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An improved SELEX technique for selection of DNA aptamers binding to M-type 11 of *Streptococcus pyogenes*

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ABSTRACT

Streptococcus pyogenes is a clinically important pathogen consisting of various serotypes determined by different M proteins expressed on the cell surface. The M type is therefore a useful marker to monitor the spread of invasive *S. pyogenes* in a population. Serotyping and nucleic acid amplification/sequencing methods for the identification of M types are laborious, inconsistent, and usually confined to reference laboratories. The primary objective of this work is to develop a technique that enables generation of aptamers binding to specific M-types of *S. pyogenes*. We describe here an *in vitro* technique that directly used live bacterial cells and the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) strategy. Live *S. pyogenes* cells were incubated with DNA libraries consisting of 40-nucleotides randomized sequences. Those sequences that bound to the cells were separated, amplified using polymerase chain reaction (PCR), purified using gel electrophoresis, and served as the input DNA pool for the next round of SELEX selection. A specially designed forward primer containing extended polyA$_{20}$/5Sp9 facilitated gel electrophoresis purification of ssDNA after PCR amplification. A counter-selection step using non-target cells was introduced to improve selectivity. DNA libraries of different starting sequence diversity (10^{16} and 10^{14}) were compared. Aptamer pools from each round of selection were tested for their binding to the target and non-target cells using flow cytometry. Selected aptamer pools were then cloned and sequenced. Individual aptamer sequences were screened on the basis of their binding to the 10 M-types that were used as targets. Aptamer pools obtained from SELEX rounds 5–8 showed high affinity to the target *S. pyogenes* cells. Tests against non-target *Streptococcus bovis*, *Streptococcus pneumoniae*, and *Enterococcus* species demonstrated selectivity of these aptamers for binding to *S. pyogenes*. Several aptamer sequences were found to bind preferentially to the M11 M-type of *S. pyogenes*. Estimated binding dissociation constants (K_d) were in the low nanomolar range for the M11 specific sequences; for example, sequence E-CA20 had a K_d of 7 ± 1 nM. These affinities are comparable to those of a monoclonal antibody. The improved bacterial cell-SELEX technique is successful in generating aptamers selective for *S. pyogenes* and some of its M-types. These aptamers are potentially useful for detecting *S. pyogenes*, achieving binding profiles of the various M-types, and developing new M-typing technologies for non-specialized laboratories or point-of-care testing.

1. Introduction

Streptococcus pyogenes is a bacterium responsible for a variety of infections, most notably pharyngitis, necrotizing fasciitis, sepsis, and the post-infection sequelae rheumatic fever and glomerulonephritis. One of the major virulence factors of invasive *S. pyogenes* isolates is the M protein present on the surface of the bacteria [1–4]. Different M-types are often but not always associated with different invasive infections [5]. In addition, the M protein can be utilized as a typing marker for understanding the epidemiology of invasive *S. pyogenes* disease [6–8].

S. pyogenes is conventionally M-typed using serological methods, which involve screening bacterial surface extracts against polyclonal antisera [9]. More recently, sequencing of the *emm* gene encoding M protein is replacing antibody-based typing methods as

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the gold standard [10] and has expanded the repertoire of M-typing for *S. pyogenes*. However, the presence of a particular gene sequence does not always correlate to the serological type. Due to the complexity of these methods, M-typing of *S. pyogenes* isolates has tended to be centralized in laboratories specializing in characterization of *S. pyogenes*. Both methods are laborious and have low throughput as each requires comparison of a bacterial isolate to myriad reference strains or databases.

We hypothesize that M proteins on the surface of *S. pyogenes* are suitable targets for selection of and binding to DNA aptamers. DNA aptamers possess key advantages over antibodies, including increased temperature stability, longer shelf-life, low variability between batches, and ease of chemical modification and synthesis once the sequences are known [11–18]. Hence, aptamers can be used in a variety of bioanalytical assays and array devices that conventionally use antibodies [18–21]. Achieving the potential benefits of aptamer-based affinity assays requires high-affinity aptamers that are specific for M proteins. The objective of this work was to generate aptamers specific for different M proteins of *S. pyogenes*. Building on our experiences of selecting aptamers for *Lactobacillus acidophilus* [22] and group A *Streptococcus* [23], we further improved the bacterial cell-SELEX technique to explore its ability to select aptamers for specific M-types. Improvements include the introduction of a specially designed primer to facilitate separation of single-stranded DNA after PCR amplification, the use of a counter-selection step to increase specificity, and testing the diversity of starting DNA library sequences to increase affinity. We describe here results from two sets of SELEX, demonstrating the selection of aptamers for *S. pyogenes* and for a specific M-type, M11.

2. Materials and methods

2.1. Bacterial strains and culture media

The procedures and growth conditions of the bacterial cultures were kept the same as those described in the previous study [23]. *Streptococcus bovis* ATCC33317 liquid cultures were grown overnight in BHI broth at 37 °C and 200 rpm shaking. Cells were harvested in the morning via centrifugation. *S. pyogenes* M type strains M1, M2, M3, M4, M6, M11, M12, M28, M77 and M89 were obtained from the National Centre for Streptococcus (Canada) strain collection. The M-types of the ten *S. pyogenes* strains used in this study were identified by sequencing of the *emm* gene as previously described [10]. All 10 isolates were from clinical cases of invasive group A streptococcal disease. The 10 *Streptococcus pneumoniae* strains used were also clinical isolates also obtained from the National Centre for Streptococcus. The pneumococcal serotypes assayed were serotypes 4, 5, 6A, 6B, 9V, 14, 18C, 19A, 19F and 23F. These serotypes were selected because they were the more common serotypes found in Canada and were contained in the 7-valent pneumococcal conjugate vaccine in use in Canada at the time of the study [24]. Pneumococcal serotypes were determined as previously described [25]. The *Streptococcus agalactiae* strains used and *Escherichia coli* DH5α have been previously described [26]. The *Enterococcus* species used in this study were obtained from American Type Culture Collection and have been previously described [27]. The enterococcus species assayed were *Enterococcus faecalis* ATCC 19433, *Enterococcus faecium* ATCC 19434, *Enterococcus gallinarium* ATCC 49573, *Enterococcus casseliflavus* ATCC 25788, *Enterococcus avium* ATCC 14025, *Enterococcus raffinosus* ATCC 49447, *Enterococcus pseudoavium* ATCC 49372, *Enterococcus mundtii* ATCC 43186, *Enterococcus malodoratus* ATCC 43197, *Enterococcus durans* ATCC 19432, *Enterococcus hirae* ATCC 8043, *Enterococcus cecorum* ATCC 43198 and *Enterococcus saccharolyticus* ATCC 43076.

2.2. DNA libraries for SELEX sets D and E

Two separate 80-nt single-stranded DNA libraries were used, each consisting of a 40-nt randomized region flanked on both sides by 20-nt primer regions. The initial ssDNA libraries and the primers used to amplify the DNA were obtained from Integrated DNA Technologies (IDT, Coralville, IA). Two sets of SELEX experiments were performed and named as set D and set E. For the SELEX set D, the single-stranded DNA library as obtained from IDT, containing a maximum of 10^{16} different sequences, was directly used as the starting DNA library in the first round of selection. For the SELEX set E, an aliquot of another single-stranded DNA library, containing 10^{14} unique sequences, was PCR-amplified for 3 cycles. Limiting the number of cycles of PCR amplification of the initial starting library to 3 was to maintain the high sequence diversity (10^{14} unique sequences) for the first round of SELEX. The reverse strand of the PCR product after 3 cycles of amplification was used as the starting DNA library in the first round of selection. Therefore, for the SELEX set E, 10^{14} sequences (of approximately 3–4 copies each) were used as the starting DNA library in the first round of selection. Prior to incubation with target cells, the DNA libraries were treated via heat denaturation at 94 °C for 5 min and subsequent cooling at 0 °C for 10 min.

2.3. Aptamer selection

Two separate sets of SELEX were carried out: SELEX set D and SELEX set E. The difference between the two sets was the number of unique sequences in the starting library: 10^{16} for SELEX set D and 10^{14} for SELEX set E.

A mixture of the 10 most prevalent *S. pyogenes* M-types was used as the target cells. Single colonies of the previously reported 10 most common *S. pyogenes* M-types in Canada [28] (M1, M2, M3, M4, M6, M11, M12, M28, M77, and M89) were grown overnight in separate liquid cultures. The growth conditions of the bacterial cultures and sub-cultures were described previously [23].

Procedures for the selection of aptamers binding to the *S. pyogenes* cells of 10 M-types are schematically summarized in Fig. 1. Briefly, an equal number of 10^7 cells from each of the 10 M-types of *S. pyogenes* were combined immediately prior to SELEX to reach a total of 10^8 cells. The cell mixture (a total of 10^8 cells) was incubated with single-stranded DNA library or the aptamer pools at room temperature (21 °C) for 60 min. Incubations were carried out under gentle rotation in order to increase the likelihood of contact between DNA and the target cells. The mixture was then centrifuged to separate the supernatant (named S_o fraction) from the cell pellets. The cell pellets were washed 3–4 times using binding buffer, and the wash solution was named W fraction. The S_o fraction and the W fraction contained the unbound or weakly bound DNA sequences. The cell pellets were resuspended in 1× PCR buffer and heated to 94 °C for 5 min to elute DNA sequences from the cells. The cell suspension was centrifuged, and the supernatant was retained. Heat-eluted DNA in the supernatant is referred to as the CA fraction. The cell pellet was resuspended in water, and PCR buffer and reagents were added directly to the cell suspension to amplify the tightly bound DNA sequences on the cells, named the Cells fraction. The cell-bound DNA sequences in both the CA fraction and the Cells fraction were separately amplified by PCR using the $polyA_{20}$/5Sp9-modifed 40-nt forward primer and 20-nt reverse primer. The PCR products were rendered single stranded by denaturing PAGE. The reverse strand (80-nt) was used as the aptamer pool for the next round of SELEX. Prior to be combined as an aptamer pool, single-stranded DNA sequences from the CA fraction and from the Cells fraction were separately tested for their binding to the target cells. A total of 8 rounds of SELEX set D and 8 rounds of SELEX set E were separately completed.

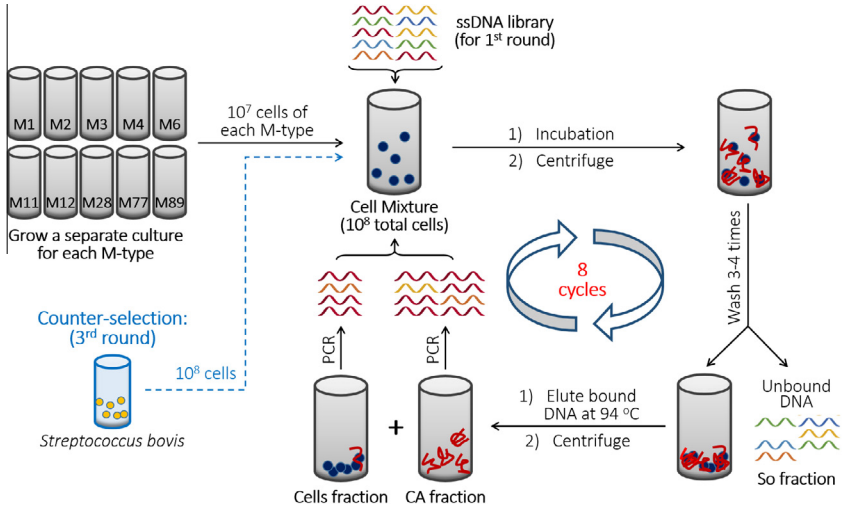

Fig. 1. Schematic showing SELEX process for generating aptamers binding to *Streptococcus pyogenes* cells of specific M-types.

Supplemental Information Fig. S1 shows example denaturing gel electrophoresis analyses of fractions collected and amplified after the first round of the SELEX procedures.

The third round of SELEX selection was a counter-selection step using 10^8 *S. bovis* cells as the non-target cells, instead of the target *S. pyogenes* cells. The introduction of the counter-selection step was to remove *non*-specific sequences from the aptamer pool. A total of 10^8 *S. bovis* cells were incubated with the aptamer pool generated from the preceding second round. After incubation at room temperature (21 °C) for 60 min, the mixture was centrifuged. The cell pellet was washed with binding buffer. The *S. bovis* cell pellet, containing DNA sequences that were bound to the non-target *S. bovis* cells, was discarded. The remaining DNA sequences in the supernatant (S_o fraction) were amplified by PCR using the polyA$_{20}$/5Sp9-modifed 40-nt forward primer and 20-nt reverse primer. Fig. S2 in Supplemental Information shows native PAGE of the amplified products from PCR. However, for generating single-stranded DNA, the PCR product was separated on denaturing PAGE. The reverse strand (80-nt) was kept and used as the input aptamer pool for the 4th round of the SELEX process.

The effects of the different parameters on the SELEX selection were previously discussed in detail [29]. An excess of tRNA and BSA (Invitrogen) were added to the incubation buffer (20-fold molar excess of each in the initial round, up to a maximum 160-fold molar excess in round 8) was added to the wash buffer. The use of increasing amounts of BSA and tRNA increased the competition between the desired target cells and non-targets (BSA molecules) for aptamer molecules. The tRNA was added to compete with the aptamer sequences for target binding sites. Incubation volumes for this set of selections were increased over previous experiments [23]. The conditions for SELEX sets D and E are summarized in Supplemental Information Table S1.

2.4. PCR amplification and gel electrophoresis

For testing the DNA sequences in the supernatant and in the wash of cells, DNA was amplified using the following primers.

Forward: 5′-AGCAGCACAGAGGTCAGATG-3′
Reverse: 5′-TTCACGGTAGCACGCATAGG-3′

After PCR, the reaction products were separated on 7.5% non-denaturing polyacrylamide gel electrophoresis (PAGE) in TBE buffer (Bio-Rad Protean III) at 60–120 V. The gels were stained with ethidium bromide, and photographed under UV light. In another set of experiments, the amplified DNA was analyzed using denaturing PAGE, as shown in lanes 4–8 of Supplemental Information Fig. S1.

For the amplification of DNA sequences bound to the target cells, a modified forward primer was used. The forward primer contained a 5′ polyA overlap of 20 adenine residues (polyA$_{20}$/5Sp9) that were joined to the remaining primer sequence via a triethylene glycol spacer (IDT Spacer 9). The addition of the polyA to the forward primer was to allow for gel electrophoresis separation of the forward strand from the reverse strand of the PCR product. We previously used heat denaturation to render single-stranded DNA, but reannealing resulted in loss of single-stranded DNA. The use of gel electrophoresis to render single-stranded DNA avoided the loss due to reannealing. The primers used to amplify the ssDNA library and the subsequent aptamer pools have the following sequences:

Forward: 5′-A$_{20}$/5Sp9/AGCAGCACAGAGGTCAGATG-3′
Reverse: 5′-TTCACGGTAGCACGCATAGG-3′

PCR conditions for amplification of the DNA aptamer pools during SELEX were optimized after each round, in order to minimize formation of mis-amplification products. The amounts of primers and nucleotides were increased in proportion to the increased amount of DNA being amplified. Standard PCR conditions used for SELEX sets D and E were: 1× PCR reaction buffer, 2 mM $MgCl_2$, 2.0 μM of each primer, 0.5 mM dNTPs, 1 E.U. of Platinum Taq DNA Polymerase, and 39.5 μL of fraction supernatant (all reagents were from Invitrogen). A MJ Mini Gradient Thermocycler (Bio-Rad Laboratories, Hercules, CA) was used for PCR. Thermocycling parameters were 94 °C for 5 min denaturation, followed by up to 20 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 20 s. A final extension step of 72 °C for 5 min was carried out following the last cycle.

After PCR, the reaction products were separated either on denaturing PAGE (Supplemental Information Fig. S1) or on native PAGE using 1× TBE buffer (Bio-Rad Protean III) at 60–120 V (Fig. S2). The gels were stained with ethidium bromide, and photographed under UV light.

2.5. Denaturing gel electrophoresis and purification of single-stranded DNA

DNA library or aptamer pools after PCR amplification were rendered single-stranded via gel electrophoresis separation on a

denaturing gel. Denaturing PAGE was carried out using 9% gels containing 8 M urea and 25% (by volume) formamide. After running the gel at 60–120 V and staining with ethidium bromide, the 100-nt forward strand was distinguished from the 80-nt reverse strand. The 100-nt forward strand was discarded, and the 80-nt reverse strand was kept. The 80-nt reverse strand was sliced from the gel. A Qiaex II Gel Extraction Kit (Qiagen) was used to purify the reverse strand collected from the gel. Upon purification, single-stranded DNA (80-nt) was used as the aptamer pool for SELEX or stored at −20 °C in 10 mM Tris–HCl buffer (pH 8.0) for later use.

2.6. Counter-selection

After two rounds of SELEX sets D and E against the target *S. pyogenes* cell mixture of 10 M-types, the third round was a counter-selection step using non-target cells. The introduction of the counter-selection step was to remove *non*-specific sequences from the aptamer pool. A total of 10^8 *S. bovis* cells were suspended in 1× binding buffer and incubated with the aptamer pool from the second round of selection. Following incubation at room temperature for 60 min, the mixture was centrifuged to separate the cell pellets from the supernatant. The cell pellets were washed three times and the wash solution was kept. The cell pellets were then discarded because the DNA sequences bound to the *S. bovis* cells were deemed not specific for the target S. pyogenes cells. The supernatant and three wash fractions, containing DNA sequences that were not bound to the *S. bovis* cells, were retained. An aliquot from each of the fractions was amplified by PCR and the amplicons were tested using native gel electrophoresis (Supplemental Information Fig. S2). The results (Fig. S2) showed that for both sets of SELEX D and E, the supernatant fraction (lane 3 for 3D and lane 5 for 3E) contained the DNA of the expected size (100 bp).

Single-stranded DNA from the supernatant and wash fractions were ethanol precipitated, resuspended in 10 mM Tris buffer (pH 8.0), pooled, and then amplified under standard conditions using a polyA–Sp9 forward primer and regular reverse primer. The resultant double-stranded DNA amplicons were separated using denaturing PAGE to render single-stranded DNA. The 80-nt reverse strand was subsequently used for the 4th round of SELEX selection.

2.7. Flow cytometry analysis

The detailed procedures and conditions of flow cytometry analysis were as previously described [23,30], with the following modifications. For testing the binding of each aptamer pool, aptamer pools were PCR amplified with 5′-FAM modified reverse primers (IDT) and the polyA–Sp9 forward primer. The resultant double-stranded DNA amplicons were purified using native PAGE to render single-stranded DNA. The fluorescently labeled reverse strand used for flow cytometry analysis. For testing the binding of individual aptamer sequences, the selected individual aptamer sequences were purchased with the fluorescent label (5′-FAM) attached (IDT). A total of 19 individual aptamer sequences identified in the aptamer pool were fluorescently labeled and tested.

All cultures used for flow cytometric screening were harvested in stationary phase in order to minimize differences in the expression of cell surface molecules. Two hundred picomoles of the fluorescently-labeled individual aptamer sequences or aptamer pools were incubated with 10^8 cells in binding buffer for 45 min at room temperature. The cells were washed and subjected to flow cytometry analysis. Parallel control experiments were performed using a fluorescently-labeled 80-nt oligonucleotide library of random sequences. The gated fluorescence intensities were compared from flow cytometry analyses of the cells incubated with the aptamer (or aptamer pool) and the cells incubated with the random library.

In the experiments of determining the binding dissociation constants (K_d) of specific aptamers, the total number of cells was kept at 10^8 and the concentrations of aptamer were varied from 0 to 150 nM. The flow cytometry measurements were performed after incubation of the aptamer with the cells, and the cells were not washed before flow cytometry analyses.

2.8. Cloning, sequencing, and structural analysis of aptamers

The highest affinity aptamer pools determined from flow cytometry analyses were chosen for sequencing analysis: pools 8D and 8E. Aptamer pools were cloned as described in our previous work [20]. A total of 40 colonies were chosen from each aptamer pool for screening, yielding 51 useable sequences. The secondary structure of each sequence both with and without primers was predicted using Oligoanalyzer 3.1 (IDT), with input conditions of room temperature (21 °C) and 1 mM $MgCl_2$. The most likely predicted structure was considered to be those having the lowest predicted free energy of formation (ΔG) (kcal/mol).

3. Results and discussion

3.1. Selection of aptamer pools for mixed S. pyogenes cells

Fig. 1 shows a summary of the selection process. Two different initial DNA libraries represented varying sequence diversities: 10^{16} unique sequences for SELEX set D and 3–4 copies of each of 10^{14} unique sequences for SELEX set E. In addition, a round of counter-selection using *S. bovis* was performed after 2 rounds of positive selection against the *S. pyogenes* mixture. To broadly and efficiently capture sequences specific to each of the 10 M-types, we used a mixture target containing an equal number of cells from each of the 10 most prevalent *S. pyogenes* M-types in Canada [28]. The mixed cells may also serve as competitive binders for each other to increase selection stringency. Subsequent screening of the selected aptamer pools was carried out to identify individual aptamers specific to each M-type.

After each round of selection sets D and E, single-stranded aptamer pools were assessed for binding affinity and selectivity to the target *S. pyogenes* cell mixture. This was done by flow cytometry analyses of the target *S. pyogenes* cell mixture incubated with fluorescently-labeled aptamer pools. Results in Supplemental Information Fig. S3 shows that the percent of the aptamer-bound cells generally increased with increasing rounds of selection. Gated fluorescence intensity from the cells incubated with the labeled aptamer pools as compared to the background from the cells incubated with the DNA random library increased to a maximum average of 39% at round 8 (Fig. S3). In separate experiments, we observed that controls composed of fluorescent aptamer pools alone and buffer with BSA and tRNA did not yield any increase in gated fluorescence above background levels. Therefore, the increase in the fluorescence signal observed in Fig. S3 can be attributed to increased binding of fluorescent aptamers to the target cells and is representative of the strength and proportion of aptamers binding to the cells.

SELEX set D, using a higher library diversity (10^{16} unique sequences), resulted in a generally higher affinity of the aptamer pool, demonstrated by a maximum of 39% gated fluorescence increase above the background (Fig. S3). SELEX set E, using a lower starting library diversity (10^{14} unique sequences), reached a maximum of 13% gated fluorescence increase above the background (Fig. S3). These results appear to suggest that the higher diversity (10^{16} vs 10^{14} unique sequences) in the starting DNA library may lead to better aptamer pools binding to the mixed *S. pyogenes* cells of ten M-types.

The aptamer pools from SELEX sets D and E were cloned and sequenced after the 8th round, when these pools displayed high affinity for the target cells. A total of 51 sequences from the aptamer pools were obtained. There was high repetition within the aptamer pools, and many sequences contained high GC content, indicative of potential formation of secondary structures. The enrichment of certain sequences with secondary structural motifs in the aptamer pool reflects the preferential selection of these structures as a result of SELEX. Many of the secondary structures formed were similar even when the sequences were not identical. Two sequences, D-Cells 9 and D-Cells 1, obtained after 8 rounds of SELEX set D, were highly repeated in the aptamer pools. Sequence D-Cells 9 was found a total of 26 times and D-Cells 1 was found 21 times in both D and E aptamer pools. Both sequences formed similar secondary structures with and without primers, one of which can be seen in Fig. 2. Other sequences sharing secondary structural motifs were often similar yet not identical, for example, 8D-Cells 9 and 8D-Cells 20 (data not shown). The presence of multiple identical sequences along with structural similarities between different sequences within the aptamer pools indicates that certain aptamer conformations are enriched by the selection. Since the aptamer pools chosen for cloning were those with the highest affinity for the target cell mixture, it is understandable that the favoured aptamer structures are also those with the highest affinities.

3.2. Screening individual aptamer sequences for their affinity to the target cell mixture

Individual aptamer sequences identified in the aptamer pool were screened for their affinity to the target cell mixture. Each aptamer was fluorescently labeled and incubated with the target mixture of M-types used for selection. The aptamer sequences that had the highest affinity for the cell mixture were D-Cells 9, 9P, D-Cells 20, 20P, and D-Cells 1, 1P. The designation P indicates the full sequence with primers. All 6 sequences showed binding to the cells, with greater than 50% increase in the average gated fluorescence intensity above background. The background was measured using a randomized library instead of the aptamer to incubate with the cells. Sequences D-Cells 1 and D-Cells 1P were the highest binders, with increases in the gated fluorescence above background by 75% and 71%, respectively. The second highest binding sequence was D-Cells 9P, with an increase of the gated fluorescence above background by 65%. D-Cells 9, D-Cells 20, and D-Cells 20P showed percent increase of 55%, 55%, and 61% respectively, in the gated fluorescence intensities above background. Sequences D-Cells 1, D-Cells 9, and D-Cells 20 have similar predicted secondary structures and sequences. The affinity of these three aptamers for the *S. pyogenes* mixture changes minimally upon inclusion or exclusion of primers in the sequence, suggesting that the target binding site is in a central region (40 nt) of the sequence.

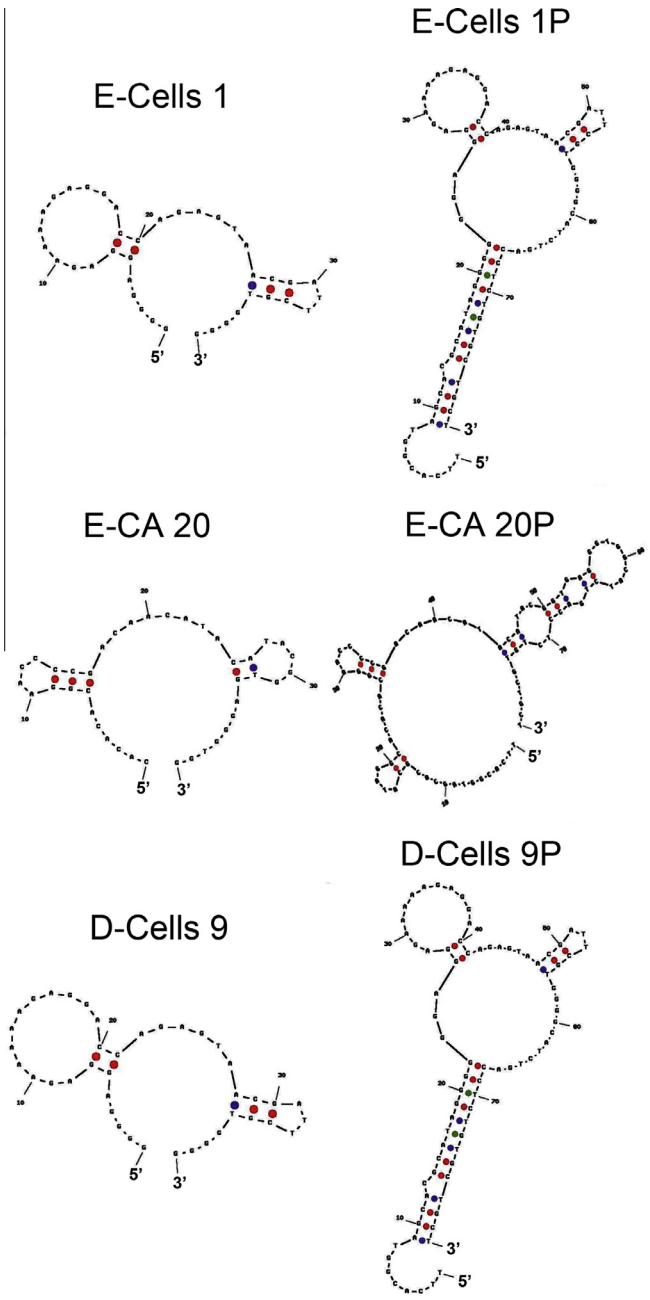

Fig. 2. Predicted structures of aptamer sequences that have high affinity and selectivity for M11 of the *S. pyogenes*. All structures were predicted using Oligoanalyzer 3.1, with conditions set to 21 °C, 100 mM NaCl, and 1 mM $MgCl_2$.

3.3. Identification of aptamers to specific M-types

Individual aptamer sequences screened against the mixed cells were also screened against separate cultures of each M-type, i.e., M1, M2, M3, M4, M6, M11, M12, M28, M77, and M89. This step was carried out to identify aptamer sequences specific to the M-types. Fluorescently-labeled aptamers were incubated with *S. pyogenes* of a specific M-type, and the gated fluorescence was measured using flow cytometry. A fluorescently-labeled randomized oligonucleotide library was used as a negative control to determine background fluorescence after its incubation with *S. pyogenes* of the specific M-type. We observed that sequences E-CA20, E-CA20P, and E-Cells 1P, obtained after 8 rounds of SELEX set E, preferentially bind to M11 (see Table 1).

We evaluated the specificity of these aptamer sequences toward M11 by testing their binding to other species of *Streptococcus*, including non-pathogenic *S. bovis*, pathogenic *S. pneumoniae* and *S. agalactiae*, and the common human flora *Enterococcus* spp. Our results show that sequences E-CA 20, E-CA 20P, and E-Cells 1P had little or no cross-reaction with these species of *Streptococcus* (Fig. 3).

We further evaluated the binding affinity (K_d) of sequences E-CA 20, E-CA 20P, and E-Cells 1P to M11. Fig. 4 shows an example of the fluorescently-labeled aptamer E-CA 20 binding to *S. pyogenes* M11. We estimated K_d values of 7 ± 1 nM. We have also generated similar binding curves for several other sequences. We tested aptamer concentrations in the incubation supernatant from 0 to 150 nM. We observed that the binding curve leveled off at the

Table 1
M11 specific sequences with and without primers. Name ending with P indicates the full sequences with primers. The primer sequences are underlined.

Name	Sequence
D-Cells 9	5′-GGGGAGGAGAAAAAGAGGACCAGAGTAACGATTCGTGGGG-3′
D-Cells 9P	5′-TTCACGGTAGCACGCATAGGGGGGAGGAGAAAAAGAGGACCAGAGTAACGATTCGTGGGGCATCTGACCTCTGTGCTGCT-3′
D-Cells 20P	5′-TTCACGGTAGCACGCATAGGGGGGAGGAGAAATAGAGGACCAGAGTAACGATTCGTGGGGCATCTGACCTCTGTGCTGCT-3′
E-CA 20	5′-CACACACGGAACCCCGACAACATACATACGGTGAGGGTGG-3′
E-CA 20P	5′-TTCACGGTAGCACGCATAGGCACACACGGAACCCCGACAACATACATACGGTGAGGGTGGCATCTGACCTCTGTGCTGCT-3′
E-Cells 1	5′-GGGGAGGAGAAAAGAGGACCAGAGTAACGATTCGTGGGG-3′
E-Cells 1P	5′-TTCACGGTAGCACGCATAGGGGGGAGGAGAAAAGAGGACCAGAGTAACGATTCGTGGGGCATCTGACCTCTGTGCTGCT-3′

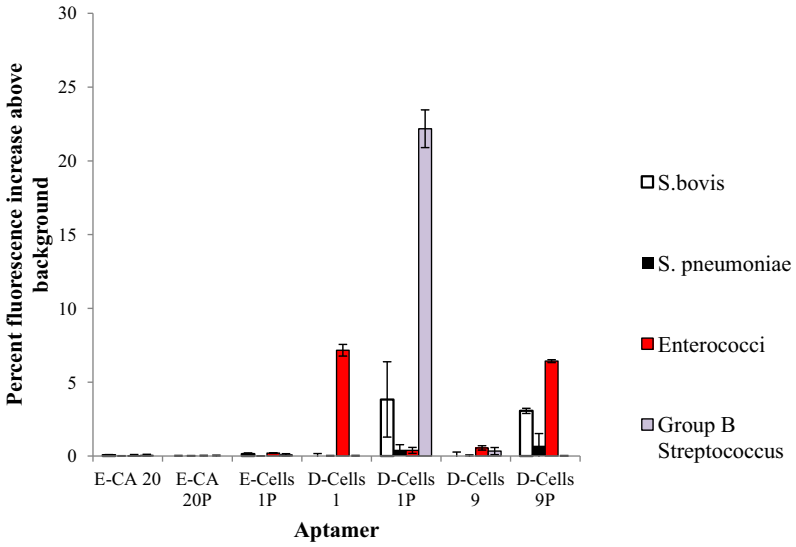

Fig. 3. Screening of the selected aptamers against non-target cells. Aptamers were labeled with 5′-FAM, and 200 pmol of aptamers were incubated with 10^8 cells. Cells were then analyzed using flow cytometry. Error bars represent standard deviation from triplicate flow cytometry analyses.

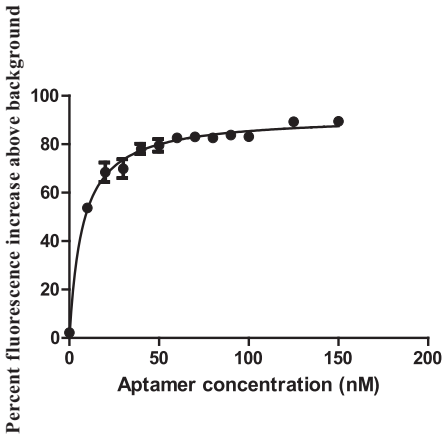

Fig. 4. Percent increase in gated fluorescence intensity above background from flow cytometry measures of M11 cells that were incubated with increasing concentrations of aptamer E-CA 20. Aptamer E-CA 20 was fluorescently labeled with 5′-FAM. The aptamer of varying concentrations (0–150 nM) was incubated with 10^8 *S. pyogenes* cells of M11-type in binding buffer for 45 min. Cell pellets were then washed twice with 1× binding buffer. Cells were resuspended in 1× binding buffer and subjected to flow cytometry analysis. A total of 10,000 events were counted and the gated fluorescence intensity over background was measured. Error bars represent standard deviation from triplicate flow cytometry analyses.

aptamer concentration below 70 nM for each sequence. We also examined the binding of a fluorescently-labeled randomized oligonucleotide library to the *S. pyogenes* cell mixture, which served as background. Table 2 summarizes the K_d values of each aptamer tested. Sequences E-CA 20 and E-CA 20P had higher affinities for M11 and both had K_d values in the low nanomolar range (K_d = 7 ± 1 nM for E-CA 20 and 12 ± 1 nM for E-CA 20P). These binding affinities are comparable to those of a monoclonal antibody for its protein target.

Table 2
Binding dissociation constants (K_d) of aptamer sequences with selectivity for *S. pyogenes* cells of the M11 M-type. SE denotes standard error.

Aptamer sequence	K_d (nM)	SE (nM)
D-Cells 9 (from Set D)	71	23
D-Cells 9P (from Set D)	44	8
E-CA 20 (from Set E)	7	1
E-CA 20P (from Set E)	12	1
E-Cells 1P (from Set E)	20	3

4. Concluding remarks

Aptamer-based affinity assays have great potential as tools in bacterial surveillance and diagnosis. The primary objective of this research was to generate aptamers for *S. pyogenes* M-types against a mixture of the 10 most prevalent M-types in Canada [23,28]. We had previously carried out a separate SELEX protocol to generate aptamers specific for *S. pyogenes* [23]. In our previous work, 13–20 rounds of SELEX against *S. pyogenes* were required to generate high affinity sequences toward target cells. In the present work, with 8 rounds of SELEX we successfully obtained several aptamer sequences that bind to specific M-types of *S. pyogenes*. Many of

the aptamer sequences have good binding affinity to the target cells, with K_d at the low nanomolar range.

The SELEX approach in the present work involved three modifications over our previous work. First, we modified a forward primer by adding a polyA$_{20}$/5Sp9 component. This facilitated denaturing gel electrophoresis separation of the forward strand from the reverse strand of the PCR amplicons. The advantage over heat denaturation is that no sequences were lost due to reannealing as in the case of heat denaturation. The purified reverse strand DNA served as the input library each subsequent round of SELEX. The more efficient separation and purification of the single-stranded DNA, combined with the use of larger portions of the input library, longer incubation time, and better mixing of the cells with DNA, were designed to minimize the loss of potential binding sequences.

Secondly, we tested the use of starting DNA libraries of two sequence diversities: 10^{16} unique sequences (SELEX set D) and 10^{14} unique sequences (SELEX set E). Limiting the number of cycles of PCR amplification of the initial starting library to 3 was to maintain the high sequence diversity (10^{14} unique sequences) for the first round of SELEX. We were able to obtain individual aptamer sequences of comparable high binding affinity from both sets of SELEX experiments. Our initial results from screening the aptamer pools against a cell mixture of ten M-types (Fig. S3) showed that the aptamer pools originated from the SELEX set D (diversity of 10^{16} unique sequences) had higher binding affinities than the aptamer pools originating from the SELEX set E (diversity of 10^{14} unique sequences). These results suggest that a higher sequence diversity in the starting DNA library is useful for generating aptamer pools that have overall high affinity in their binding to mixed cells (ten M-types of *S. pyogenes*). We then cloned and identified 51 sequences in the aptamer pools of SELEX sets D and E after 8 rounds of selection. When the selective aptamers were tested against a specific M-type, M11, individual aptamers from both sets of SELEX showed similar high affinity binding to M11 of *S. pyogenes* (Table 2). These results suggest that a diversity of 10^{14} unique sequences in the starting DNA library is sufficient for generating a specific DNA aptamer binding to a single M-type of *S. pyogenes*.

Thirdly, we incorporated a counter-selection step after the first two rounds of positive selection. The counter-selection, using the non-target *S. bovis* cells, was designed to remove sequences that are not specific to *S. pyogenes*. The selected aptamer sequences showed specificity for *S. pyogenes* over other species of bacteria, such as *S. bovis*, *S. pneumoniae*, *S. agalactiae*, and the *Enterococcus* species. While the SELEX process used a mixture of M-types, three sequences were found to bind selectively to M11 cells with high affinities (low nanomolar K_d). The most promising highest affinity and specificity sequence for M11 cells was E-CA 20, with a K_d of 7 ± 1 nM. Further screening of more clones may yield aptamers specific to different M-types. In addition, further selection with cells of a single M-type may be used to obtain aptamers specific for each of the 10 most prevalent M-types circulating in the Canadian population. Future work can also include the introduction of counter-selection against unwanted M-types.

Aptamers specific for M11 of *S. pyogenes* cells have potential applications for detecting this bacterium and could improve upon current M-typing technologies and possibly extend this to development of point-of-care assays. Antibody-based serotyping, the gold standard method for streptococcal typing, has been phased out worldwide to be replaced by sequencing of the *emm* gene (the gene encoding the M protein). However, comparison of sequences to reference strain databases is still required. M-type specific aptamers could substitute for antibodies in latex agglutination or immunochromatographic antigen tests, both improving sensitivity and negating the need for confirmatory culture. M-type specific aptamers could also be affixed to a solid support to develop an aptamer array for high-throughput M-typing of clinical isolates. The aptamers on the array could be used in either a direct assay or a sandwich assay to capture whole cells or the extracted M-proteins out of solution. The direct assay would involve using the array to capture *S. pyogenes* cells of a specific M-type out of solution, and then fixing and counting the number of cells captured. In the sandwich assay, the captured cells or protein could be visualized with a second fluorescently-labeled aptamer against the M protein or a fluorescently-labeled antibody recognizing the non-variable M-protein C-terminus or an *S. pyogenes* cell wall component common to all M-types, such as the group A carbohydrate or peptidoglycan.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ymeth.2015.12.005.

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