, Li D, Chen H, Zhang XL (2009) CS-SELEX generates high-affinity ssDNA aptamers as molecular probes for hepatitis C virus envelope glycoprotein E2. PLoS One 4:e8142

Chen F ,Zhou J, Luo F, Mohammed AB, Zhang XL (2007) Aptamer from whole-bacterium SELEX as new therapeutic reagent against virulent *Mycobacterium tuberculosis*. Biochem Biophys Res Commun 357:743-748

De LN, Doran G, Connor JO, Mammina C, Cormican M (2006) Use of pulsed-field gel electrophoresis for comparison of similar but distinguishable isolates of *Shigella sonnei* collected in Ireland and Italy. J Clin Microbiol 44:3808-3810

Heier BT, Nygard K, Kapperud G, Lindstedt BA, Johannessen GS, Blekkan H (2009) *Shigella sonnei* infections in Norway associated with sugar peas, May-June 2009. Euro Surveill 14:pii:19243

Huang DB, Vu D, Cassiday LA, Zimmerman JM, Maher LJ, III, Ghosh G (2003) Crystal structure of NF-kappaB (p50)2 complexed to a high-affinity RNA aptamer. Proc Natl Acad Sci U S A 100:9268-9273

Huang PJ, Liu J (2010) Flow cytometry-assisted detection of adenosine in serum with an immobilized aptamer sensor. Anal Chem 82:4020-4026

Jenison RD, Gill SC, Pardi A, Polisky B (1994) High-resolution molecular discrimination by RNA. Science 263:1425-1429

Kotloff KL, Winickoff JP, Ivanoff B, Clemens JD, Swerdlow DL, Sansonetti PJ, Adak GK, Levine MM (1999) Global burden of *Shigella infections*: implications for vaccine development and implementation of control strategies. Bull World Health Organ 77:651-666

Lan R, Lumb B, Ryan D, Reeves PR (2001) Molecular evolution of large virulence plasmid in *Shigella* clones and enteroinvasive *Escherichia coli*. Infect Immun 69:6303-6309

Lautner G, Balogh Z, Bardoczy V, Meszaros T, Gyurcsanyi RE (2010) Aptamer-based biochips for label-free detection of plant virus coat proteins by SPR imaging. Analyst 135:918-926

Niyogi SK (2005) Shigellosis. J Microbiol 43:133-143

Pan Q, Zhang XL, Wu HY, He PW, Wang F, Zhang MS, Hu JM, Xia B, Wu J (2005) Aptamers that preferentially bind type IVB pili and inhibit human monocytic-cell invasion by *Salmonella enterica* serovar typhi. Antimicrob Agents Chemother 49:4052-4060

Preston MA, Borczyk AA (1994) Genetic variability and molecular typing of *Shigella sonnei* strains isolated in Canada. J Clin Microbiol 32:1427-1430

Sansonetti PJ (2006) Shigellosis: an old disease in new clothes? PLoS Med 3:e354

Shangguan D, Li Y, Tang Z, Cao ZC, Chen HW, Mallikaratchy P, Sefah K, Yang CJ, Tan W (2006) Aptamers evolved from live cells as effective molecular probes for cancer study. Proc Natl Acad Sci U S A 103:11838-11843

Sur D, Ramamurthy T, Deen J, Bhattacharya SK (2004) Shigellosis : challenges & management issues. Indian J Med Res 120:454-462

Tuerk C, Gold L (1990) Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. Science 249:505-510

Ulrich H, Trujillo CA, Nery AA, Alves JM, Majumder P, Resende RR, Martins AH (2006) DNA and RNA aptamers: from tools for basic research towards therapeutic applications. Comb Chem High Throughput Screen 9:619-632

Vivekananda J, Kiel JL (2006) Anti-Francisella tularensis DNA aptamers detect tularemia antigen from different subspecies by Aptamer-Linked Immobilized Sorbent Assay. Lab Invest 86:610-618

Wu Y, Sefah K, Liu H, Wang R, Tan W (2010) DNA aptamer-micelle as an efficient detection/delivery vehicle toward cancer cells. Proc Natl Acad Sci U S A 107:5-10

Yang F, Yang J, Zhang X, Chen L, Jiang Y, Yan Y, Tang X, Wang J, Xiong Z, Dong J, Xue Y, Zhu Y, Xu X, Sun L, Chen S, Nie H, Peng J, Xu J, Wang Y, Yuan Z, Wen Y, Yao Z, Shen Y, Qiang B, Hou Y, Yu J, Jin Q (2005) Genome dynamics and diversity of *Shigella* species, the etiologic agents of bacillary dysentery. Nucleic Acids Res 33:6445-6458