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Original Article

Utilizing Cell-SELEX, as a Promising Strategy to Isolate ssDNA Aptamer Probes for Detection of *Staphylococcus aureus*

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Abstract

Staphylococcus aureus is one of the most important pathogens in hospital-acquired infections. Annually, many people are infected with *S. aureus* in hospitals. Rapid detection of this bacterium is extremely helpful in preventing and managing this bacterium mediated diseases. Aptamers are powerful probes, which can be used as a target explorer in a wide range of diagnostic systems.To isolate a specific aptamer against *S. aureus*, a library of single-stranded DNA molecules was designed, and enriched through Cell-SELEX procedure. In the Cell-SELEX, the DNA library was exposed to the *S. aureus* bacterium in 8 reiterative quadruple rounds including: binding, separation, elution and amplification. After 8 rounds, the PCR product was cloned and sequenced. Cloned aptameric sequences were evaluated through enzyme-linked oligonucleotide assay (ELONA), and a sequence with the best outcomes was selected as ideal aptamer. Eight rounds of Cell-SELEX procedure led to isolation of a specific ssDNA aptamer against *S. aureus* and named as "*STAPT*" (conflation of *STAphylococcus* and *APTamer*). Using ELONA technique, the detection limit of this aptamer was determined as 4×10^3 CFU/ml. The aptamer "STAPT" showed the promising and potent abilities and features to be utilized as a bio-detection element likely in advanced detection systems. Although more extended researches are needed for this purpose.

Keywords: *Staphylococcus aureus*, Detection, Aptamer, Cell-SELEX, ELONA

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Introduction

Staphylococcus aureus is a pathogenic gram-positive, facultative anaerobe bacterium that can cause infection in humans and animals, ranging from simple skin infections to life-threatening disease. *S. aureus* is considered as one of the five common causes of nosocomial infections [1, 2]. Many people annually are infected with *S. aureus* in hospitals. Currently traditional methods which are often time-consuming and cost-intensive, applied to diagnose *S. aureus*. For instance LCR (ligase chain reaction) and PCR (polymerase chain reaction) account as highly sensitive diagnostic systems [3] that are able to track a small number of bacteria within a few hours but they require some obstacles such as isolation of bacterial DNA, post amplification procedure and expensive instruments to amplify nucleic acid. Such handicapping barriers limit the widespread use of these technologies for clinical diagnosis. Latex Immuno-agglutination Assay (LIA) and ELONA are also employed which are insensitive or time-consuming methods [4, 5]. Accordingly it is essential to develop a fast, inexpensive and nevertheless accurate method to identify *S. aureus* contaminated samples. Aptamer is a single stranded oligonucleotide of DNA or RNA which have an equal or higher sensitivity than antibodies. Aptamers are able to bind specifically to the targets in quantities of pico to nano molar ranges [6, 7]. Unlike antibodies, aptameric probes are not immunogenic, and have long half-life and stability under different conditions. Aptamers are smaller in size, which leadsthem to create more surface density on the receptors and easier binding to the analytes [8]. These probes are isolated via a systematic evolution procedure so-called SELEX (systematic evolution of ligands by exponential enrichment). Indeed SELEX is an in vitro method to isolate target-specific single-stranded oligonucleotides among a random sequence library. SELEX conditions can be massively altered to enrich aptameric sequences with desired characteristics. Developing different SELEX procedures have led to the isolation of large variety of aptamers dedicated to many targets, ranging from small organic molecules to a protein complex or even whole cells. Cell-SELEX is a term that explains the pathway in which whole live cells are employed as target to isolate aptamers (Fig. 1). Thus, it is useful enrichment strategy for lots of applications, including bacterial detection [9]. Aptamers bind to their targets with a specific three-dimensional structure [10].

In the dimensions of research, aptamers are broadly utilized for manufacturing various biosensors so-called apta-sensors. In the past few recent years, aptamers have been exhibited to be worthy substitute for antibodies in serological diagnostic techniques with higher merits. Aptamer probes are appropriate alternative tools to detect interested targets in multifarious formats such as [11], Surface plasmon resonance (SPR), enzyme-linked oligonucleotide assay (ELONA) [12-14], aptamer-linked immobilized sorbent assay (ALISA), lateral flow assay (LFA) [15], fluorescent labeled aptamers and electrochemical and other biosensors [11, 16, 17].

However, the prerequisite for manufacturing an efficient biosensor is to have a capable aptamer. Therefore, in this study we tried to design a Cell-SELEX procedure to isolate a specific aptamer against *S. aureus* bacterium.

Materials and Methods

Materials

To run Cell-SELEX procedure all biological and chemical agents were prepared from reliable companies. Some of the most important agents include; PCR ready master-mix from Topaz gen Co. Lambda exonuclease from Thermo Fisher Inc. HRP-conjugated streptavidin was purchased from Invitrogen Co. PTG19-T vector and other cloning requirements were prepared from Sinaclon Co. *S. aureus* strain, as target, (ATCC 25923), *Streptococcus epidermidis*, (ATCC 12228) and *Streptococcus pyogenes* (ATCC 19615), as counter SELEX, were prepared from reference laboratory.

DNA Library and primers

The ssDNA library (5'-GCCTGTTGTGAGCCTCCTAAC (N38) CATGCTTATTCTTGTCTCCC-3') and primers were designed and synthesized by Metabion Co, Germany which below is shown:

Primer 1	Forward	5′-GCCTGTTGTGAGCCTCCTACC-3′
	Reverses	5′-GGGAGACAAGAATAAGCATG-3′
Primer 2	Forward	5′-biotin-GCCTGTTGTGAGCCTCCTACC-3′
	Reverses	5′-phosphate GGGAGACAAGAATAAGCATG-3′

Cell-SELEX procedure

Synthetic ssDNA library (2 nM) was denatured by heating at 90°C for 5 min and immediately cooled on ice for 10 min. After running the negative SELEX step (removal of cross-reactive aptamers), the denatured ssDNA library was mixed with 1 × 10^7 CFU/ml of *S. aureus* resuspended in 300 ml of screening buffer (Tris-HCl 25 mM, KCl 50 mM, Nacl 200 mM, EDTA 0.2 mM, Glycerol %5, Dtt 0.5 mM)

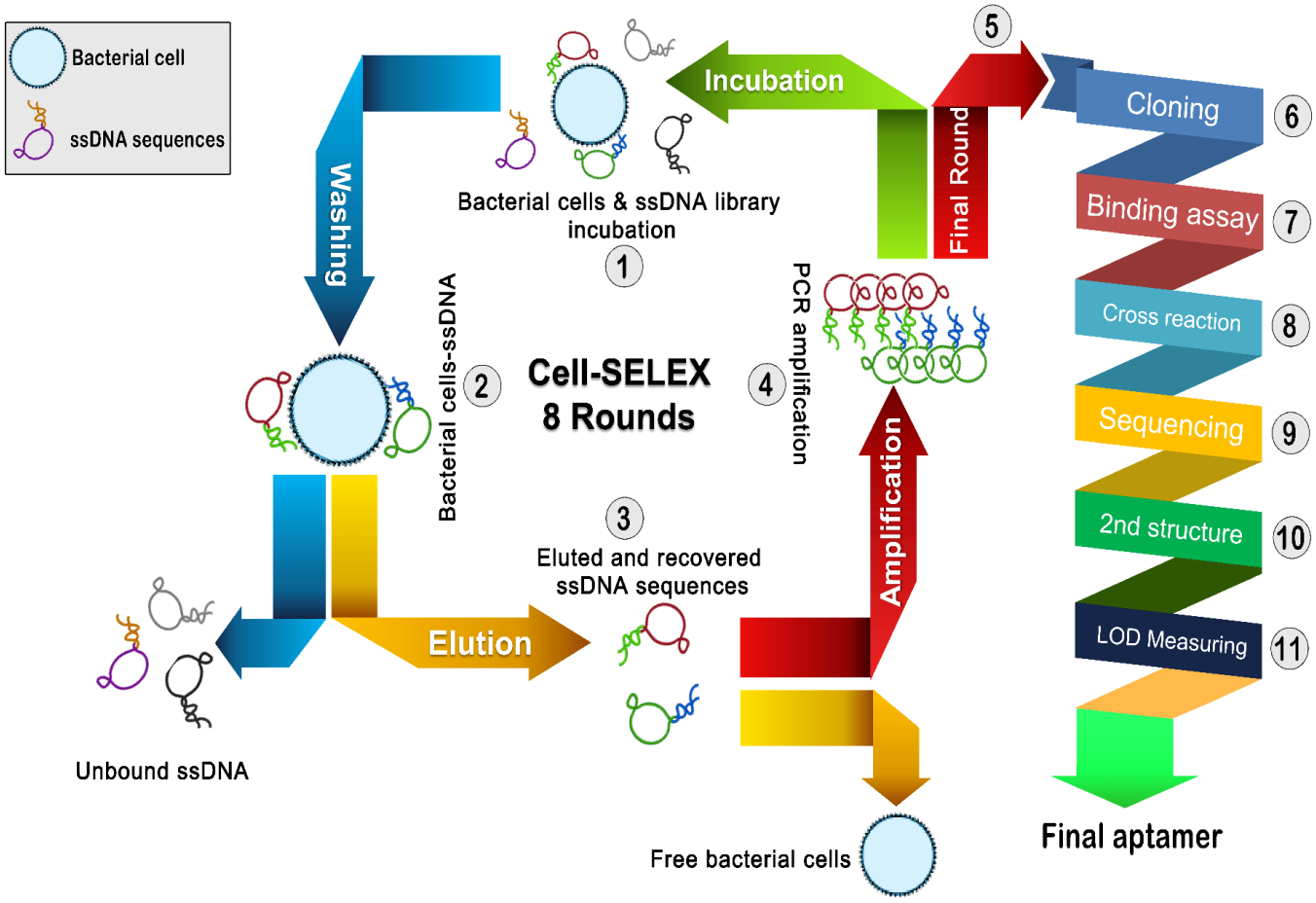

Figure 1. SELEX schematic procedure. Synthesized ssDNA random pool was mixed with bacteria cells at the first round. Unbounded aptamers were discarded and bound aptamers were collected and amplified via asymmetric-PCR. PCR products were served as secondary pool for the second round. After 8 reiterative rounds, the final pool was cloned and then analyzed by enzyme-linked oligonucleotide assay (ELONA). Finally, high binding clones were sequenced and their affinities were analyzed by SPR system.

and was incubated at 37°C for 1 h. In the following, the mixture was centrifuged and the supernatant containing unbound oligonucleotides was discarded. The pellet containing aptamer-bacteria complex was washed 3 times with 200 ml of washing buffer (TBST buffer: PBS buffer plus Tween 0.05%), then 100 ml of distilled water was added and incubated at 80°C for 10 min. Following the centrifugation, the supernatant containing eluted ssDNA was transferred into a new tube and recovered using alcohol-glycogen precipitation procedure (ethanol: 2.5 fold, acetate sodium 0.1 fold and glycogen 0.05 fold of supernatant) and the ssDNA concentration was assessed using optical densitometry in the wavelength of 260 nm.

The recovered ssDNA was amplified by PCR procedure, and after conversion to the single-stranded oligonucleotide (by Lambda exonuclease enzyme), used for the next round of Cell-SELEX. Every SELEX round condition became restricted through reducing the amount of ssDNA pool at each round (2 to 0.1 nM), reducing the incubation time (from 1 h to 45 min), and increasing the number of rounds. The aptamer enrichment repetitive cycles were followed and monitored by ssDNA binding quality. To evaluate the cell-SELEX process, the binding quantity of ssDNA was determined based on the ratio of used to recovered concentration of ssDNA in each Cell-SELEX round. According to this checkpoint assessments, 8 rounds of Cell-SELEX were continued and then the procedure was entered the next step (Fig. 1). The final ssDNA pool were amplified and cloned into *E. coli* DH5α, as cloning host, by means of the PTG19-T vector. In order to identify the cloned aptameric DNA sequences, several randomly selected colonies were sequenced (BioneerDNA sequencing service).

Evaluation of the specificity and binding properties (Quality, cross-reactivity and LOD) of isolated aptamer

Isolated aptamer was biotinylated by 5′-Biotin-forward primers using PCR. Then the indirect ELONA was used to measure the limit of detection (LOD) of aptamers as follows: the microplate was first covered with *S. aureus* (9 consecutive dilutions from 0.5 McFarland with reducing concentration steps of $^1/_{10}$ in each well), then the plate was washed (3 times with 200 ml of PBST buffer). Afterwards, the wells were blocked using blocking buffer (commercially purchased from Roche Co). In the following, 2 µl aptamer (with the concentration of 1 ng/µl PBS) was added in each well and incubated at 37°C with gently shaking for 60 minutes. After washing, 100 µl streptavidin-HRP conjugate was added (1/5000 in PBST buffer) and again incubated at 37°C with gently shaking for 60 minutes. After washing, 100 ml of TMB substrate was added to each well and the microplate was transferred to a dark place. The reaction was stopped by addition of 50 µl of 2.5 M sulfuric acid after 15 min. Finally, the optical density of appeared yellow color was read by ELISA reader at the wavelength of 450 nm. A well without bacteria was considered as negative control. As the final ELONA evaluation, the aptamer cross reactivity with similar bacteria was also assessed against *S. epidermidis* and Streptococcus through the same procedure. All experiments for evaluation of binding quality, cross-reactivity and LOD calculation were repeated at least 3 times and the average values were used as final outputs.

Determination of the secondary structure of aptamers

Secondary structure of the isolated sequence was determined by using of mfold online software (http://mfold.RNA.albany.edu/q=mfold) at the folding temperature of 37°C. The concentration of Na^+ and Mg^{2+} were also adjusted in the base of screening buffer.

Results

Cell-SELEX and PCR

Aptamers obtained at each round of Cell-SELEX need to be amplified for the next round. One of the difficulties in this case was the optimization of PCR conditions at every round. In a general conclusion, reducing the number of PCR cycles, increasing the annealing temperature and diluting the template oligonucleotide pool, resulted in the elimination of smear and unwanted products. By considering these key items, the PCR procedure with the most optimized condition was performed for amplification of recovered ssDNA at the each round (Fig. 2).

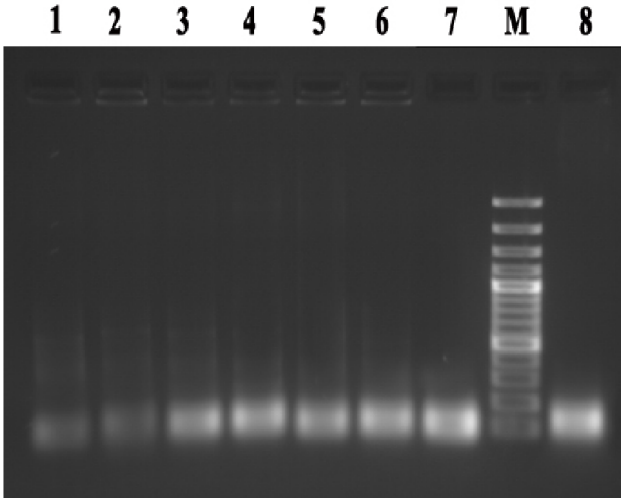

Figure 2. Lane 1 to 8: amplification of recovered ssDNA pool at the end of each round (round 1 to 8), M: DNA marker.

Investigation of SELEX performance

In order to ensure about how the SELEX works and to confirm that the SELEX procedure enriched the oligonucleotide fragments with the higher levels of affinity to bacterial cells, the binding quantity of the all oligonucleotide pools were measured based on the ratio of used to recovered amount of ssDNA for each round of the Cell-SELEX (Fig. 3). According to the chart illustrated at Figure 3, the ssDNA pool of round five (R5) showed the highest binding quantity compared to other rounds even rounds of six (R6), seven (R7) and eight (R8). It could be due to the maximum tendency of oligonucleotides to the target. These findings somehow indicated the end of SELEX in round eight (R8).

Evaluation of binding quality of isolated aptamers

The pool of ssDNA obtained from round eight were cloned and sequenced. Randomly selected cloned were synthesized and their binding qualities were independently

evaluated by ELONA procedure. Analytical results demonstrated that more aptamers could significantly raise the optical density in ELONA (Fig. 4). Meanwhile, the aptamer with the highest binding properties was selected to continue research procedure. The selected aptamer was named "STAPT" which is a logical conflation of STAphylococcus and APTamer.

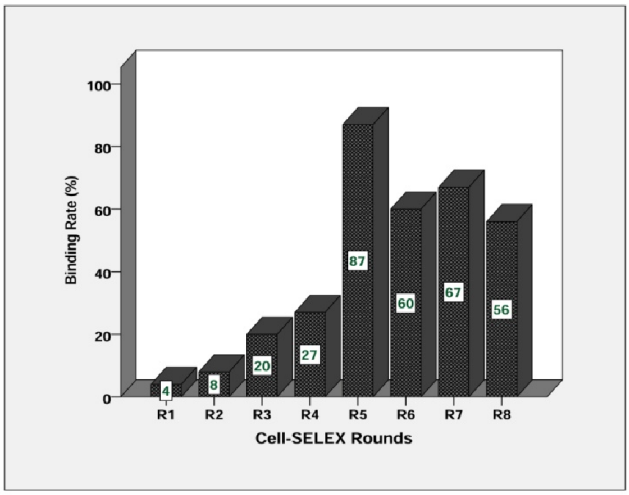

Figure 3. Binding quantity of ssDNA pool in the SELEX rounds (ratio of used amount to recovered amount of ssDNA) from primary library to round 8.

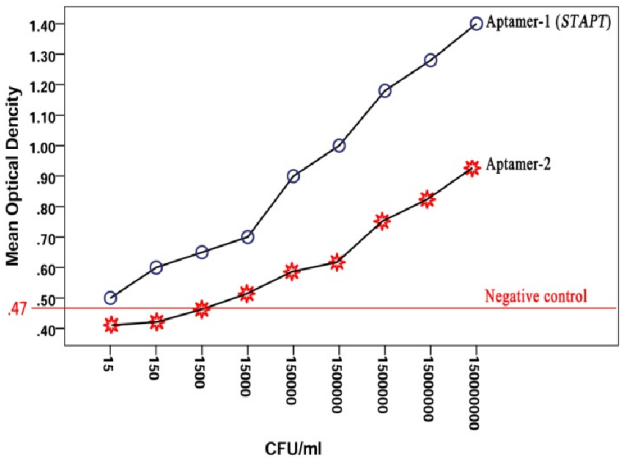

Figure 4. Binding quality of sequences obtained from final pool of Cell-SELEX (Round 8).

STAPT cross-reactivity analysis

In order to study the cross reactivity of selected aptamer, it was employed against some other bacteria such as *S. epidermidis* and *Streptococcus* in ELONA. Results revealed that the STAPT is definitely dedicated to *S. aureus* with a powerful feature (Fig. 5).

Calculation of ATAPT aptamer LOD

At the first step, according to the formulas 1 and 1′, the limit of blank (LOB) (the highest optical density, expected to be recorded in replications of a blank sample without analyte) was calculated. Based on LOB, and by use of formulas 2 and 2′, the limit of detection (LOD) (the lowest number of bacteria at which detection is reliably feasible)was estimated [18]. Calculations revealed that the aptamer detection limit was approximately at the OD = 0.67. The OD corresponding point on the logarithmic scale and subsequently bacterial number (CFU/ml) revealed that the LOD of *STAPT* was ~ 4×10^3 CFU/ml. it means that the minimum number of bacteria that can determine by *STAPT* is ~ 4×10^3 CFU/ml (Fig. 6).

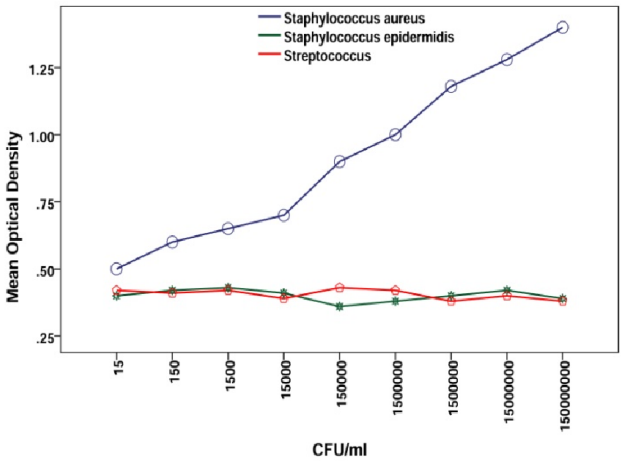

Figure 5. *STAPT* aptamer cross-reactivity test. *S. aureus* used as specific and *S. epidermidis* and Streptococcus as non-specific targets.

$$Formula\ 1\text{: } LOB\ (Limit\ of\ Blank) = mean_{blank} + 1.645\ (SD_{blank})$$

$$Formula\ 1'\text{: } LOB\ (Limit\ of\ Blank) = 0.47 + 1.645\ (0.061) = 0.57$$

$$Formula\ 2\text{: } LOD\ (Limit\ of\ Detection) = LOB + 1.645\ (SD_{low\ concentration\ sample})$$

$$Formula\ 2'\text{: } LOD\ (Limit\ of\ Detection) = 0.57 + 1.645\ (0.065) = 0.67$$

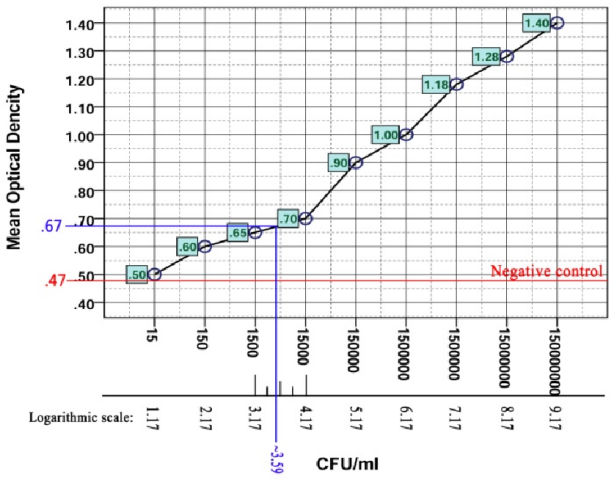

Figure 6. The graphical illustration for calculation of *STAPT* LOD.

Secondary structure of STAPT aptamer

The secondary structures of selected aptamer (*STAPT*) were determined by using mfold online software. This software predicted the secondary structure of *STAPT* with

and without constant regions. Illustration of ATAPT secondary structure revealed that both core region of *STAPT* (aptamer without constant regions) and full length *STAPT* (aptamer with constant regions) have two functional loops (Fig. 7).

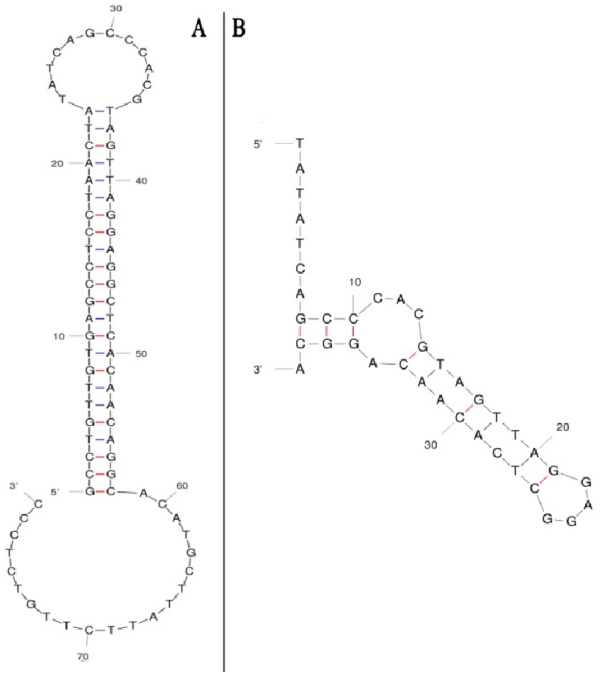

Figure 7. Secondary structure of aptamers predicted by mfold. A) Full length *STAPT* with the minimum energy of -27.65 Kcal/mol. B) Core region of *STAPT* with the minimum energy of -1.50 Kcal/mol.

Discussion

Staphylococci can be normally isolated from skin, nasopharynx and also public places specially hospitals [19]. Even when have no symptoms, they can show the potential to cause disease. Three major pathogens of this gram-positive genus of bacteria include *S. aureus*, *S. epidermitis*, and *S. saprophyticus*. Today, *S. aureus* is known as a leading cause of disease in hospital [1]. S. aureus causes a wide range of diseases which can be classified into two categories including: 1) Diseases caused by exotoxin release such as gastroenteritis (food poisoning) and toxic shock syndrome, 2) Diseases caused by direct bacterial onset such as dermal infection and bacteremia/sepsis [20]. Accordingly, the diagnosis of *S. aureus* can pave the way for disease prevention and management. How to detect *Staphylococci* from *Streptococci* is very important because most *Staphylococci* are resistant to penicillin G. There are three methods for their diagnosis: Gram-positive test, catalase test, and direct culture. It is more important that we can identify *S. aureus* as the most pathogenic species from other species of *Staphylococci*. Aptamer, known as chemical antibody [21], is a potent probe which can be served as a nimble bio-detector element and a replacement for antibody. SELEX with all its derivatives is a high throughput strategy to isolate aptamer through a nucleic acid pool with a large diversity. In this study, the Cell-SELEX method was employed for the isolation of specific aptamers against *S. aureus* whole cell. Performing consecutive Cell-SELEX rounds is aimed to increase the affinity and proprietary performance of aptamer. Rising trend in the affinity of aptamers during the SELEX process will confirm and verify the accuracy of SELEX for aptamers isolation. For this purpose, different methods have been developed, such as capillary electrophoresis [22], flow cytometry [23] affinity matrix [24] surface plasmon resonance (SPR) etc [25]. In the present study, the Cell-SELEX accuracy was monitored by measuring round binding quantity, whereby the ratio between the used and recovered ssDNA was calculated at the end of each round. Here we also used ELONA to investigate binding quality, cross-reactivity and LOD of isolated aptamer. Finding proved that our isolated aptameric probe named "*STAPT*" has acceptable binding quality and LOD (~ 4×10^3 CFU/ml) and also a promising specificity for S. aureus. A large number of studies entitled bacterial detection technique based on aptamer have been published, and readers will be involved in a confusing diversity of methods, findings and conclusions. However, we tried to simplify some of them and evaluate our *STAPT* aptamer. Lavu and colleagues [26], isolated ssDNA aptamers against *Salmonella enterica* serovar *Typhimurium* using Cell-SELEX. They reported that their beat isolated aptamer (SAL 26) had sensitivity limit of 102 CFU/ml. Wang et al. [27], used two DNA aptamers against *S. typhimurium* to manufacture a label-free detection system by SPR. They immobilized aptamers on the gold chips in SPR experiment and operated the detection procedure. Finally, they determined that the LOD of their aptamer-based sensor was as 3×10^4 CFU/ml. Hu and colleagues [28] isolated, a single-stranded DNA aptamer against *Bifidobacterium breve* through 12 rounds of whole-cell-SELEX. Their findings showed that the LOD of their aptamer with the highest affinity (BB16-11f) was 103 CFU/ml. Duan *et al*., [29] isolated and characterized some aptamers against *S. typhimurium* using Cell-SELEX. By a superficial looking at the structure of their best aptamer (ST2P) its complexity is revealed. Of course, this aptamer with a quad-blade structure and with five complete loops, establishes themore complicated and stronger binding. As expected, this aptamer was very desirable and its LOD was equal to 25 CFU/ml. Savory and colleagues [30] developed a colorimetric apta-sensor for detection of *Streptococcus mutans*. They demonstrated that their immobilized aptamer on gold colloids, in the form of a flow-through diagnostic system, could detect *S. mutans* with a LOD of 1×10^5 CFU/ml. There are many examples with various outcomes, however, in comparison with other similar studies, *STAPT* aptamer is placed in the middle class position with satisfactory properties. The relatively large discrepancies between the aptamers are often rooted in the quality of the implementation of SELEX, its own inherent characteristics and binding assessment techniques.

Conclusions

Manufacturing a sensitive and specific diagnostic system for any agent is of researchers interests. Different types of aptamers are desirable choices that can pave the way to reach this goal. Our aptamer with the promising features of

what shows itself is no exception and can play a decisive role in manufacturing of biosensors.

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