Title: Staggered Target SELEX, a novel approach to isolate non-cross-reactive aptamer for detection of SEA by apta-qPCR

Authors: Hamid Sedighian, Raheleh Halabian, Jafar Amani, Mohammad Heiat, Mohsen Amin, Abbas Ali Imani Fooladi

PII: S0168-1656(18)30641-2
DOI: https://doi.org/10.1016/j.jbiotec.2018.09.006
Reference: BIOTEC 8278

To appear in: *Journal of Biotechnology*

Received date: 24-9-2017
Revised date: 1-5-2018
Accepted date: 15-9-2018

Please cite this article as: Sedighian H, Halabian R, Amani J, Heiat M, Amin M, Fooladi AAI, Staggered Target SELEX, a novel approach to isolate non-cross-reactive aptamer for detection of SEA by apta-qPCR, *Journal of Biotechnology* (2018), https://doi.org/10.1016/j.jbiotec.2018.09.006

Isolation of specific non-cross-reactive aptamers against staphylococcal enterotoxin type A using a novel approach: Staggered Target SELEX

According reviewer suggestion New Title:

Staggered Target SELEX, a novel approach to isolate non-cross-reactive aptamer for detection of SEA by apta-qPCR

Hamid Sedighian[1], Raheleh Halabian[1], Jafar Amani[1], Mohammad Heiat[2], Mohsen Amin[3] and Abbas Ali Imani Fooladi*[1]

[1]Applied Microbiology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran.

[2]Applied Biotechnology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran.

[3]Department of Drug and Food Control, and Pharmaceutical Quality Assurance Research Center, Faculty of Pharmacy, Tehran University of Medical Sciences, Tehran, Iran

[1]*[1]Corresponding Author:

***Abbas Ali ImaniFooladi**, Applied Microbiology Research Center, Systems Biology and Poisonings Institute, Baqiyatallah University of Medical Sciences, Tehran, Iran. Email: imanifouladi.a@gmail.com and imanifouladi.a@bmsu.ac.ir*

Abstract:

Background and objective:

Aptamers or chemical antibodies are oligonucleotides (DNA or RNA) that are able to bind to various targets with high specificity and affinity such as toxins which are isolated by an *in vitro* method known as SELEX. To date, there are many SELEX procedures for the isolation of novel aptamers against proteins. However not all modified SELEX are suitable for similar protein based on sequence homology

such as staphylococcal enterotoxins. Staphylococcal enterotoxin type A (SEA) is the most prevalent toxin involved in staphylococcal food poisoning (SFP) worldwide. SEA is homologous to Staphylococcal enterotoxin type D (SED) and Staphylococcal enterotoxin type E (SEE) about 50% and 83%, respectively. Here, we have developed Staggered Target SELEX (ST-SELEX) as a novel designed SELEX procedure to acquire specific non-cross-reactive aptamers against SEA as a model protein.

Methods:

In this study, isolated ssDNA aptamers by ST-SELEX were used for detection of SEA via apta-Real time PCR (apta-qPCR). After *in silico* analysis of SEA protein with SEE and finding the specific region on the surface of protein, ST-SELEX was carried out in two steps (classical SELEX and Second SELEX). Finally, after isolating high specific aptamers, the apta-qPCR was used for the detection of the SEA. In this technique, poly-clonal antibody against SEA was immobilized on protein G sepharose beads (Ab-PGs). Then, the SEA protein was captured by poly clonal antibody as the target that immobilized on sepharose beads. The isolated aptamers were bound on the surface of SEA protein that captured by Ab-PGs. Finally, the heat-released aptamers were amplified by qPCR.

Result:

Our investigation showed that the aptamers were generated *in vitro* by a ten-round selection process based on ST-SELEX procedure with dissociation constant (K_D) value 7.44± 0.6 nM and limit of detection (LOD) of 146.67 fM.

Discussion and conclusion:

The advantage of ST-SELEX compared to other SELEX methods was to select a specific non cross-reactive aptamer against two or more proteins with high sequence homology. These aptamers can be used in sensitive detection methods such as apta-qPCR.

Keywords:

Staggered Target SELEX, Aptamer, Apta-qPCR, Staphylococcal enterotoxin A

Introduction:

Staphylococcus aureus (S. aureus) is a Gram positive bacterium that causes a wide range of human infections, including pneumonia, endocarditis, toxic shock syndrome, and food poisoning (Fooladi et al., 2015). Staphylococcal food poisoning (SFP) caused by *S. aureus* enterotoxins is among the most common food borne illnesses worldwide (Fooladi et al., 2010). Staphylococcal enterotoxins (SEs) cause

gastroenteritis with a short incubation period (about 6-8 hours) and symptoms include abdominal pain, vomiting, nausea, and diarrhea. Enterotoxins also act as superantigens in immune system response through their ability to activate T cell proliferation (Balaban and Rasooly, 2000; Yousefi et al., 2015). SEA is the most common staphylococcal enterotoxin and is responsible for more than 70% of food intoxications (Agheli et al., 2016).

Enzyme-linked immunosorbent assay (ELISA) has been used routinely for the detection of SEs. This technique relies on polyclonal or monoclonal antibodies (Wu et al., 2016). Despite the many advantages of using antibodies, especially monoclonal antibodies, they still suffer from some drawbacks. For example, the production of antibodies is complicated and is time consuming, they have a short half-life and they are easily denatured at high temperature and by extreme pH. Indeed, their properties could be influenced by biological environment causing batch to batch variations (Kong and Byun, 2013).

Aptamers are oligonucleotides (DNA or RNA) that are able to bind to various targets with high specificity and affinity including proteins, amino acids, nucleic acids, drugs, and other molecules (Xian Tan, 2016). They are isolated by *in vitro* selection and amplification methods from combinatorial libraries through a process known as the systematic evolution of ligands by exponential enrichment (SELEX) (Rhouati et al., 2013). Aptamers have several advantages over antibodies, including their cost-effectiveness, lack of immunogenicity and toxicity, high stability, ability to adapt various modifications and chemical synthesis with high purity (Lu et al., 2015).

To date, aptamers have been widely applied for the diagnosis and treatment of various targets in combination with other novel techniques such as, flow cytometry, microfluidic cell separation, graphene oxide (GO), electrochemical biosensor and etc. (Wang et al., 2011; Xiong et al., 2018; Zhuo et al., 2017). A few years after the introduction of aptamers, many studies have been carried out using aptamers technology to diagnose staphylococcal enterotoxins (Bruno and Kiel, 2002; DeGrasse, 2012; Hedayati Ch et al., 2016a; Liu et al., 2013; Sedighian et al., 2018). For example, Huang *et al.* reported an aptamer against SEA enterotoxin using water-soluble GO method (Huang et al., 2014). Although GO has some positive aspects, it also has some negative properties such as limited material selection (strong attraction

between components and water solubility of all components), high setup cost and has complexity (Putz et al., 2010).

In the 1990s, Sano *et al*. reported a very sensitive method for detection of a target with quantitative polymerase chain reaction (qPCR) and was called Immuno-PCR (Sano et al., 1992). In this technique, a specific antibody as a receptor was conjugated to DNA marker and after formation of a sandwich-type immune-complex, the marker served as a template for the qPCR reaction.

Aptamer-qPCR is a technique that can be used for a variety of applications ranging from clinical diagnostics to food quality control. This approach takes advantage of the combination of the specificity of aptamers with the sensitivity of real-time PCR (Civit et al., 2016). There are four kinds of apta-qPCR assay including direct, indirect, sandwich and competitive (Civit et al., 2016; Pinto et al., 2014; Svobodova et al., 2014).

A fundamental problem in the development of sensitive technique for the detection of SEA protein is the risk of cross-reactivity with SEE. Protein BLAST analysis indicates that SEA is similar to SEE about 83% (Schlievert et al., 1995). For resolving this problem, we designed Staggered Target SELEX (ST-SELEX) as a novel modified approach to isolate specific aptamers against two or more proteins with high sequence similarity. Especially, the three-dimensional conformation of SEA protein was simulated based on bioinformatics analysis before SELEX, which aims at screening specific non-cross-reactive aptamers against SEA. Finally, the selected aptamer and antibody have been used in an apta-qPCR (sandwich approach) to detect SEA. In the sandwich apta-qPCR approach, an analyte is measured between two layers of aptamers or in the combination of antibody and aptamer (as a capture and detection biomolecule). Either antibody or aptamer can be used as the capture biomolecule in the sandwich apta-qPCR (Lee et al., 2009).

In the present study, at first, bioinformatics analysis of SEA and SEE enterotoxins were performed to select a specific sequence of SEA and to isolate an ssDNA aptamer against these unique sequences of protein using ST-SELEX (The term “staggered”, means using ambiguous targets in a periodic schedule). Then, polyclonal mouse antibody versus SEA was produced in our laboratory and was conjugated to the

protein G sepharose to capture the target SEA and the specific aptamer pool was further bound to the captured protein in a sandwich method. Finally, the released DNA aptamers were amplified by real-time PCR for the sensitive detection of staphylococcal enterotoxin type A. After 10 repetitive rounds, indirect enzyme-linked oligonucleotide assay (ELONA) was used to evaluate the ssDNA pool-binding capability (R7, R8, R9, and R10) then round 9 was selected and cloned in pJET vector. Our investigation showed that the dissociation constant (K_D) value and the limit of detection (LOD) of the generated aptamers were 7.44± 0.6 of nM and 146.67 fM, respectively, using ST-SELEX in combination with qPCR.

Material and methods

Material

Escherichia coli Dh5α and BL21 (DE3) strains, staphylococcal enterotoxin Type C1, D, E and pET28a vector were kindly provided by Applied Microbiology Center, BMSU, Iran. Staphylococcal enterotoxin Type A and B from *Staphylococcus aureus*, CNBr-activated Sepharose 4B, Protein G Sepharose, HRP-conjugated streptavidin, kanamycin, complete and incomplete Freund's adjuvant, bovine serum albumin (BSA), isopropyl β-D-1-thiogalactopyranoside (IPTG) and HEPES were purchased from Sigma, USA. Agarose, 100 bp DNA ladder and prestained protein marker were obtained from Thermo Fisher Science, USA. Bacterial Plasmid DNA Purification Kit was purchased from Intron Company, Korea. DNA library, biotinylated and non-biotinylated primers for isolating ssDNA aptamer were commercially synthesized from Metabion Company, Germany. Primers for detection and cloning of SEA were commercially synthesized from SinaClon, Iran (Table 1 illustrates all synthesized acid nucleic sequences). Peptides were commercially synthesized from ShineGene Bio-Technologies, Inc, China. SYBR® Green Master Mix and *Taq* PCR master Mix were purchased from Ampliqon, Denmark. Five week old BALB/c mice were obtained from BMSU, Iran. All other reagents were of analytical grade.

Bioinformatics analysis

Bioinformatics analysis for detection of SEA specific sequences was used. Related sequences of SEA and SEE were obtained from the National Centre for Biotechnology Information (Sayers et al., 2011). Multiple sequence alignments were accomplished using Clustal Omega software of the European

Bioinformatics Institute Website (http://www.ebi.ac.uk/Tools/msa/clustalo). PDB files of proteins were obtained from the protein data bank (NCBI) (Berman et al., 2000). Accelrys Discovery Studio 2.5 software was used to visualize the modeled 3D structures and the availability of selectable areas on the surface of the protein. Finally, the physico-chemical parameters such as, peptide weight, total numbers of positive and negative residues and hydrophobic indices were computed using the ExPASY ProtParam tool (http://web.expasy.org/protparam/protparam-doc).

Synthesis of SEA aptamer by ST-SELEX

Figure 1 illustrates the whole project to set up apta-qPCR for detection of enterotoxin type A. In this study, isolation of ssDNA aptamer against SEA was selected using ST-SELEX.

The modified SELEX procedure included 2 phases. In the first phase, which contained four rounds, the isolation of aptamers was carried out similar to classic SELEX (Hedayati Ch et al., 2016b). For the first round of selection, 1 nM (26 µg) of whole SEA protein (positive SELEX) and SEB, SEC and SED proteins (counter SELEX) were immobilized on separate CNBr-activated sepharose 4B as described by the manufacturer. Then 3 nM (75 µg) of the ssDNA library (Table 1) diluted in 300µL of binding buffer (100 mM $NaHCO_3$, 0.5 M NaCl, 1.47 mM $MgCl_2$, 2.7 mM KCl pH 8.3-8.5) was incubated with non-immobilized sepharose beads (negative SELEX) for 15 min at room temperature. The unbound oligonucleotides were isolated by centrifugation at 10,000 rcf for 1 min and were incubated with counter column (SEB, SEC and SED) for 15 min at room temperature. Again the unbound oligonucleotides were isolated by centrifugation at 10,000 rcf for 1 min and then incubated with the SEA immobilized sepharose beads for 30 min with rotation at RT. To remove unbound and weakly bonded aptamer, sepharose beads were washed five times with phosphate buffer saline (PBS 1X, 137mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 2 mM KH_2PO_4, pH 7.4). In the next step, the bound DNA was eluted by the addition of 400µL of glycine-HCl (100 mM, pH 2.5) for 30 min with rotation at RT. The eluted complexes were extracted through precipitation with ethanol (Schneider et al., 2017).The collected aptamers were amplified by the asymmetric polymerase chain reaction (PCR) (Heiat et al., 2016), and the process was repeated up to the fourth round as described before.

In the second SELEX, each round consisted of two steps, and specified peptide 1 and 2 were immobilized on separated CNBr-activated sepharose 4B. In the first step, the recovered and amplified ssDNA libraries from the previous round were incubated with "peptide 1 column" for 30 min with rotation at room temperature (RT). The bound ssDNAs were recovered and amplified as described above. Then the same process was repeated for the second step via incubation of amplified ssDNA with "peptide 2 column" for 30 min with rotation at RT. The process was repeated until the final round of SELEX with increasing stringency of washing, decreasing concentrations of ssDNA oligonucleotides and shortening the incubation time. Asymmetric PCR method for amplification of oligonucleotide was done with a total volume of 25 µL including 1 µM of forward and reverse primer (Table 1), 12.5 µL Taq DNA polymerase (Master Mix 2X) and 3 - 0.37 nM DNA template. The DNA was then amplified by 19 successive cycles of denaturation at 95°C for 45 s, primer annealing at 59°C for 30 s, and DNA chain extension at 72°C for 30 s.

ssDNA pool binding assay

Four rounds of ST-SELEX (R7, R8, R9 and R10) were selected for evaluation of the ssDNA pool binding ability by ELONA. Each enriched pool was amplified with biotinylated forward SEA aptamer primers (ratio 40/1) by the asymmetric PCR. The products were denatured at 94 °C for 10 min and subsequently cooled in an ice-bath for 5 min. For the binding assay, Maxi-binding 96 wells strips plates (SPL Company, Korea) were coated overnight with 2 µg of SEA protein in 100 µL coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6) at 4 °C (except $Protein^-$ and $Aptamer^-/Protein^-$ wells as controls). On the following day, wells were blocked with 200 µL blocking buffer (3% BSA) and incubated for 2 hours at 37°C. Then the wells were washed 3 times with 300 µL PBS+1% Tween 20 (PBST). The amplified libraries from each selection rounds were diluted to 100 nM with binding buffer and were added to each well (except $Aptamer^-$ and $Aptamer^-/Protein^-$ wells as controls) and incubated for 1 hour at room temperature with shaking at 100 rcf. Subsequently, each well was washed 3 times with 300 µL of 1% PBST and coated with 1:1500 diluted HRP-conjugated streptavidin in PBST and incubated for 40 min on a shaker incubator at RT. Finally, the wells were washed 8 times and TMB substrate was added and

incubated for 15 min. The reaction was stopped with 0.3 M H_2SO_4. The color assay was measured at 450 nm with reference measurement at 620 nm using the (BIORAD) microplate reader.

Cloning, sequencing and bioinformatic analysis

After measurement binding assay of the four rounds of SELEX, round 9 ssDNA pool was selected, purified and cloned using the ClonJET PCR Cloning kit as described by the manufacturer and then transformed into *Escherichia coli* DH5-α host. On the following day, 20 colonies were randomly selected for colony screening by digestion. The extract plasmids were analyzed by single digestion with *BglII* that was available in both sides of aptamer cloning site in the vector map. Subsequently, Positive colonies (14 colonies) were selected and binding affinity to SEA protein was measured with ELONA (as described before). Five colonies with high affinity were sequenced using a pJET Forward primer. The aptamer sequences were analyzed by CLC sequence viewer software. The folding structure and hybridization secondary structure of candidate aptamer was predicted by Mfold web server (http://unafold.rna.albany.edu/).

Measurement of the dissociation constants (K_D)

The dissociation constant (K_D) of candidate aptamer was measured by surface plasmon resonance (SPR). In the SPR measurement (Autolab ESPRIT instrument), HEPES buffer was used as the running buffer and 50 µg/mL of SEA protein (dissolved in 10 mM acetate buffer pH 6.0) was immobilized onto SPR-chip. Aptamers at various concentrations (100, 50, 25 and 12.5 nM) were then injected as analytes. The association and disassociation of the aptamer–SEA conjugates were observed for 900 and 600 seconds each. The regeneration of the protein coated surface was achieved with a 120s pulse (200 mM glycine/HCl buffer pH 2.5). Statistical analysis of the K_D values was measured by using kinetic Evaluation Software.

Detection and cloning of the SEA enterotoxin

160 *staphylococcus aureus* strains were isolated from 200 patients with a skin infection and all samples were collected from an educational hospital in Tehran, Iran. The Pattern of antimicrobial resistance was

examined by previously reports that described the process (Habibian et al., 2014). The genomic DNA was extracted using the DNA extraction kit (Roche, Germany) according to manufacturer's instruction. The presence of *sea* gene was analyzed with SEA specific primers and PCR technique. A PCR method was performed with a total volume of 25 µL including 2 pM of forward primer, 2 pM of reverse primer (Table 1), 12.5 µL *Taq* PCR Master Mix (2X) and 100 ng DNA template. Each mixture was heated at 94 °C for 3 min before 25 cycles of amplification (denaturation at 94 °C for 1 min, annealing at 57 °C for 30 s, extension at 72 °C for 1 min) in a S1000™ Thermal Cycler (BioRad USA). After detection, cloning of *sea* gene was done with SEA cloning primer (Table 1). PCR was performed in a final volume of 25 µl containing 1X PCR buffer, 0.2 Mm dNTPs mix, 4 mM $MgSO_4$, 2 pM of each primer and 5 U *Pfu* DNA polymerase (Thermo Fisher Scientific, USA)**.** The PCR program was as follows: initial denaturation at 94 °C for 5 min, denaturation at 94 °C for 1 min, annealing at 59 °C for 30 s and extension at 72 °C for 90 s (35 cycles), with the final extension at 72 °C for 5 min. The PCR products were digested with the restriction enzymes *Eco*RI and *Hin*dIII and finally confirmed by sequencing (SinaClon, Iran), then cloned in pET28a expression vector and transformed into *E.coli* strain BL21 (DE3) by electroporation (BioRad, USA).

Expression and purification of the recombinant SEA

A single colony of *E.coli* BL21 (DE3) containing pET-SEA was picked up and grown in Luria Bertani (LB) broth supplemented with 40 µg/ml kanamycin. Expression of the SEA protein was induced at OD600 of 0.8 by the addition of 1 mM IPTG and was analyzed by using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The protein was purified by batch purification of His-tagged proteins from *E. coli* under denaturing conditions and using Ni–NTA affinity chromatography (Qiagen, USA). The denaturant (8 M urea) was removed by stepwise dialysis.

Western blot analysis, animal immunization and quantification of IgG antibody

Western blot analysis, animal immunization and quantification of IgG antibody were carried out according to our previous study (Sedighian Rad et al., 2013). Briefly, for western blot analysis, the purified protein (rSEA) was transferred to nitrocellulose membrane and after blocking with 5% bovine

serum albumin (BSA) the membrane was incubated with anti-His-tag-peroxidase antibody. Finally, detection was carried out using BM Blue pod precipitation (Roche-Germany)

For animal immunization and IgG quantification, five-week-old BALB/c mice were divided into test and control groups and animals were housed and treated in compliance with regulations of the International Council on Animal Care. In the test group, each mouse was injected subcutaneously 4 times at 2-week intervals with recombinant protein (SEA) and complete and incomplete Freund's adjuvant. After collecting blood samples from the mice (a week after each booster dose), antigen-specific antibody responses were determined by an indirect enzyme linked immune sorbent assay (ELISA). Polystyrene 96-well plates were coated with 5 µg of rSEA protein and after blocking nonspecific binding sites with 5% BSA, serum samples were added to the ELISA plates. Then anti-goat IgG-HRP was added to all wells. Finally, a TMB substrate was added to each well and after stopped with 0.3M H_2SO_4, the color absorbance was measured at 450 nm.

Preparation of antibody-conjugated Protein G sepharose beads for SEA capture

To conjugate polyclonal SEA antibody to Protein G sepharose beads (Ab-PGs), 100 µL of Protein G sepahrose beads were washed three times with 500 µL of 100 mM Tris-HCl pH 8 and 10 mM Tris-HCl pH 8. After washing, 20 µl of mouse serum diluted in 100 µl of 10 mM Tris-HCl pH 8 was added and incubated at 37 °C under shaking (120 rcf) for 60 min. After incubation, serum solution was removed by centrifugation at 10,000 rcf for 1 min and Ab-PGs were washed three times with 500 µl of 10 mM Tris-HCl pH 8.

Detection of SEA using Ab-PG and real-time PCR

20 µL of Ab-PGs were dispersed in 1 ml of SEA protein in various concentrations (4.63, 2.31, 1.18, 0.587, 0.286, 0.146 and 0.073pmol) containing PBS solution. For detecting cross binding of aptamer with other enterotoxin, Ab-PGs were also dispersed in 1 mL of SEE, SED and SEB (50 ng/ml). The mixture was incubated at 37 °C under shaking (120 rcf) for 1 h. After incubation, protein solution was removed by centrifugation at 10,000 rcf for 1 min and Ab-PG-SEA was washed three times with 500 µL of 10 mM Tris-HCl pH 8. Ab-PG–SEA complex was resuspended in 100 µL of 25 nM aptamer (optimum

concentration) and incubated at 37 °C under shaking (120 rcf) for 1 hour. Next the mixture was removed by centrifugation at 10,000 rcf for 1 min and Ab-PG-SEA-Aptamer complex was washed three times with 500 µL of 10 mM Tris-HCl pH 8. Then, the bound aptamer was heat-eluted in 50 µL of ultrapure water at 95 °C for 20 min. Finally, 3 µL of the eluted aptamer was used for quantification through qPCR. Finally, aptamer was amplified with *Taq* DNA polymerase using a SYBR® Green master mix. Samples were heated at 95 °C for 3 min followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 59 °C for 20 s, extension at 72 °C for 25s and hold on 4 °C. The data were analyzed with the Rotor-Gene 6000 Series Software (Corbett Life Science, USA).

Statistical analysis

All statistical analysis was performed using SPSS 21.0 statistical program. Student *t*-test was used to analyze the data for antibody responses between immunized and non-immunized groups. A p-value of < 0.05 was considered statistically significant.

Result

Bioinformatics analysis

The amino acid (AA) sequences of SEA, SEE were retrieved from online gene banks. Blast and sequence alignment comparison by Clustal Omega, illustrated the highly conserved sequences between SEA and SEE (about 83%) and there were just two unique short peptides (8 amino acids) on the surface of SEA protein (Fig. 2). The amino acid sequences of RYLQEKYN and TSTEPSVN were selected for the ST-SELEX. The position of the peptide, the number of unique amino acids, hydrophobicity index and the number of positively and negatively charged residues of selected peptide are shown in Table 2.

Staggered Target SELEX (ST-SELEX)

In this study, we used a library containing approximately 10^{14} (40 random nucleotides) different ssDNAs. In the first step (4 rounds) of *in vitro* ST-SELEX selection, we incubated the library with whole SEA protein, but in the second step, we incubated the enriched library of ssDNAs with both peptides 1 and 2, separately. We recovered and amplified ssDNAs that bound to either proteins with asymmetric PCR and

repeated this step during rounds 4–10 of SELEX using increasing stringency of washing and decreasing concentrations of ssDNA aptamer and shortening the incubation time. The details were shown in table 3.

ssDNA pool binding assay with ELONA

Indirect ELONA was used for evaluation of pool binding assay (Fig. 3). After 10 rounds of ST-SELEX, four rounds of R7, R8, R9 and R10 were selected and our result showed that round 9 with optical density (OD) of 1.814 ± 0.212 and CV = 7.8 % had the best binding capacity.

Sequencing and bioinformatic analysis of isolated aptamer

After that the enriched ssDNA pool of the 9th round was cloned, the isolated plasmids were analyzed by single digestion with *BglII* which is available in both side of aptamer cloning sites in the vector map (Fig. 4-A). We isolated 14 colonies for affinity analysis. According to high binding capacity, 5 clones (C5, C7, C10, C13 and C16) were chosen (Fig. 4-B) and sequenced. 2-D structures of the candidate aptamers, obtained from Mfold web server (Fig. 5).

Measurement of KD values by SPR analysis

The SPR analysis was performed to check the affinity of selected aptamer to SEA. The interaction of various concentrations of aptamers with 50 µg/mL immobilized SEA is demonstrated in Fig.6. The K_D for the interaction of round 9 with SEA was estimated to be 7.44 ± 0.6 of nM.

Detection, cloning, expression and purification of the SEA

After analyzing 160 *staphylococcus aureus* strains with SEA detection primers and PCR technique (Fig. 7-A), 12 strains were isolated by the presence of *sea* gene. We amplified *sea* by PCR using specific primers and the PCR product (720 bp) is shown in Fig 7-B. The gene was cloned in pET28a vector and then transformed into *E. coli* Dh5α and BL21 (DE3). Then, pET28a-SEA plasmids were extracted and digested by *Eco*RI/*Hin*dIII and analyzed by 1 % agarose gel (Fig. 7-C). The recombinant SEA protein with N-terminal His-tag was purified by Ni-NTA affinity chromatography. SDS-PAGE analysis of the purified product is demonstrated in Fig. 7-D.

Western blot analysis and quantification of IgG antibody

The expression of SEA recombinant protein was confirmed using anti-His-Tag antibody with western blotting (Fig. 8-A). Immunized mice with a purified SEA protein showed significant SEA-specific IgG antibodies (Fig. 8-B). Compared to control antisera, the anti-SEA IgG antibody titer was shown to be significantly high ($p < 0.05$), diluted 1:128,000.

Preparation of antibody-conjugated beads, sandwich aptamer binding and real-time PCR of aptamer

The anti-SEA polyclonal antibody was conjugated to sepharose beads by using protein G as a linker. The amount of conjugated antibody was evaluated by the difference of the antibody contents, measured by the Bradford protein assay, before and after the conjugation. About 0.5 mg of antibody was conjugated onto 100 µL PGs, then Ab-PGs were dispersed in 1 ml solution with various concentrations of SEA protein. After incubation for 1 hour, sepahrose beads with captured SEA (Ab-PGs-SEA) were washed using 10 mM Tris-HCl pH 8 and the complex was incubated with SEA specific aptamers for a sandwich binding of aptamers onto captured SEA. The aptamers bound to SEA were released from the Ab-PGs-SEA complex by heating and the released aptamers were quantified by qPCR (Fig. 9). The threshold cycle (CT), defined by the number of amplification cycles to get the fluorescent signal in 0.10, was used as an indicator for the concentration of SEA in this experiment. The limit of detection (LOD) of SEA using this method was 146.67 fmol.

Discussion

In this paper, we propose an efficient SELEX approach to isolate non-cross-reactive aptamers against similar proteins such as Staphylococcal enterotoxins. SELEX is a process for high affinity isolating DNA or RNA aptamer against molecular targets from random sequence libraries (Stoltenburg et al., 2007). A few years after the presentation of SELEX methods, its modifications were investigated for isolated novel and versatile aptamers with regard to optimizing binding to the target or to increase the stability of the selected aptamers. For instance, Burke and Willis, used two different pools of RNA oligonucleotides to obtain chimeric dual-function RNAs aptamer for a wide variety of applications (Burke and Willis, 1998). Vater *et al*. developed Tailored-SELEX that identified aptamers which were useful for Spiegelmers

without primer binding sites (Vater et al., 2003). Wang *et al*. designed subtractive SELEX for specific isolated ssDNA aptamer that could distinguish a subtype of cells from cells of homologous origin (Wang et al., 2003). Bianchini *et al*. switched between the native and the denatured proteins in order to obtain aptamers called oligobodies by using only one selection step (Bianchini et al., 2001). Rhodes *et al*. protected the RNA aptamers from degradation using nucleases to substitute the 2´-OH groups of ribose with 2´-O-methyl (Rhodes et al., 2000) or Green *et al.* and Ruckman *et al.* substituted the 2´-OH groups of ribose with 2´-NH 2 groups and 2-F(Green et al., 1995; Ruckman et al., 1998). White *et al*. designed a SELEX (Toggle SELEX) to selected aptamer with cross-reactivity to two homologous targets that isolated aptamers could bind to both proteins (White et al., 2001).

From the short review above, the paper present an innovative SELEX procedure (ST-SELEX). The results obtained from this technique confirm that this is able to isolate non-cross-reactive aptamers against two homologous proteins such as staphylococcal enterotoxins.

The applicability of these obtained results is then tested by qPCR. For this purpose, the sandwich apta-PCR was performed using polyclonal antibody as the capture and the aptamer was used as the detection biomolecules to identify SEA. According to our result shown in figure 9, the cross-reactivity of the isolated anti-SEA aptamers by ST-SELEX with SEB, SED, and SEE were insignificant.

The function of ST-SELEX was very similar to subtractive SELEX (Wang et al., 2003), but this modified SELEX can be used for distinguishing between similar proteins. This method is a complete inspiration of other SELEX methods including Toggle SELEX (White et al., 2001), Tailored-SELEX (Vater et al., 2003), Multi-stage-SELEX (Wu and Curran, 1999) and Switching-SELEX (Bianchini et al., 2001) to isolate the specific non-cross-reactive aptamers against two or more proteins with high sequence similarity. However, this method had some drawbacks. It was time-consuming and labor-intensive in comparison with other modified SELEX techniques mentioned above.

Using ambiguous targets in a periodic schedule was the major application of this method. Therefore “staggered” with the dual concept (ambiguous and periodic) was selected to denominate this new designed SELEX method.

ST-SELEX required preliminary studies such as *in silico* analysis or experimental data to find specific sequences (Mahboobi et al., 2017). Herein, we used the bioinformatics study for determination of specific short sequences on the surface of target proteins because the placement of selected region on the surface of protein is very important to isolate efficient aptamers. According to our *in silico* analysis shown in figure 2, there were just two short peptides toward the SEA protein (position 161-168 and 188-195) with the condition of interest.

In the current study, in order to isolate high affinity and high specificity aptamers without significant cross-reactivity, we used both peptides during the SELEX procedure, separately. Then the ST-SELEX was carried out in two steps described in details in table 3.

In this research, based on our results shown in figure 3 and 4, we could isolate aptamers against SEA via ST-SELEX. Additionally, because of simplicity to use, label-free and real-time detection of aptamer–ligand interactions of SPR method (Subramanian et al., 2013), affinity of the selected aptamers from round 9 SELEX was determined by SPR binding assays.

In this research, according to our results shown in figure 6 and 9, we could increase the dissociation constants (K_D) and the limit of detection (LOD) about 6.5 and 2.5 fold, respectively which was in contrast with a prior study (Huang et al., 2014). This contradiction is likely due to the different methods of SELEX used, as noted above. On the other hand, apta-real time PCR has been used for ultrasensitive detection of targets such as, proteins and cells especially, cancer cells and even bacteria with various strategies with more than 20000-fold improvement in the limit of detection (Civit et al., 2016; Lee et al., 2009; Pinto et al., 2014).

Finally, according to the afore mentioned problems including cross-reactivity and sensitive detection of the current approaches, the ST-SELEX and qPCR methods were used simultaneously for detection of staphylococcal enterotoxins for the first time. Based on comparing our results to those of older studies (Huang et al., 2014), it seems that apta-real time PCR for LOD is simpler and more sensitive than water-soluble GO sensor used in the Huang et al. study.

Conclusion:

In this study a novel modification of SELEX process (ST-SELEX) was used to isolate specific aptamers against two or more proteins with high sequence similarity. The ssDNA aptamers from round 9 SELEX were characterized to have high affinity and specificity to their target without significant cross-reactivity that was evaluated by real-time PCR, thus, validating the methodology for ST-SELEX. This strategy facilitates the isolation of specific aptamers for diagnostic and therapeutic applications. However this method had some disadvantages such as being time-consuming and labor-intensive. To the best of our knowledge, this study is the first to use apta-real time PCR for the detection of SEA enterotoxin. The dissociation constant (K_D) value and the limit of detection (LOD) of the isolated aptamer were determined as 7.44 ± 0.6 nM and 146.67 fM.

Funding

This study was funded by grant from Iranian National Sciences Foundation (INSF) Number: 93041908

Conflict of Interest

No conflict of interest declared.

Acknowledgments

This article has been extracted from a PhD thesis, which approved and supported by applied Biotechnology Research Center and applied microbiology Research Center, Baqiyatallah University of medical Sciences, Tehran, Iran. The authors would like to express their gratitude to all researchers and colleagues atBaqiyatallah University of Medical Sciences for their sincere support during carrying out this research.

References:

Agheli, R., Emkanian, B., Halabian, R., Mehrabadi, J.F., Fooladi, A.A.I., (2016) Recombinant Staphylococcal Enterotoxin Type A Stimulate Antitumoral Cytokines. Technology in Cancer Research & Treatment, 1533034616679344.

Balaban, N., Rasooly, A., (2000) Staphylococcal enterotoxins. International journal of food microbiology 61, 1-10.

Berman, H.M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T.N., Weissig, H., Shindyalov, I.N., Bourne, P.E., (2000) The protein data bank. Nucleic acids research 28, 235-242.

Bianchini, M., Radrizzani, M.ج., Brocardo, M.G., Reyes, G.B., Solveyra, C.s.G., Santa-Coloma, T.s.A., (2001) Specific oligobodies against ERK-2 that recognize both the native and the denatured state of the protein. Journal of immunological methods 252, 191-197.

Bruno, J.G., Kiel, J.L., (2002) Research report use of magnetic beads in selection and detection of biotoxin aptamers by electrochemiluminescence and enzymatic methods. Biotechniques 32, 178-183.

Burke, D.H., Willis, J.H., (1998) Recombination, RNA evolution, and bifunctional RNA molecules isolated through chimeric SELEX. Rna 4, 1165-1175.

Civit, L., Pinto, A., Rodrigues-Correia, A., Heckel, A., O'Sullivan, C.K., Mayer, G., (2016) Sensitive detection of cancer cells using light-mediated apta-PCR. Methods 97, 104-109.

DeGrasse, J.A., (2012) A single-stranded DNA aptamer that selectively binds to Staphylococcus aureus enterotoxin B. PLoS One 7, e33410.

Fooladi, A.A.I., Ashrafi, E., Tazandareh, S.G., Koosha, R.Z., Rad, H.S., Amin, M., Soori, M., Larki, R.A., Choopani, A., Hosseini, H.M., (2015) The distribution of pathogenic and toxigenic genes among MRSA and MSSA clinical isolates. Microbial pathogenesis 81, 60-66.

Fooladi, A.I., Tavakoli, H., Naderi, A., (2010) Detection of enterotoxigenic Staphylococcus aureus isolates in domestic dairy products. Iranian journal of microbiology 2, 135-140.

Green, L.S., Jellinek, D., Bell, C., Beebe, L.A., Feistner, B.D., Gill, S.C., Jucker, F.M., Janjiژ‡, N.a., (1995) Nuclease-resistant nucleic acid ligands to vascular permeability factor/vascular endothelial growth factor. Chemistry & biology 2, 683-695.

Habibian, R., Khameneie, M.K., Sedighian, H., Daneshi, F., Moghadam, M.B., Mahboobi, M., (2014) Virulence Factor Diversity Between Imipenem Resistant and Imipenem Susceptible Strains of Escherichia coli Isolated from Hospitalized Patients with Severe Urinary Tract Infections. Biosc Biotech Res Asia 11, 469-477.

Hedayati Ch, M., Amani, J., Sedighian, H., Amin, M., Salimian, J., Halabian, R., Fooladi, I., Ali, A., (2016a) Isolation of a new ssDNA aptamer against staphylococcal enterotoxin B based on CNBrâ€ےactivated sepharoseâB affinity chromatography. Journal of Molecular Recognition.

Hedayati Ch, M., Amani, J., Sedighian, H., Amin, M., Salimian, J., Halabian, R., Fooladi, I., Ali, A., (2016b) Isolation of a new ssDNA aptamer against staphylococcal enterotoxin B based on CNBrâ€ےactivated sepharoseâ€4ےB affinity chromatography. Journal of Molecular Recognition 29, 436-445.

Heiat, M., Ranjbar, R., Latifi, A.M., Rasaee, M.J., Farnoosh, G., (2016) Essential Strategies to Optimize Asymmetric PCR Conditions as a Reliable Method to Generate Large Amount of ssDNA Aptamers. Biotechnology and applied biochemistry.

Huang, Y., Chen, X., Xia, Y., Wu, S., Duan, N., Ma, X., Wang, Z., (2014) Selection, identification and application of a DNA aptamer against Staphylococcus aureus enterotoxin A. Analytical Methods 6, 690-697.

Kong, H.Y., Byun, J., (2013) Nucleic Acid aptamers: new methods for selection, stabilization, and application in biomedical science. Biomol Ther (Seoul) 21, 423-434.

Lee, H.-J., Kim, B.C., Kim, K.-W., Kim, Y.K., Kim, J., Oh, M.-K., (2009) A sensitive method to detect Escherichia coli based on immunomagnetic separation and real-time PCR amplification of aptamers. Biosensors and Bioelectronics 24, 3550-3555.
Liu, A., Zhang, Y., Chen, W., Wang, X., Chen, F., (2013) Gold nanoparticle-based colorimetric detection of staphylococcal enterotoxin B using ssDNA aptamers. European Food Research and Technology 237, 323-329.
Lu, C., Tang, Z., Liu, C., Kang, L., Sun, F., (2015) Magnetic-nanobead-based competitive enzyme-linked aptamer assay for the analysis of oxytetracycline in food. Analytical and bioanalytical chemistry 407, 4155-4163.
Mahboobi, M., Sedighian, H., CH, M.H., Bambai, B., Soofian, S.E., Amani, J., (2017) Applying bioinformatic tools for modeling and modifying type II E. coli L-Asparginase to present a better therapeutic agent/drug for acute lymphoblastic leukemia. International Journal of Cancer Management 10.
Pinto, A., Polo, P.N., Henry, O., Redondo, M.C.B., Svobodova, M., and Sullivan, C.K., (2014) Label-free detection of gliadin food allergen mediated by real-time apta-PCR. Analytical and bioanalytical chemistry 406, 515-524.
Putz, K.W., Compton, O.C., Palmeri, M.J., Nguyen, S.T., Brinson, L.C., (2010) High-Nanofiller-Content Graphene Oxide–Polymer Nanocomposites via Vacuum-Assisted Self-Assembly. Advanced Functional Materials 20, 3322-3329.
Rhodes, A., Deakin, A., Spaull, J., Coomber, B., Aitken, A., Life, P., Rees, S., (2000) The generation and characterization of antagonist RNA aptamers to human oncostatin M. Journal of Biological Chemistry 275, 28555-28561.
Rhouati, A., Yang, C., Hayat, A., Marty, J.-L., (2013) Aptamers: A promising tool for ochratoxin A detection in food analysis. Toxins 5, 1988-2008.
Ruckman, J., Green, L.S., Beeson, J., Waugh, S., Gillette, W.L., Henninger, D.D., Claesson-Welsh, L., Janjic, N., (1998) 2â€²-Fluoropyrimidine RNA-based aptamers to the 165-amino acid form of vascular endothelial growth factor (VEGF165) Inhibition of receptor binding and VEGF-induced vascular permeability through interactions requiring the exon 7-encoded domain. Journal of Biological Chemistry 273, 20556-20567.
Sano, T., Smith, C.L., Cantor, C.R., (1992) Immuno-PCR: very sensitive antigen detection by means of specific antibody-DNA conjugates. Science 258, 120-122.
Sayers, E.W., Barrett, T., Benson, D.A., Bolton, E., Bryant, S.H., Canese, K., Chetvernin, V., Church, D.M., DiCuccio, M., Federhen, S., (2011) Database resources of the national center for biotechnology information. Nucleic acids research 39, D38-D51.
Schlievert, P.M., Bohach, G.A., Ohlendorf, D.H., Stauffacher, C.V., Leung, D.Y., Murray, D.L., Earhart, C.A., Jablonski, L.M., Hoffmann, M.L., Chi, Y.-I., (1995) Molecular structure of staphylococcus and streptococcus superantigens. Journal of clinical immunology 15, S4-S10.
Schneider, D., Wemheuer, F., Pfeiffer, B., Wemheuer, B., (2017) Extraction of Total DNA and RNA from Marine Filter Samples and Generation of a cDNA as Universal Template for Marker Gene Studies. Metagenomics: Methods and Protocols, 13-22.
Sedighian, H., Halabian, R., Amani, J., Heiat, M., Taheri, R.A., Fooladi, A.A.I., (2018) Manufacturing of a novel double-function ssDNA aptamer for sensitive diagnosis and efficient neutralization of SEA. Analytical biochemistry 548, 69-77.
Sedighian Rad, H., Mousavi, S.L., Rasooli, I., Amani, J., Nadooshan, M.R.J., (2013) EspA-Intimin chimeric protein, a candidate vaccine against Escherichia coli O157: H7. Iranian journal of microbiology 5, 244.
Stoltenburg, R., Reinemann, C., Strehlitz, B., (2007) SELEX—a (r) evolutionary method to generate high-affinity nucleic acid ligands. Biomolecular engineering 24, 381-403.

Subramanian, P., Lesniewski, A., Kaminska, I., Vlandas, A., Vasilescu, A., Niedziolka-Jonsson, J., Pichonat, E., Happy, H., Boukherroub, R., Szunerits, S., (2013) Lysozyme detection on aptamer functionalized graphene-coated SPR interfaces. Biosensors and Bioelectronics 50, 239-243.
Svobodova, M., Mairal, T., Nadal, P., Bermudo, M.C., and Sullivan, C.K., (2014) Ultrasensitive aptamer based detection of β-conglutin conglutin food allergen. Food chemistry 165, 419-423.
Vater, A., Jarosch, F., Buchner, K., Klussmann, S., (2003) Short bioactive Spiegelmers to migraine-associated calcitonin gene-related peptide rapidly identified by a novel approach: Tailored-SELEX. Nucleic acids research 31, e130-e130.
Wang, C., Zhang, M., Yang, G., Zhang, D., Ding, H., Wang, H., Fan, M., Shen, B., Shao, N., (2003) Single-stranded DNA aptamers that bind differentiated but not parental cells: subtractive systematic evolution of ligands by exponential enrichment. Journal of biotechnology 102, 15-22.
Wang, Y., Li, Z., Wang, J., Li, J., Lin, Y., (2011) Graphene and graphene oxide: biofunctionalization and applications in biotechnology. Trends in biotechnology 29, 205-212.
White, R., Rusconi, C., Scardino, E., Wolberg, A., Lawson, J., Hoffman, M., Sullenger, B., (2001) Generation of species cross-reactive aptamers using a Toggle- SELEX. Molecular Therapy 4, 567-573.
Wu, L., Curran, J.F., (1999) An allosteric synthetic DNA. Nucleic acids research 27, 1512-1516.
Wu, S., Duan, N., Gu, H., Hao, L., Ye, H., Gong, W., Wang, Z., (2016) A Review of the Methods for Detection of Staphylococcus aureus Enterotoxins. Toxins 8, 176.
Xian Tan, K., (2016) Aptamer-mediated polymeric vehicles for enhanced cell-targeted drug delivery. Current Drug Targets 17, 1-11.
Xiong, X., Shi, X., Liu, Y., Lu, L., You, J., (2018) An aptamer-based electrochemical biosensor for simple and sensitive detection of staphylococcal enterotoxin B in milk. Analytical Methods.
Yousefi, F., Mousavi, S.F., Siadat, S.D., Aslani, M.M., Amani, J., Sedighian Rad, H., Fooladi, A.A.I., (2015) Preparation and In Vitro Evaluation of Antitumor Activity of TGFαL3-SEB as a Ligand-Targeted Superantigen. Technology in Cancer Research & Treatment 15, 215-226.
Zhuo, Z., Yu, Y., Wang, M., Li, J., Zhang, Z., Liu, J., Wu, X., Lu, A., Zhang, G., Zhang, B., (2017) Recent Advances in SELEX Technology and Aptamer Applications in Biomedicine. International journal of molecular sciences 18, 2142.

Figure legends:

Figure 1: A scheme of conceptual project to set up apta-qPCR for detection of enterotoxin type A. five milestones including 1) ST-SELEX (Staggered target-SELEX) for isolated specific aptamers, 2) preparation of recombinant protein with cloning in bacteria, 3) production of mouse polyclonal antibody, 4) assembling of affinity chromatography column with immobilized antibody on protein G sepharose and 5) finally qPCR are decisive steps in development of this diagnostic tool. See text for details.

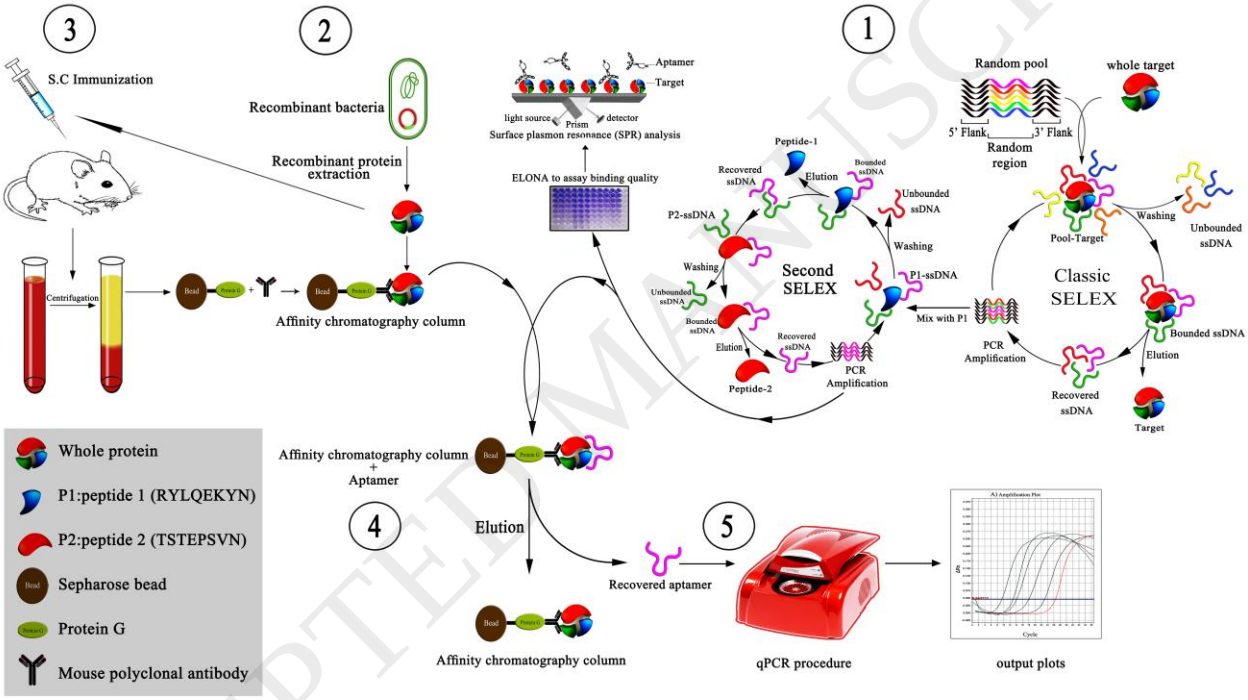


Figure 2. Peptides mapped on the SEA structure. The unique surface accessible peptides are shown as VDM on the structure of SEA.

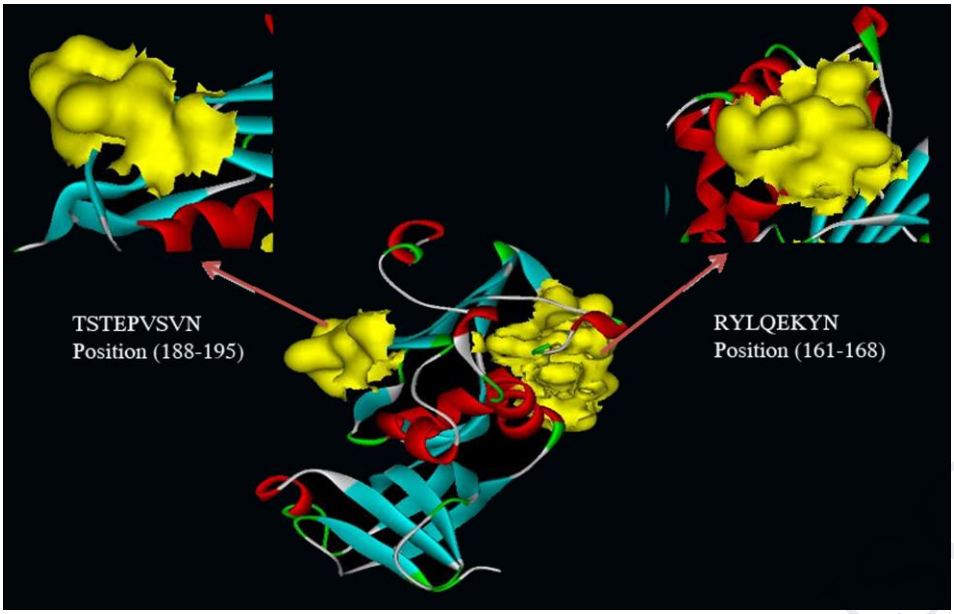


Figure 3. Pool binding assay. Indirect ELONA was carried out for rounds R7, R8, R9 and R10 and round 9 contained the best binding capacity with SEA protein.

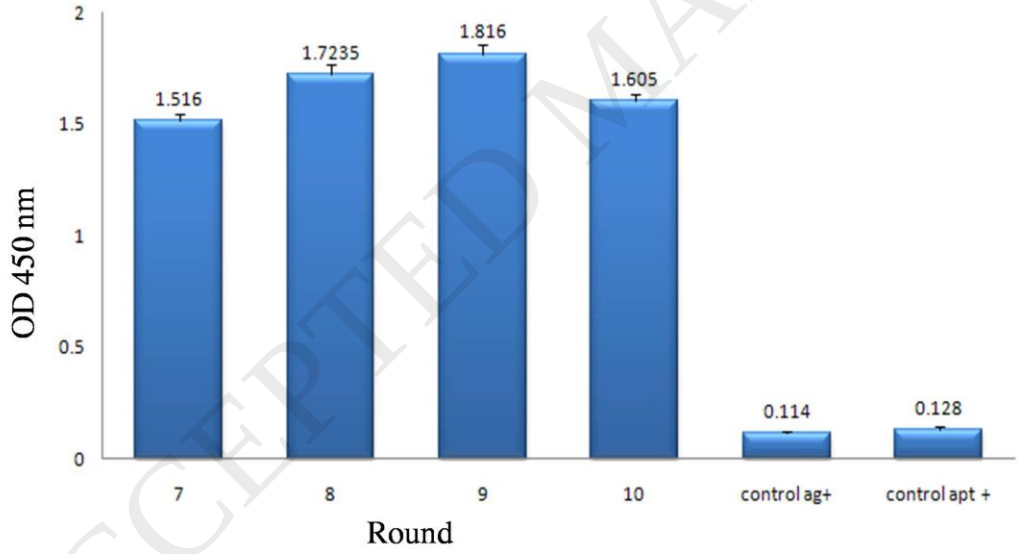


Figure 4. A) Digestion of isolated plasmid. Result show that, clone C2, C3 and C4 are positive clone and clone C1 and C2 are negative colon **B) Binding affinity results.** Binding affinity results of positive clones of Round 9th showed that 5 clones (C5, C7, C10, C13 and C16) are the best clones according to their affinity to SEA and were chosen for sequencing.

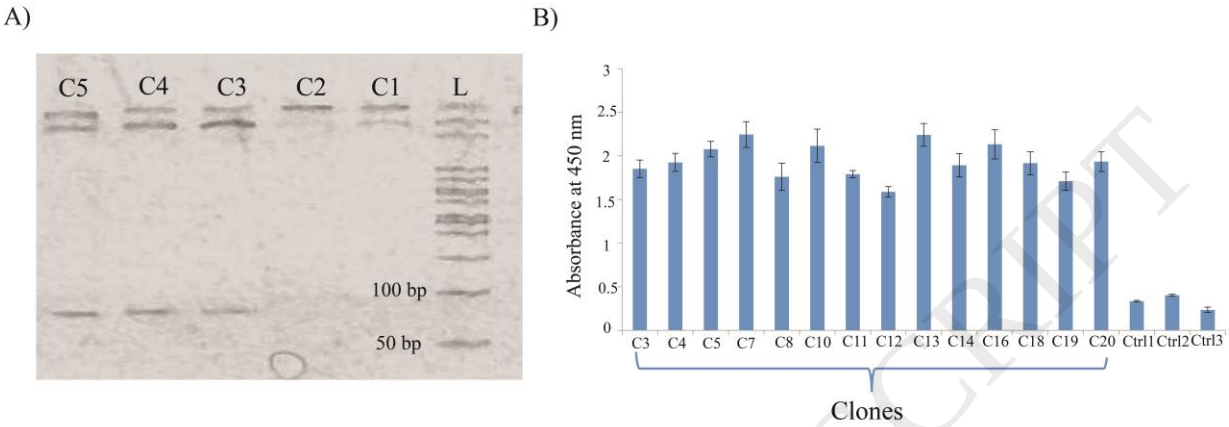


Figure 5. 2D structure of isolated aptamers. Structure of selected clones after sequencing, predict by Mfold Web server and possible structure with best dG were showed.

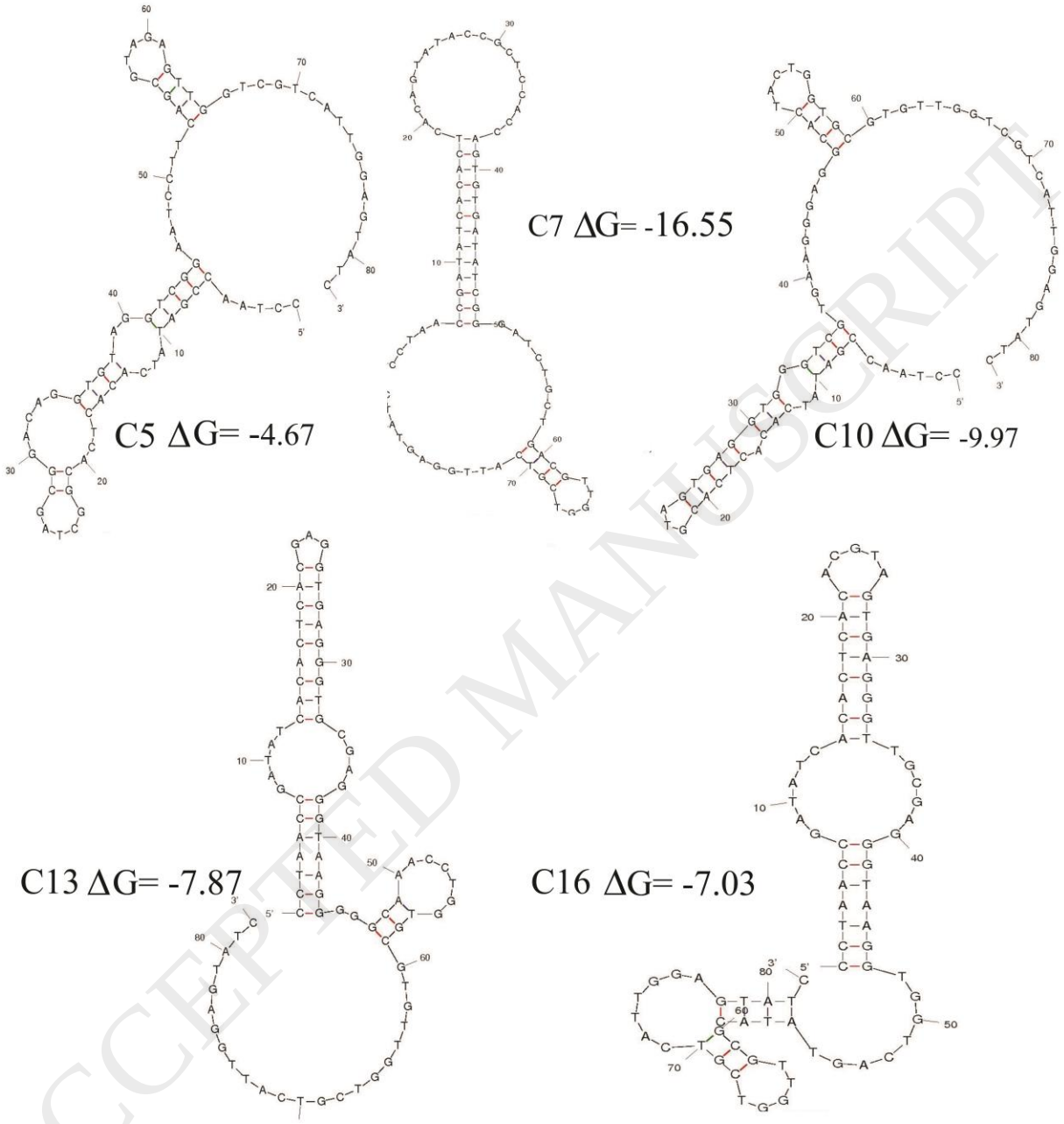


Figure 6: Determination of dissociation constants by SPR. A) SPR sensor response overlay plot for the interaction of different concentrations (12.5–100 nM) of aptamers with 50 ug/mL immobilized SEA protein. B) Langmuir isotherm plot of equilibrium angle (Req) versus aptamers concentration. C) Summery of equilibrium analysis

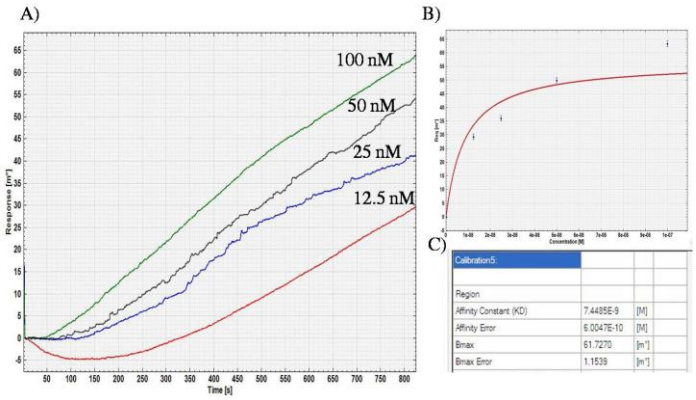


Figure 7: A) Detection of *sea* gene with detection primer and PCR technique. B) Amplify of *sea* gene in order to cloning in expression vector. C) Double digest of recombinant vector by *EcoRI* and *HindIII* restriction enzymes. D) Purification of SEA protein with N-terminal His-tag by Ni-NTA affinity chromatography

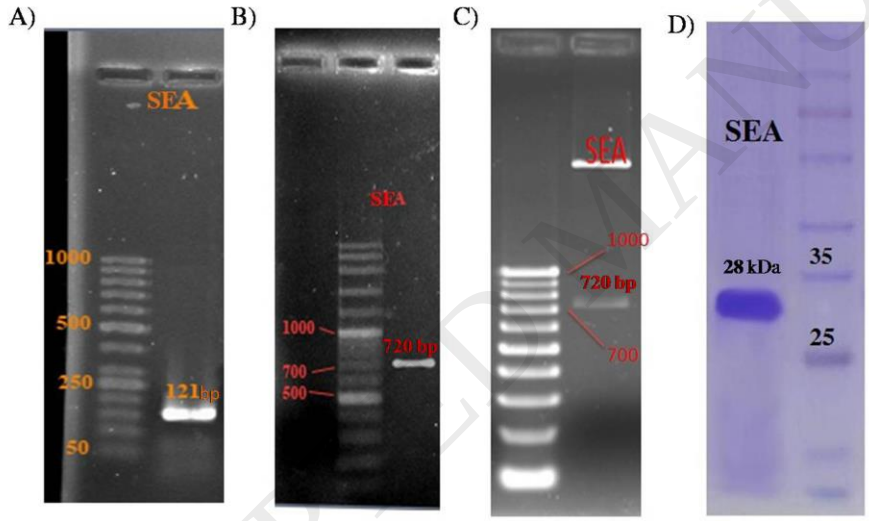


Figure 8: A) Western blotting analysis of SEA protein. T1 purified chimeric recombinant protein and T2 supernatant of induced transformed BL21DE3. B) Serum antibody response of BALB/c mice immunized with recombinant SEA proteins

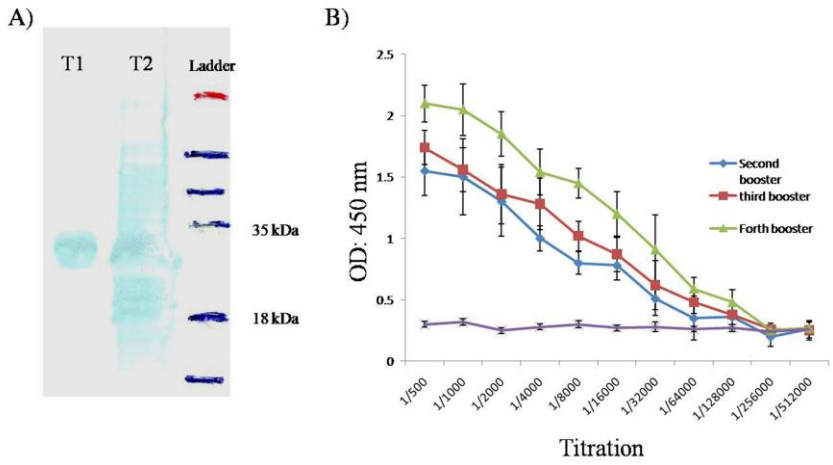
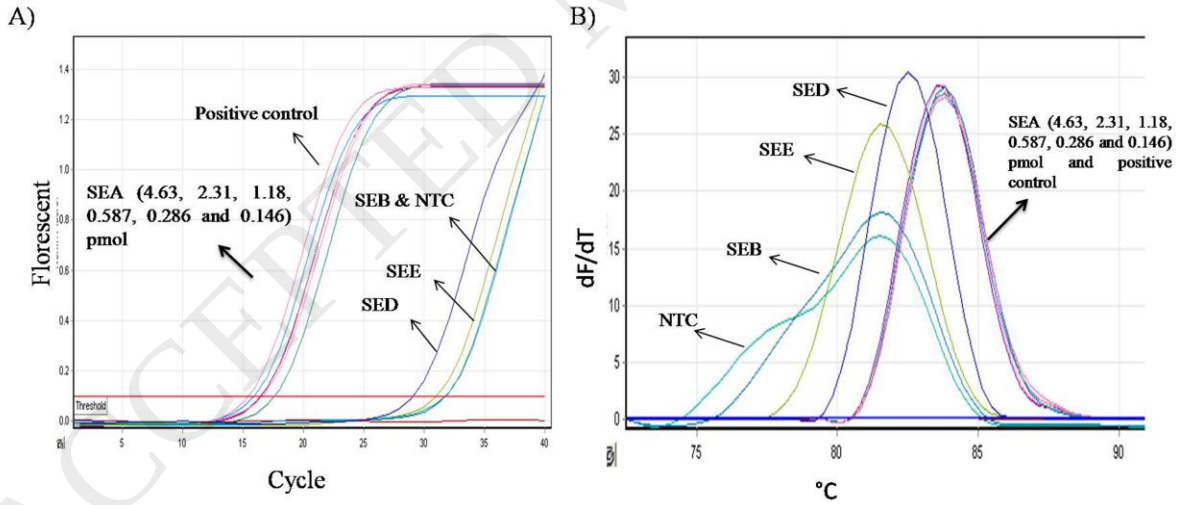


Figure 9: A) Amplification curves of apta-real time PCR. The threshold cycle (CT) value was determined from the number of amplification cycles to reach the fluorescence signal of 0.10. B) Melting curves of apta-real time PCR. The peak of positive control and SEA (4.63, 2.31, 1.18, 0.587, 0.286 and 0.146 pmol) were determined as 83.7 ^{oc}. The peak of SED was determined as 82.5 ^{oc} and the peak of SEE, SEB and NTS were determined as 81.5 ^{oc}. These result indicate that the isolated Aptamer can detected SEA until 146 fmol without any cross-reactivity.



Table

Table -1. Synthesized acid nucleic sequences of were used in these studies

	Name	Seq.	Enzyme	Length	Tm(°c)
	Library ssDNA	CCTAACCGATATCACACTCAC-40N-GTTGGTCGT CATTGGAGTATC	*EcorV*	82	-
	Forward SEA aptamer	CCTAACCGATATCACACTCAC	*EcorV*	21	59.0
	Reverse SEA aptamer	GATACTCCAATGACGACCAAC	-	21	59.0
	Forward SEA detection	TTGGAACGGTTAAAACGAA	-	19	58.5
	Reverse SEA detection	GAACCTTCCCATCAAAAACA	-	20	58.5
	Forward SEA cloning	TATAGAATTCATGAGCGAGAAAAGCGAAG	*EcorI*	29	61
	Reverse SEA cloning	TGCCAAGCTTTCAACTTGTATATAAATATATATC	*HindIII*	34	61

Table 2. The characterization of peptide 1 and 2.

Name	sequence	Position	number of unique AA	Hydrophobicity index	number of positive residues	number of Negative residues	2-D Structure
Peptide 1	RYLQEKYN	161-168	5	0.38	2	1	α helix
Peptide 2	TSTEPSVN	188-195	6	0.19	0	1	Turn

Table-3. Details of 10 rounds STS for isolation of ssDNA aptamers against SEA target

Round	Counter	Time of counter	Amount of ssDNA	Time of incubation	Washing	Number of washing	Time of elution	Amount of recovery

									DNA
1		Negative/ SE(B,C,D,E)	15/15	3 nmol or 75 µg	30 min	PBS	5	30 min	500 ng
2		-	-	2.25 nmol or 60 µg	30 min	PBS	5	30 min	420 ng
3		-	-	2.07 nmol or 55 µg	25 min	PBS	5	25 min	700 ng
4		Negative/ SE(B,C,D,E)	15/15 min	1.88 nmol or 50 µg	22 min	PBS	7	25 min	550 ng
5	1	-	-	1.88 nmol or 50 µg	30min	PBS	5	30 min	200 ng
	2	-	-	1.88 nmol or 50 µg	20 min	PBS	5	30 min	280 ng
6	1	-	-	1.69 nmol or 45 µg	15 min	PBS	7	25 min	725 ng
	2		-	1.69 nmol or 45 µg	15 min	PBS	7	25 min	985 ng
7	1	-	-	1.32 nmol or 35 µg	15 min	PBS+0.02% Tween20	5	20 min	1 µg
	2		-	1.32 nmol or 35 µg	15 min	PBS+0.02% Tween20	5	20 min	1.25 µg
8	1	-	-	0.94 nmol or 25 µg	12 min	PBS+0.02% Tween20	10	12 min	1.1 µg
	2	-	-	0.94 nmol or 25 µg	12 min	PBS+0.02% Tween20	10	12 min	1.8 µg
9	1	Negative/ SE(B,C,D,)	15/10 min	0.75 nmol or 20 µg	10 min	PBS+0.05% Tween20	5	12 min	726 ng
	2	-	-	0.75 nmol or 20 µg	10 min	PBS+0.05% Tween20	5	12 min	1.12 ng
10	1	-	-	0.37 nmol or 10 µg	10 min	PBS+0.05% Tween20	10	10 min	1.2 µg
	2	-	-	0.37 nmol or 10 µg	10 min	PBS+0.05% Tween20	10	8 min	1.4 µg