Development of specified DNA aptamer binding to *group A Streptococcus* serotype M3

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Abstract

Group A streptococcus (GAS) is an important gram-positive pathogen causes various human diseases ranging from peripheral lesions to invasive infections. The M protein is one of the main virulence factors presents on the cell surface, associated with invasive GAS infections. The serotype M3 compared to other M types has a predominant role in lethal infections and represents epidemic behaviors, including streptococcal toxic shock syndrome, bacteremia and necrotizing fasciitis. Traditional methods for M typing are time-consuming, grinding, contradictory and generally restricted to reference laboratories. Therefore, development of a new technique is worth full. Aptamers with the capability of detecting targets with high accuracy and specificity can be ideal candidates used for specific M-typing of *S. pyogenes*. In this research high binding affinity DNA aptamer against *S. pyogenes* serotype M3 were selected through 12 iterative rounds of Systematic Evolution of Ligands by EXponential (SELEX) enrichment procedure using live cells as a target. Development of a SELEX procedure for highest binding aptamer is monitored by flow cytometry analysis. Out of several aptamer sequences analyzed, 12L18A showed the highest binding efficiency toward *S. pyogenes* type M3 with lowest Kd of 7.47 ± 1.72 pM. Therefore the isolated aptamer can be used in any tool like biosensor for detection of *S. pyogenes* as well as developing a novel M-typing system.

Keywords: Serotyping, Flow Cytometry, Cell-SELEX, Aptamers, *Streptococcus pyogenes*

Introduction

Group A Streptococcus, an important gram-positive pathogen, causes a wide range of diseases such as streptococcal pharyngitis, streptococcal toxic shock syndrome (STSS), scarlet fever, invasive systemic infections, necrotizing fasciitis (NF) and endocarditis. Among M-types, serotypes M1 and M3 play a significant role in invasive diseases especially in NF and STSS cases. These two serotypes bring about an increased mortality rate in the USA and Canada (Beres et al. 2004; Nakagawa et al. 2003). Patient samples, for GAS analysis, usually are throat and skin swabs as well as wound aspirates where antibody-based rapid antigen detection (RAD) or culture are the currently available methods for the detection. Two-site sandwich immunoassay is the most common type of RAD. This method relies on the recognition of Group A carbohydrate present in the cell wall (Upton et al. 2014). Although RAD is more rapid compared to culture, but it is not sensitive enough and negative results need culture test validation (Hamula et al. 2011). The sensitivity of both methods depends on the presence of adequate amount of live cells in the inoculum.

Emm gene encoding the M surface protein is also used as a typing method, but some disadvantages such as: the presence of a *emm* sequence does not always correlate to the serological type (Beall et al. 1996; Hamula et al. 2016; Yoonim et al. 2005) and requirement of a specific probes for each *emm* allele leads to expensive DNA labelling reagents, are present with this method (Beall et al. 1996). Moreover, type M1 and M3 isolates from Atlanta have little variation between *emm1* and *emm3* alleles according to the Bernard et al. (Beall et al. 1997). Therefore, developing an accurate and sensitive method is needed.

Aptamers are short (35-1oo nucleotides) single-stranded DNA or RNA oligonucleotides with highest binding affinity toward targets due to their 3D conformations (Acquah et al. 2015; Darmostuk et al. 2015; Gold 1995). Compared to antibodies, aptamers have various features such as low cost and easy generation, high bioavailability and stability due to their small size, wide range of targets and handed modifications (Chen and Yang 2015; Hamula et al. 2008). Therefore, a detection method based on an aptamer can be a good choice where oligonucleotides are selected through a procedure called SELEX. To date, several numbers of aptamer sequences against pathogens have been characterized like *Streptococcus mutans* (Savory et al. 2014), *Haemophilus influenzae* type b (Bitaraf et al. 2016), *Staphylococcus aureus* (Moon et al. 2015), *Listeria monocytogenes* (Liu et al. 2014) and a mixture of the 10 most prevalent GAS M-types using whole cell-SELEX technique (Hamula et al. 2011). For detection of pathogens, the whole cell-SELEX where live bacterial cells are used as a target, is preferred. This is because the selected aptamer can bind more efficiently to its target since

surface proteins are in their physiological conditions (Cerchia and de Franciscis 2010; Dua et al. 2011; Meyer et al. 2011; Meyer et al. 2013; Moon et al. 2015; Ohuchi 2012).

In the present research, we have characterized a high potency DNA aptamer toward the GAS serotype M3 using whole cell-SELEX technique. These aptamer candidates can be used in rapid diagnostic tools for detection of GAS serotype M3.

Material and Methods

Bacterial strains and cultures

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The GAS serotype M3 used as a target for aptamer selection and GAS serotype M1 used for counter-SELEX were gifted from the Kerman University of Medical Science. GAS (ATCC 10403), GAS (ATCC 19615) and Group B *streptococcus* (GBS) (ATCC 12386) were purchased from the Culture Collection of Iranian Research Organization for Science and technology. *Streptococcus pneumonia* (ATCC 33400), *Bacillus subtilis* (ATCC 6633), *Escherichia coli* O1: K1: H7 (ATCC 11775), *Acinetobacter baumannii* (ATCC 19606) *Staphylococcus aureus* (ATCC 25923), *Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus epidermidis* (ATCC 12228), *Bacillus cereus* (ATCC 1015) were obtained from microbial collection center of the Shahed University. These strains were used for counter -SELEX and selectivity test. *E. coli* TOP10 competent cells were used for transformation procedure. All strains were cultured in brain heart infusion broth (BHI) except Serotypes M3, M1, GAS (10403 ATCC), GAS (ATCC 19615) and *S. pneumonia* , which were cultured in the 5% defibrinated sheep–blood agar in aerobic condition at 37° C. Viable cell numbers were specified using the standard plate counting method to assess the number of colony forming units (CFU). All the bacterial strains harvested in the mid-log growth phase (OD_{600} of about 0.55) by centrifugation at 6000 ×g for 10 min. The cells were washed 3 times in 1x washing buffer (phosphate-buffered saline (PBS) and 0.05% tween20) and the pellet was suspended in 200 µl of binding buffer [washing buffer plus 1% bovine serum albumin (BSA)]. Approximately 10^7-10^8 CFU/ml was used for each round of SELEX.

Oligonucleotides and DNA library

A randomized 38-mer DNA library flanked by the constant sequence at 3′ and 5′ end for primer binding (5′-GCCTG TTGTGAGCCTCCTAAC (N 38) CATGCTTATTCTTGTCTCC-3') was synthesized by Metabiom (Steinkirchen, Germany). The modified and free labeled primers were synthesized by TAG Copenhagen A/S (Frederiksberg, Denmark). The primer sequences are given below:

Forward primer: 5'-GCCTGTTGTGAGCCTCCTAAC-3' and 5'- FITC labeled-forward primer

Reverse primer: 5′- GGAGACAAGAATAAGCATG-3′ and 5′- Phosphorylated- reverse primer

Preparation of ssDNA aptamer

The DNA library with approximate diversity of 10^{23} is amplified by the following condition: initial denaturation at 94°C for 4 min and 35 cycles with denaturation at 94°C for 30 sec, annealing at 62 °C for 30 sec, extension at 72 °C for 30 sec and a final extension step of 72 °C for 7 min in a total reaction volume of 25 µl using free label forward and 5′- phosphate reverse primers. The most abundant PCR product was obtained using 10mM of dNTP mix, approximately 900ng of template and 10 pM concentration of each primer in a total reaction volume of 50 µl. All PCR amplicons were analyzed with 2% agarose gel electrophoresis. The amplified dsDNA library was digested with Lambda exonuclease (Thermo SCIENTIFIC Company, USA), a highly processive exodeoxyribonuclease that selectively digests the dsDNA from 5′- phosphorylated strand to 3′. Five units of λ exonuclease with 5µg of dsDNA was incubated in a total reaction volume of 30µl in 1× lambda exonuclease reaction buffer at 37 °C. The reaction was terminated after 20 min by deactivating λ exonuclease at 80°C for 10 min and the mixture was flash cooled on ice.

SELEX procedure:

Two nanomols of ssDNA aptamer pool was incubated with 10^8 CFU/ml of *S. pyogenes* M3 in 500 µl of binding buffer for 1h at room temperature with gentle mixing. Concentrations of DNA aptamer were measured with Pico200 (Picodrop, Hinxton, United Kingdom). To remove the unbound or poorly bound ssDNA, the cells were washed twice with 1ml of washing buffer. Aptamer bound cells were recovered by adding 100 µl of DNAase-free water and heated at 85°C for 10 min then placed on ice for 10 min. The cells, now free of aptamers, were

centrifuged at 6000 ×g for 10 min. The supernatant containing the eluted DNA aptamers were used as the template for PCR to obtain the aptamer pool for the next round of selections. PCR products of each step was purified by ethanol precipitation before going for SELEX procedure. The stringency of the selection reactions was increased by increasing the washing frequency from 2 to 5 times, decreasing the incubation time from 60 min to 15 min and volume of the binding buffer from 500 µl to 200 µl. In order to eliminate nonspecific aptamers from the library pool and direct selection toward the target, three rounds of counter-SELEX were carried out. The 3rd, 5th and 9th rounds of ssDNA aptamer pool were incubated with 10^8 CFU/ml nontarget bacterial cocktail. Preparation of the bacterial cocktail for counter SELEXs was similar to that of the target cells. One ml aliquots (10^8 CFU/ml) from each of *S.pneumonia*, *B.subtilis*, *E.coli* O1: K1: H7, *A.baumannii*, *S. aureus*, *P.aeruginosa*, *S.epidermidis, B.cereus,* GAS (ATCC10403 and19615), GAS serotype M1and Group B *streptococcus* were pooled together, and washed 3 times with wash buffer and suspended in 10 ml of PBS and used as a bacterial cocktail. One ml of this bacterial cocktail was used in counter-SELEX. The ssDNA pools were incubated with the cocktail. The supernatant was collected after 1h for the next round of selection.

Binding efficiency

The binding efficiency of the ssDNA aptamer pools after 2nd, 4th, 6th, 7th, 8th, 10th, 11th and 12th rounds of selection are estimated by flow cytometry using FITC-labelled aptamer. For this purpose, 200 pM of ssDNA from each round of SELEXs with 10^8 CFU/ml *S. pyogenes* serotype M3 in 200 µl of binding buffer was incubated at room temperature for 45 min. The mixtures were then washed and resuspended in 1 ml of binding buffer, and the cells were taken for flow cytometry analysis. The binding efficiency of the counter-SELEXs was also monitored. This method, which is generally called fluorescence-activated cell sorting analysis is used for measuring cell-assorted fluorescence (Fischer et al. 2008). The fluorescent activated cell sorting (FACS) introduces a special type of flow cytometry where the instruments analytical are merged with the capability to isolate certain cells from the analyzed population. This is done by sorting the cells of interest, according to their FITC-fluorescence labelling. The fluorescent intensity was estimated by SysmexPartec GmbH flow cytometer (Görlitz, Germany). The *S. pyogenes* serotype M3 stained with FITC-labelled aptamer were analyzed by flow cytometry. Stained cells were analyzed with marker analysis where less than 1% unlabeled cells were found in the marker region.

Binding affinity of selected aptamer

Following the 12 rounds of selection, the enriched ssDNA aptamer pools were amplified with free-labeled primers and were cloned into the pTG19-T vector and transformed into the *E.coli* TOP 10 competent cells. The colonies containing the vector were selected by overnight incubation at 37°C on LB plates containing 50 µg/ml ampicillin. The colonies were tested with colony PCR using specific simple primer of DNA aptamer and the positive clones were selected for further characterization. The positive clones containing aptamer sequences individually amplified by FITC–labeled primers and the fluorescent intensity was estimated by flow cytometry analysis. The aptamers showing high binding affinity toward *S. pyogenes* M3 were selected for the cross-reactivity test to check the specificity of the aptamer candidate. The cross reactivity was tested with 200 pM of FITC-labeled aptamer candidates incubated with the bacterial cocktail (10^8 CFU/ml) as well as individual samples of *S. pyogenes* ATCC 10403, ATCC 19615 and GAS serotype M1. The cells were collected by centrifugation and fluorescent intensity was estimated by flow cytometry.

Sequencing and structural analysis of selected aptamers

Two aptamers with the highest binding affinity toward *S. pyogenes* serotype M3 were selected and sent for sequencing (Bioneer, Daejeon, South Korea). The secondary structure analysis and Quadruplex forming G-Rich Sequences (QGRS) in nucleotide sequences were performed by Mfold web server (Zuker 2003) and QGRS Mapper (Kikin et al. 2006) respectively. The secondary structure prediction analysis were carried out under the condition set up of 144 mM Na^+ and 1 mM mg^{+2} at 21°C.

Determination of dissociation constant

The apparent dissociation constant (Kd) was determined by fluorescence-based affinity assay. The constant amount of target cells incubated with various concentrations of FITC-labeled ssDNA aptamer candidates ranging from 0 to 200 pM in 300 µl of binding buffer for 45 min at room temperature. The cells were washed and resuspended in 1ml of binding buffer and the fluorescent intensity of each concentration was measured (Duan et al. 2013; Kim et al. 2013; Mondal et al. 2015). The Kd was calculated by plotting the fluorescence intensity obtained from the different concentration of ssDNA. The data points were fitted by nonlinear

regression analysis in GraphPad Prism 6.01. According to Y = Bmax .X/ (Kd + X) equation. Where Y, Bmax and X are the fluorescence intensity, maximum fluorescence intensity, and aptamer concentration respectively

Results

PCR amplification and SELEX optimization

The PCR protocol for the amplification of aptamers eluted from SELEXs was slightly different from that of the original library. The Mg concentration and annealing temperature changed from 0.75mM and 62°C applied for the amplification of original library to 0.45mM and 58°C respectively. The number of the cycles was also reduced from 35 to 25 on cycle fifth onward. The results on 2% agarose gel electrophoresis showed a single band with the proper size.

A total of nine rounds of positive SELEX and three rounds of counter-SELEX were carried out until the ssDNAs binding to targeting *S. pyogenes* serotype M3 was obtained.

Binding efficiency

The amount of FITC-labeled aptamer pool bound to the target was increased from 2.04% in the second round to 41.08% in the twelfth round of SELEX (Fig 1). The percentage of cells found in the marker region are considered as positive. The results of marker analysis is displayed in Fig 2.

The binding efficiency during the SELEX rounds showed an impressive growth except in rounds 11^{th} and 12^{th}. Since there was no any significant difference between the rounds 11^{th} and 12^{th}, the SELEX process was terminated at round 12^{th}. The results of SELEX 12^{th} showed an improvement in binding efficiency of about 20-fold compared to the second round of selection (Fig 3). Our data showed descending binding affinity toward counter cells. The aptamer pool of round 12^{th} was cloned and transferred into the *E.coli* TOP 10 cells. A total of 87 aptamer transformants were achieved, of which 48 were confirmed as positive clones with colony PCR. All positive clones were analyzed by flow cytometry and three clones with the highest fluorescent intensity of 74%, 63% and 58% toward *S. pyogenes* serotype M3 were selected for further analysis (Fig 4). These aptamers were named 12L18A, 12L12A and 12L4A, respectively.

Binding Specificity

The three aptamers showing the highest binding affinity were further tested with bacterial cocktail used in counter-SELEX as well as with *S. pyogenes* serotype M1, *S. pyogenes* (ATCC 19615) and *S. pyogenes* (ATCC 10403) individually. The histogram resulting from the FITC fluorescence by flow cytometry analysis presented a clear specificity of the aptamer to the target cells. Due to the high binding affinity and specificity of aptamers 12L18A and 12L12A, these two were selected for Kd calculation (Fig 5).

Dissociation constants

The strength of the binding affinity between the aptamer and its target as a function of different aptamer concentrations from 0 to 200 pM upon the constant amount of 10^8CFU/ml^{-1} of *S. pyogenes* serotype M3 cells was estimated. The dissociation constant values (Kd) of 12L18A and 12L12A based on a nonlinear regression analysis were calculated to 7.47 ± 1.72 pM and 8.25 ± 1.43 pM respectively. The binding saturation curve of both aptamers is presented in Fig 6.

Sequence analysis of aptamer candidates

The sequence of the variable regions is given in Table 1. The proposed secondary structures of the aptamers 12L18A and 12L12A are shown in Fig 7. The Gibb's free energy of each sequence calculated by UNAFold was -11.05 and -12.71 cal/mol, respectively. The QGRS analysis of aptamer candidates showed the 12L18A is guanine-rich and have a G4 motif.

Discussion

High-level binding affinity and specificity toward target made aptamers as an appropriate tool in bacterial diagnosis (Bruno et al. 2012; Groff et al. 2015; Hamula et al. 2016; Toh et al. 2015a; Wandtke et al. 2015). The previous analysis demonstrates no difference between RNA and DNA aptamers in terms of affinity and

specificity (Gold 1995). However, DNA aptamers have specific advantages over RNA aptamers, because in every SELEX procedure RNA aptamers initially need reverse transcription before PCR amplification, which makes the selection process complex. In addition, DNA aptamers exhibit much higher stability than RNA aptamers.

Various reports are available for the isolation of aptamers candidate to develop an aptasensor for pathogen diagnosis including *Campylobacter jejuni* (Dwivedi et al. 2010), *Francisella tularensis* (Vivekananda and Kiel 2006), *Mycobacterium tuberculosis*(Chen et al. 2007), *Acinetobacter baumannii* (Rasoulinejad and Gargari 2016) and *Staphylococcus aureus* (Chang et al. 2013). In the present work we carried out the cell-SELEX procedure to isolate high-affinity aptamers toward *S. pyogenes* type M3, using Flow cytometry to track the enrichment progress of SELEX, where a cell bound aptamers can be sorted (Cerchia and de Franciscis 2010; Dua et al. 2011; Dwivedi et al. 2010, 2013). This assay not only has reproducibility and sensitivity but also present a rapid and great level of statistical precision due to a large number of cells being counted (Davis et al. 1996; Davis et al. 1998). Accessibility of the target in physiological conformation which plays an important role in high efficiency and functionality of the binding aptamer is the advantage of cell-SELEX to protein SELEX. In other word in the traditional protein SELEX we cannot strictly conclude that the aptamer binding site of the target is the one which is available on the physiological condition and that is why aptamers selected against recombinant targets have often failed postselection to bind to their targets on the surface of cells (Dassie et al. 2014).

Our isolated aptamers toward *S. pyogenes* serotype M3 showed higher affinity (7±1 and 8±1 pM) which is incompatible with our previous reports where aptamers have been generated for different target through whole-cell SELEX (Bitaraf et al. 2016; Rasoulinejad and Gargari 2016), indicating the efficiency of our selection procedures. Hamula et al. reported aptamer based identification for the mix M-types of *Streptococcus pyogenes* with whole cell-SELEX with a Kd = 7 ± 1 nM for E-CA 20 and 12 ± 1 nM for E-CA 20P (Hamula et al. 2011; Hamula et al. 2016).

The reason for higher affinity of our aptamers could be attributed to the presence of a single target in the reaction mixture against the mix type used by others. Appropriate selection of bacteria for counter-SELEX which significantly removes a non-target, non-specific and poor binding cells along with ascending stringency during the SELEX procedures could be the other reasons to reach for high efficient aptamers. Amplification of whole DNA pool after each SELEX which covers all possible binders without losing any, the way of preparing ssDNA aptamers and washing strategy are the other reasons for obtaining high affinity binders. Since, in the

initial library there might be only one copy from each sequence, therefore, the entire aptamer pool after each SELEX must be amplified before going for the next SELEX. This reduces the possible loss of any sequences (possibly high binder). Single stranded DNA preparation has crucial importance in the combinatorial chemistry technique SELEX for aptamer isolation and many other molecular biology approaches (Avci-Adali et al. 2010; Bitaraf et al. 2016). Therefore, in this research we prepared the ssDNA using Lambda exonulease digestion which offers better results compared to heat denaturation in which some sequences can be lost due to reannealing.

The 12L18A aptamer showed highest specificity toward *S. pyogenes* serotype M3 with no affinity toward *S. pyogenes* ATCC 19615, *S. pyogenes* ATCC 10403, GAS serotype M1, *S. agalactiae*, *S. pneumonia* and other related bacteria used in the counter-SELEX. Therefore, we can assume that the selected aptamers are directed toward M-type protein which is specifically expressed on the *S. pyogenes* cells and hence the developed aptamer could be against *S. pyogenes* serotype M3. The aptamer 12L18A showed a remarkable binding efficiency toward *S. pyogenes* serotype-M3 with Kd in a picomolar range. In a similar study, Shipley et al. reported the Kd value of 96.98 pM by flow cytometry against *E.coli*. (Shipley et al. 2010). Chen et al. obtained the aptamer sgc8 toward HeLa cells with a binding affinity in a picomolar range (Chen et al. 2009). Aptamers with the highest specificity and sensitivity have also been reported by Alves et al. and Kimoto et al (Alves Ferreira-Bravo et al. 2015; Kimoto et al. 2013).

Flow cytometry analysis of the selection process revealed 74% efficiency toward the *S. pyogenes* serotype M3. The cell-SELEX procedure does not show 100% efficiency towards any target. This is due to the different expression level of cell surface proteins in the bacterial populations through the Log phase (Dwivedi et al. 2010, 2013).

Duan et al. and Dwivedi et al. demonstrated that, the existence of multiple hairpins, stems- and –loops can be key domains and potential binding sites in the secondary structure (Duan et al. 2013; Dwivedi et al. 2013). The 12L18A is guanine-rich and contains a G4 motif [G x Ny1G x Ny2G x Ny3G] where, x indicates the number of guanine-quadruplex and y1, y2 and y3 denote the gaps (loops). Aptamers with these G-quadruplex have been reported for a different range of targets from purified protein to live cells (Savory et al. 2014; Yoshida et al. 2013). The existence of G4-motif can help the aptamers as a scaffold in their stabilities.

In this research, we isolated specific aptamer against *S. pyogenes* serotype M3 cells. The selected aptamer has the potentiality for identifying this pathogen and hence could improve on M-typing system. There are several reasons where the aptamers are preferred to antibodies (Toh et al. 2015b). Therefore, our selected aptamer can

be replaced with antibodies used in immunochromatographic antigen tests or latex agglutination. In addition, the specified aptamer can be used in different diagnostic tools such as biosensors or ELASA for detection of *S. pyogenes* serotype M3 in the clinical isolates.

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Compliance with ethical standards

Conflict of interest: The authors have declared that no competing interests exist.

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Figure legends

Fig 1 The binding efficiency was calculated compared to the initial library toward *S. pyogenes* M3. The binding efficiency increased from 2.04% of the 2nd SELEX to 41.08% of SELEX 12th.

Fig 2 Marker analysis. Unstained *S. pyogenes* cells (a). *S. pyogenes* stained by the FITC- labelled aptamer (b). The events (percentage count) of cells found in the M1 region are defined as positive.

Fig 3 Fluorescent binding efficiency to the target cells through the SELEX procedures. Flow cytometric analysis of the binding capacity of the FITC-labeled aptamer pools to *S. pyogenes* M3 from the 2nd, 6th, 11th and 12th rounds of whole cell selection. There is a significant increase in the binding efficiency of 11th and 12th SELEX rounds compared to that of the initial library.

Fig 4 Histogram overlays for the individual aptamer compared to the initial library. Flow cytometric analysis of the binding capacities of three candidate aptamers (4, 12 and 18) with highest affinity compared to initial library toward *S. pyogenes* M3

Fig 5 Comparison of the fluorescent intensity toward target cells, other bacteria as individual or cocktail labelled with three aptamers candidates. Flow cytometric analysis of three aptamer candidates (4, 12 and 18)

with the highest affinity toward the *S. pyogenes* serotype M3, *S. pyogenes* (ATCC 10403 and 19615), *S. pyogenes* serotype M1 and the bacterial cocktail.

Fig 6 The equilibrium dissociation constant (Kd) of FITC-labeled aptamer 12L18A and 12L12A to *S. pyogenes* serotype M3. Various concentration of aptamers (0 to 200 pM) incubated with 10^8 *S. pyogenes* serotype M3 cells. A nonlinear regression curve was plotted by GraphPad Prism 6.01.

Fig 7 Predicted secondary structures of aptamer candidates. Secondary structures of aptamers 12L12A and 12L18A is predicted based on the folding algorithm of zuker using Mfold.

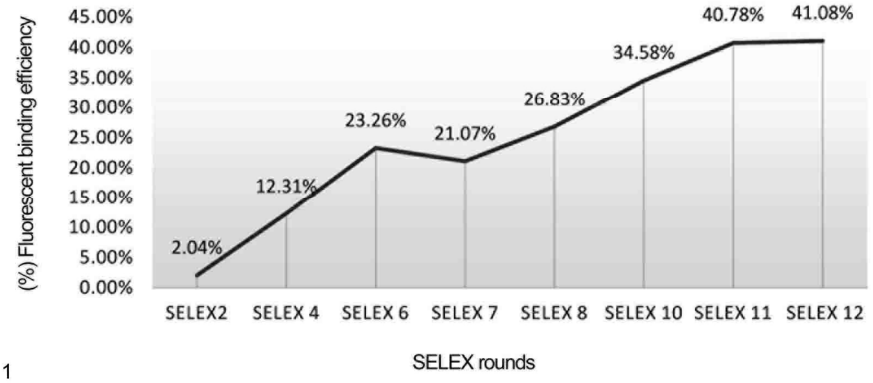

Fig. 1

Fig 1 The binding efficiency was calculated compared to the initial library toward S. pyogenes M3. The binding efficiency increased from 2.04% of the 2nd SELEX to 41.08% of SELEX 12th.

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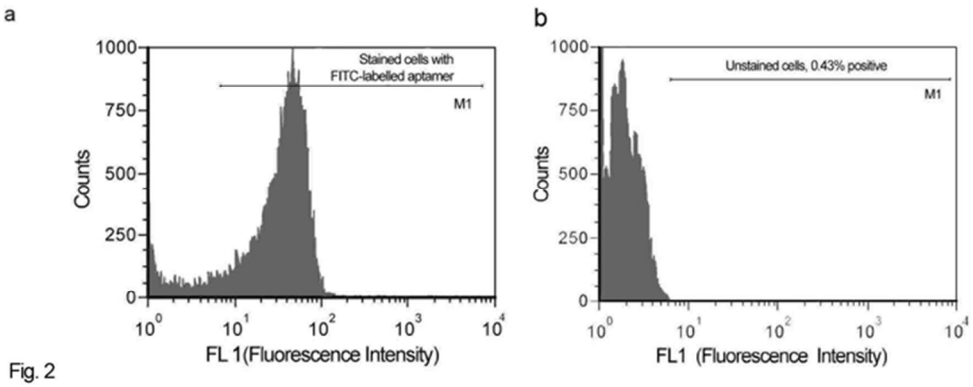

Fig 2 Marker analysis. Unstained S. pyogenes cells (a). S. pyogenes stained by the FITC- labelled aptamer (b). The events (percentage count) of cells found in the M1 region are defined as positive.

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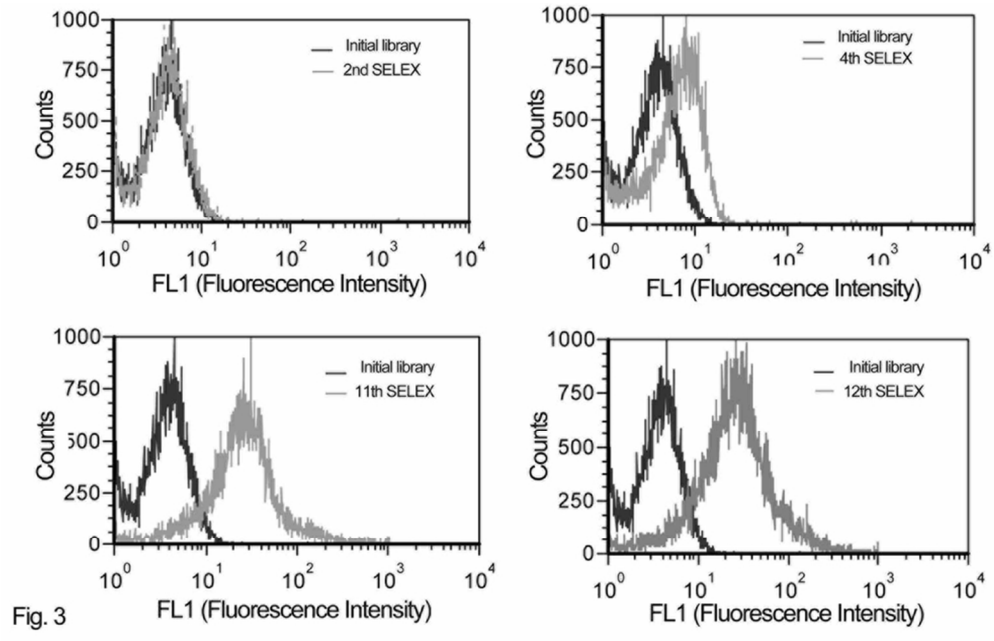

Fig 3 Fluorescent binding efficiency to the target cells through the SELEX procedures. Flow cytometric analysis of the binding capacity of the FITC-labeled aptamer pools to S. pyogenes M3 from the 2nd, 6th, 11th and 12th rounds of whole cell selection. There is a significant increase in the binding efficiency of 11th and 12th SELEX rounds compared to that of the initial library.

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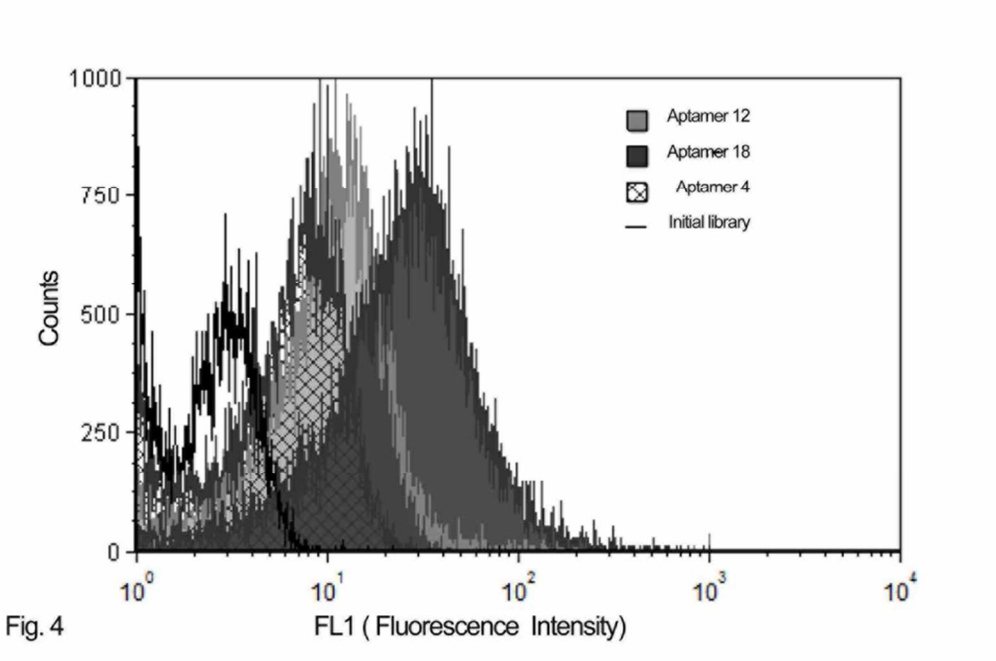

Fig 4 Histogram overlays for the individual aptamer compared to the initial library. Flow cytometric analysis of the binding capacities of three candidate aptamers (4, 12 and 18) with highest affinity compared to initial library toward S. pyogenes M3

79x52mm (300 x 300 DPI)

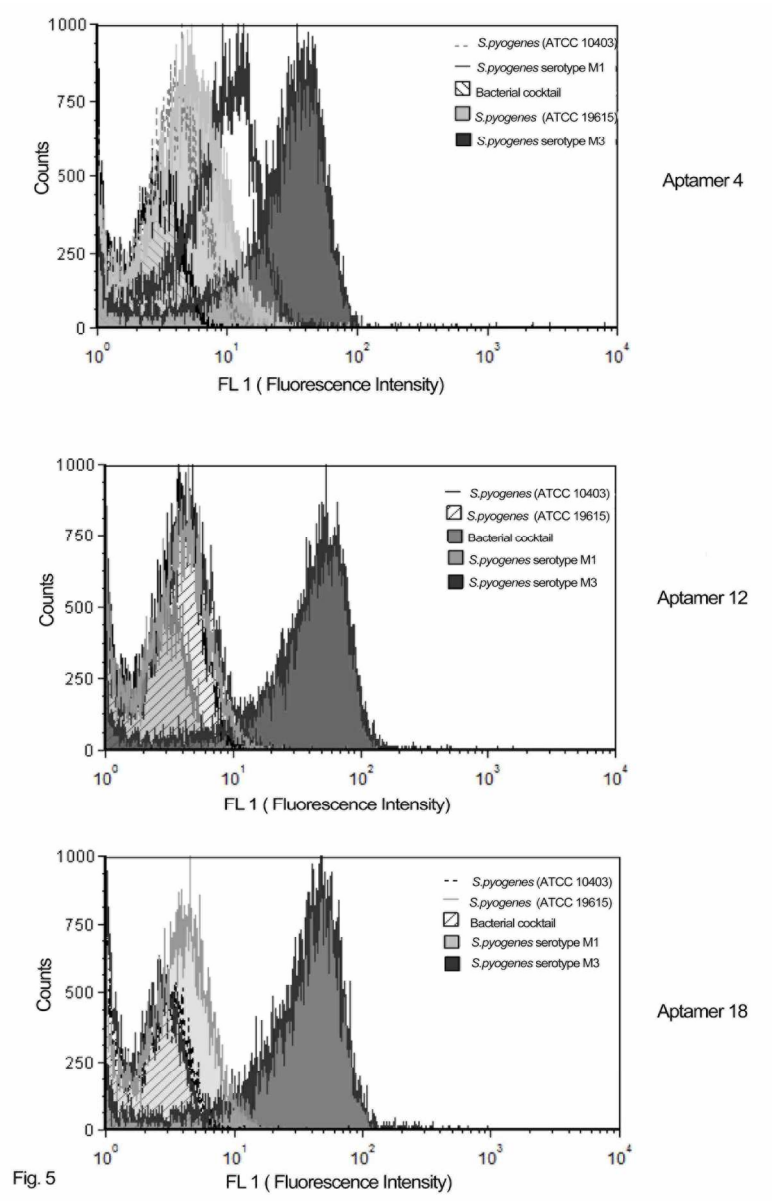

Fig 5 Comparison of the fluorescent intensity toward target cells, other bacteria as individual or cocktail labelled with three aptamers candidates. Flow cytometric analysis of three aptamer candidates (4, 12 and 18) with the highest affinity toward the S. pyogenes serotype M3, S. pyogenes (ATCC 10403 and 19615), S. pyogenes serotype M1 and the bacterial cocktail.

186x290mm (300 x 300 DPI)

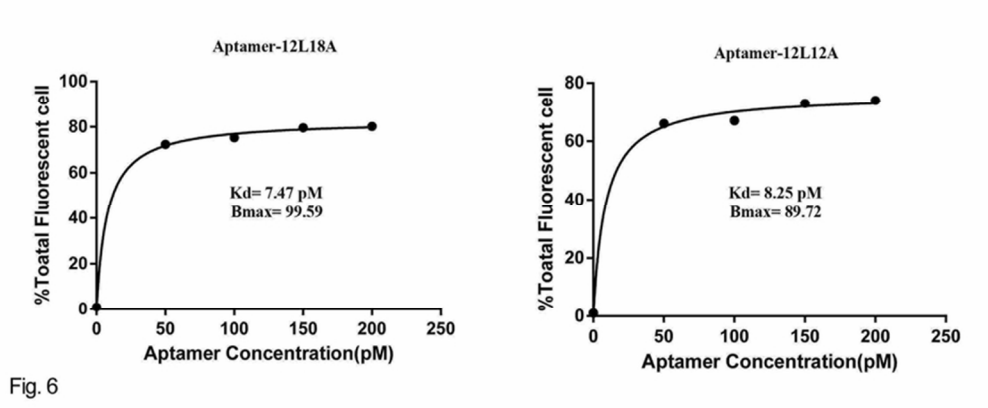

Fig 6 The equilibrium dissociation constant (Kd) of FITC-labeled aptamer 12L18A and 12L12A to S. pyogenes serotype M3. Various concentration of aptamers (0 to 200 pM) incubated with 108 S. pyogenes serotype M3 cells. A nonlinear regression curve was plotted by GraphPad Prism 6.01.

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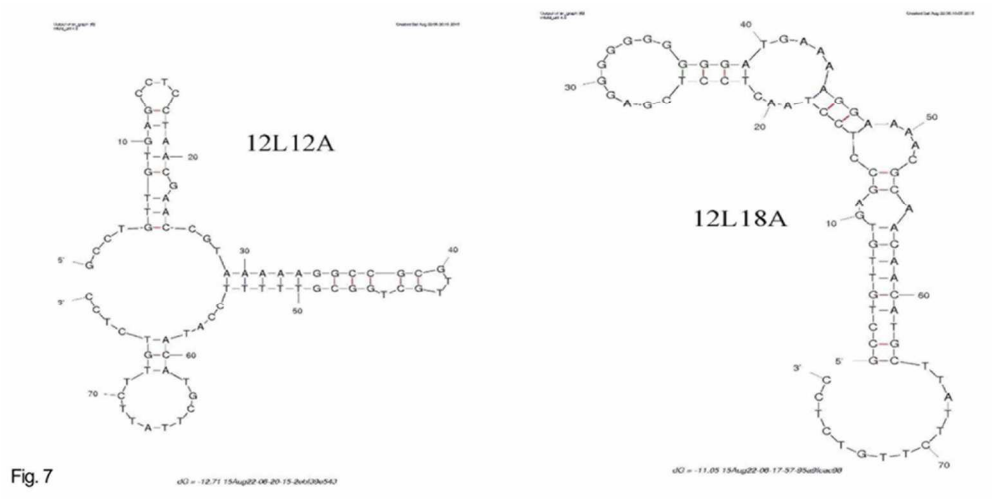

Fig 7 Predicted secondary structures of aptamer candidates. Secondary structures of aptamers 12L12A and 12L18A is predicted based on the folding algorithm of zuker using Mfold.

83x41mm (300 x 300 DPI)

Table 1. The variable sequences of highest binding affinity of aptamers

Aptamer No.	Sequence (without primers)
12L12A	GAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATA
12L18A	TCCTCGAGGGGGGGGGGATGAAAAGGAAAACGCAACAA