ORIGINAL ARTICLE

Veterinary Research Forum. 2026; 17 (4): 225 - 231
doi: 10.30466/vrf.2025.2065775.4838
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Cell SELEX-based DNA aptamer selection targeting *Streptococcus agalactiae* using high-throughput sequencing: toward multifunctional applications in aquaculture

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Article Info

Article history:

Received: 16 July 2025
Accepted: 15 November 2025
Available online: 15 April 2026

Keywords:

Aptamer
Aquatic pathogens
High-throughput sequencing
Streptococcus agalactiae
Whole-cell SELEX

Abstract

Streptococcus agalactiae is a Gram-positive bacterial pathogen accountable for disease outbreaks in tilapia, resulting in substantial economic losses in aquaculture. In this study, a 10-round whole-cell Systemic Evolution of Ligands by Exponential Enrichment procedure was conducted to isolate and characterize single-stranded DNA aptamers with specific binding affinity to *S. agalactiae*. The enriched single-stranded DNA libraries were sequenced by high-throughput sequencing, yielding a total of 180,987 reads, of which 126,933 included unique aptamer sequences. The most frequent sequences were selected for further analysis. Among them, SA1 had the highest frequency, with 231 reads, whereas SA2 showed a lower number of 16 reads. These findings underscored the validity of combining whole-cell Systemic Evolution of Ligands by Exponential Enrichment, high-throughput sequencing and aptamer-structure analysis to explore the interaction between aptamers and bacterial cell surfaces. The selected aptamers not only showed promise for the detection of *S. agalactiae* but also served as molecular probes for identifying surface-exposed proteins, studying host-pathogen interactions and guiding targeted drug delivery in aquaculture-related applications.

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Introduction

As aquaculture becomes a crucial contributor to global food security amid a steadily growing population, Asia has evolved into the world leading hub for aquatic animal production.[1] Nonetheless, climate change and the increased occurrence of disease outbreaks are undermining the stability and long-term sustainability of the industry.[2] Rising global temperatures have been shown to significantly impact the occurrence and mortality of infectious diseases. A 1.00 ˚C rise in water tempertature might lead to an increase in mortality due to pathogens such as *Edwardsiella spp.*, *Lactococcus spp.*, *Aeromonas spp*., *Vibrio spp.*, *Flavobacterium columnare*, *Streptococcus spp.* to be between 2.82 - 4.12%.[3] Of the many bacteria that thrive in warm water conditions, *Streptococcus agalactiae* has raised concern for its ability to cause serious disease outbreaks in Nile Tilapia (*Oreochromis niloticus*) which is one of the main aqua culture species in Thailand and other Southeast Asia countries. *Streptococcus agalactiae* significantly impacts the aquaculture industry because it can lead to high mortality within a short period.[4,5] This pathogen has a wide host range, as not only fish but also humans and other mammals have been documented with such infections,[6] while virulence is often increased due to rising temperatures linked to climate change.[7] These unpredictable epidemiological and biological changes warrant much more attention for flexible approaches including vaccines, rapid immunodiagnosis technologies, platforms for the early detection of pathogens, antibacterial approaches and the development of targeted therapeutics.

One of promising biotechnologies is the development of aptamers, which are short strands of DNA or RNA that can bind selectively to biological targets such as bacteria, viruses, antibiotics, proteins, small molecules, or even whole cells and form stable complexes, making them potential candidates for both detection and treatment of

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bacterial infections.[8] Aptamers are three-dimensional single-stranded oligonucleotides (DNA or RNA) selected through the Systematic Evolution of Ligands by Exponential Enrichment (SELEX) process, known for their high affinity and specificity.[9,10] Aptamers as compared to antibodies offer several notable advantages which include ease of synthesis, low production cost, high stability under harsh conditions, along with being easily labeled or integrated into detection systems.[11,12] In aquaculture, aptamers have shown significant progress in pathogen detection, biosensor development and as antibacterial strategies.[13-17]

In this study, SELEX process was utilized to isolate single-stranded DNA (ssDNA) aptamers that can specifically bind to *S. agalactiae*. Mostly, the final selected aptamer library is cloned and sequenced by Sanger sequencing to identify individual aptamer sequences.[14,18] Nowadays, high-throughput sequencing, combined with bioinformatics, enables rapid identification of high-affinity aptamers and provides a comprehensive analysis of sequence abundance, nucleotide composition and predicted secondary structures.[19-21] Furthermore, after the selection phase, the enriched aptamers binding *S. agalactiae* were processed for high throughput sequencing to identify the most abundant sequences, which are presumed to have high binding affinity to *S. agalactiae*. Additionally, the aptamers developed in this study have potential for broader applications such as monitoring pathogens in an aquatic environment, aiding the isolation of specific bacteria and serving as molecular probes for investigating the mechanisms of diseases. Overall, this research contributed to establish foundation for the development of accurate, adaptable and field-deployable biological tools in aquaculture.

Materials and Methods

Bacterial isolates. *Streptococcus agalactiae* was isolated from Nile Tilapia (*O. niloticus*) showing clinical signs of streptococcosis, including darkened skin, abdominal distention, bilateral exophthalmos, hemorrhage, spleen enlargement/swelling, gallbladder enlargement (Fig. 1).[22] Moribund fish were collected from commercial fish farm and transported to the laboratory by the farm owner. For bacterial isolation, samples were collected from mucosal surfaces, gill tissue, brain, head, kidney, and liver. Isolation of *S. agalactiae* was carried out using tryptic soy agar (HiMedia Laboratories, Nashik, India) supplemented with 5.00% sheep blood (sourced from Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom, Thailand) and incubated at 28.00 ˚C for 24 hr. Pure bacterial colonies were then subject to genomic DNA extraction using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany). Species was identified using the MALDI-TOF mass spectrometry (Bruker Daltonics GmbH, Bremen, Germany) and confirmed by amplifying the full-length *16S rRNA* gene using universal primers with a target size of approximately 1.50 kb.[23] The obtained sequences were analyzed and compared to reference sequences available in GenBank® using Basic Local Alignment Search Tool (BLAST) algorithm.

Single strands DNA library and primer. The initial DNA library (1.50 nmol) used in the SELEX process was designed with a central region of 40 random nucleotides, flanked by two fixed primer-binding sites for PCR amplification. This library was synthesized by Synbio Technologies Company (Monmouth Junction, USA) and had the following structure: 5’ – TAATACGACTCACTATA GGGCCAGGCAGCGAG – N_{40} – CCGACCACACGCGTCCGAGA– 3’. The forward (5’-TAATACGACTCACTATAGGGCCAGGC AGCGAG-3’) and reverse primer (5’-TCTCGGACGCGTGTGG TCGG-3’) were used to amplify aptamer sequences during each SELEX cycle.

***In vitro* selection of the ssDNA aptamers for *S. agalactiae*.** The whole-cell SELEX aptamer process in this study began with culturing *S. agalactiae* in tryptic soy agar broth at 28.00 ˚C with gentle shaking until a minimum optical density (OD_{600}) of 0.30 was reached. Cell was centrifuged at 5,000 rpm at 4.00 ˚C to remove the culture medium and washed with 1.00 X phosphate-buffered saline, pH 7.40 (137 mM NaCl, 2.70 mM KCl, 8.00 mM Na_2HPO_4, and 2.00 mM KH_2PO_4). Simultaneously, an ssDNA aptamer library was prepared by mixing with 1.00 X binding buffer including 2.50 mM $MgCl_2$ (Merck, Darmstadt, Germany), 0.02% Tween-20 (Sigma-Aldrich, St. Louis, USA) in phosphate-buffered saline (HyClone, Logan, USA), denatured by heating to 95.00 ˚C for 10 minutes, then rapidly snap-cooled on ice for 10 min, followed by a slow return to room temperature for 1 hr to promote aptamer folding. For the first round, the folded ssDNA aptamer solution was incubated with whole bacteria cells at OD_{600} = 0.30 in binding buffer at RT for

Fig. 1. Lesions caused by *Streptococcus agalactiae* in Nile Tilapia (*Oreochromis niloticus*), showing **A)** abdominal distention (red arrow), **B)** bilateral exophthalmos (red arrow), and **C)** spleen enlargement/swelling, gallbladder enlargement (red arrows).

1 hr with slow rotation to promote binding. After incubation, the mixture was centrifuged at 5,000 rpm at 4.00 ˚C for 5 min and the bacterial pellet (containing cell-bound aptamers) was collected. The cell pellet was washed twice with washing buffer including 1.00 mM $MgCl_2$, 40.00 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; HyClone), 4.00 mM KCl (Merck), 2.50 mM $CaCl_2$ (Merck), 140 mM NaCl (Merck), 0.02% Tween-20 in 1.00 X phosphate-buffered saline) to remove any loosely bound aptamers, centrifuging each time at 5,000 rpm at 4.00 ˚C for 5 min. The cleaned bacterial pellet was then re-suspended in 100 µL of distilled water and heated at 95.00 ˚C for 5 min to release the cell-bound aptamers. A final centrifugation at 5,000 rpm at 4.00 ˚C for 5 min was performed to collect the supernatant, which contained the enriched, cell-binding aptamers. These aptamers were then amplified by PCR and used as the input for the next round of SELEX. The PCR was carried out in a 20.00 µL reaction containing 5.00 µL of ssDNA templates, 0.50 µL of 10.00 µM forward primer, 0.50 µL of 10.00 µM reverse primer, 10.00 µL of 2X GoTaq® MasterMix (Promega, Madison, USA), 4.00 µL Nuclease-free water. The PCR amplification was performed using 10, 15, 20, and 25 cycles to identify the optimal number of cycles that yielded specific products without generating off-target amplification. The thermal cycling conditions included an initial denaturation at 95.00 ˚C for 5 min, followed by 15 cycles of 95.00 ˚C for 30 sec, 55.00 ˚C for 30 sec and 72.00 ˚C for 30 sec, with a final extension at 72.00 ˚C for 5 min. The amplified products were purified using GF-1 AmbiClean Kit (Gel and PCR; Vivantis, Subang Jaya, Malaysia). This process was repeated for 10 rounds, with each round aiming to enhance the selection of aptamer with high specificity and affinity toward the target bacterial cells. A negative control was conducted under identical conditions, except that the bacterial cells were incubated with binding buffer in the absence of the ssDNA library to account for nonspecific interactions (Fig. 2).

High-throughput sequencing and structure analysis of aptamer. Aptamers with high specificity and affinity, obtained after SELEX selection, were sequenced using paired-end 150 bp reads on the Illumina MiSeq platform (Illumina, San Diego, USA) through sequencing services provided by Vishuo Biomedical, Thailand. Raw reads were quality-checked with FastQ quality control (version 0.11.9; Babraham Bioinformatics, Cambridge, UK) and processed using Fastp to remove adapters and primers, retaining only those with a Phred quality score > 30.00.[24,25] Clean reads were merged using Fast Length Adjustment of Short reads (FLASH) with a minimum overlap of > 90 bp and a maximum of ≤ 100 bp to reconstruct full-length sequences.[26] The merged sequences were filtered again with Fastp to retain only those with lengths between 90 - 94 bp. Subsequently, unique sequences with 100% similarity were clustered, and their frequencies were quantified using VSEARCH (version 2.28.1; University of Oslo, Oslo, Norway) implemented in QIIME2 (version 2024.10; Northern Arizona University, Flagstaff, USA).[27,28] The two most abundant unique sequences from forward and reverse strands were selected for further analysis. Gibbs free energy (ΔG) and secondary structures were predicted using mfold (https://www.unafold.org/mfold/applications/dna-folding-form.php) under SELEX-equivalent conditions, including 2.50 mM Mg^{2+} at 25.00 ˚C, matching the composition of the binding buffer.

Results

Selection of high-affinity aptamers targeting *S. agalactiae*. The whole-cell SELEX process was successfully performed ten iterative rounds to enrich ssDNA aptamers with high specificity and affinity for *S. agalactiae* cells. In each round, the recovered ssDNA was successfully amplified by PCR. The number of PCR cycles was adjusted based on the intensity of DNA bands and

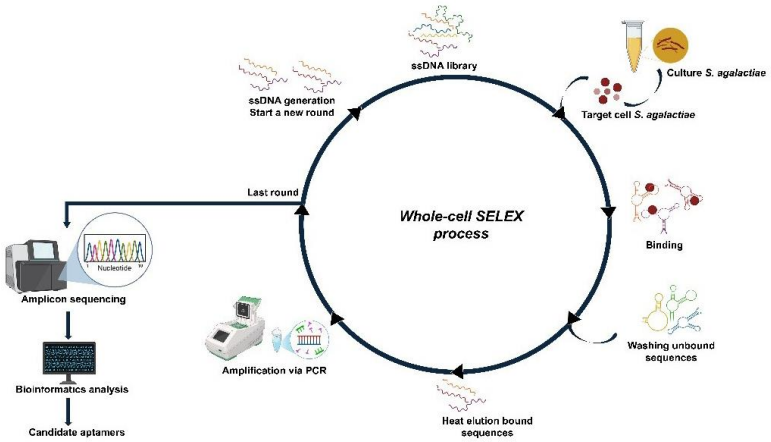

Fig. 2. Schematic representation of the whole-cell Systemic Evolution of Ligands by Exponential Enrichment (SELEX) process used to isolate aptamers targeting *S. agalactiae*.

expected size observed by agarose gel electrophoresis. Based on PCR cycle testing, both 10 and 15 cycles yielded the specific target, while 15 cycles was determined to be optimal for efficient ssDNA amplification without the appearance of non-specific products, which were observed at 20 and 25 cycles. The specific amplification products were approximately 100 bp in size, including primer regions. Therefore, the products around 200bp observed at higher cycles were considered non-specific (Fig. 3A). Gel electrophoresis of PCR products from SELEX rounds 1 to 10 indicated successful enrichment of target-binding aptamers throughout the selection process (Fig. 3B). The final enriched pool was subsequently sequenced using high-throughput sequencing.

Characterization of aptamer candidates using next-generation sequencing. Following ten rounds of SELEX, the enriched aptamer pool was subjected to amplicon sequencing to identify ssDNA sequences with high specificity and affinity for *S. agalactiae*. The sequencing yielded a total of 180,987 reads. Among these, serveral sequences were highly abundant, which represented aptamers with strong binding capacity for the target cells. Sequencing analysis identified a total number of 126,933 reads. Among these, the SA1 sequence had the highest frequency with 231 reads, followed by SA2 had 16 reads while the remaining sequences were found at lower frequencies (Table 1).

Aptamer sequences with high copy numbers were considered strong candidates, potentially possessing high binding affinity and specificity toward the target cells. The most frequent of both forward and reverse strands were selected for secondary structure prediction. Among the top analyzed aptamers, SA2_reverse strand exhibited the most negative ΔG value. Conversely, SA1_Forward strand had the least negative ΔG value (Fig. 4).

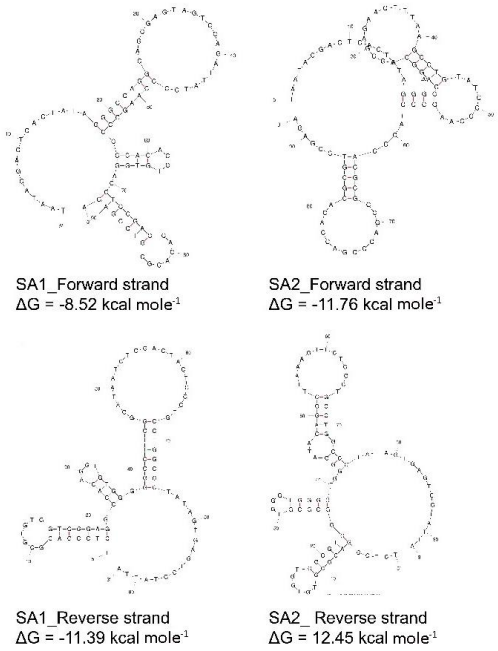

Fig. 4. Secondary structure with minimum Gibbs free energies (ΔG) of top single-stranded DNA aptamers predicted by the mFold Software (http://www.unafold.org). Predictions were generated at 25.00 ˚C in the presence of 2.50 mM Mg^{2+}.

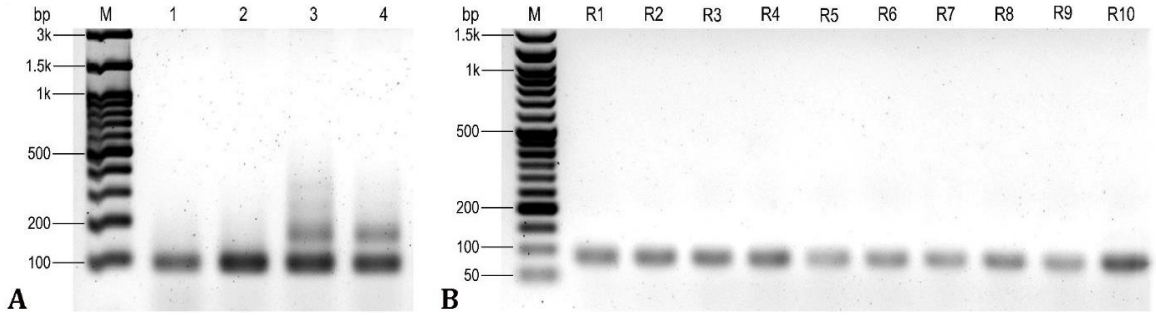

Fig. 3. A) Gel electrophoresis showing single-stranded DNA (ssDNA)enrichment via PCR amplification from 10 cycles to 25 cycles. Lane M: 3.00 kb DNA ladder; Lane 1: 10 cycles, Lane 2: 15 cycles, Lane 3: 20 cycles, Lane 4: 25 cycles. **B)** Gel electrophoresis showing ssDNA enrichment across Systemic Evolution of Ligands by Exponential Enrichment rounds (SELEX). Lane M: 1.50 kb DNA ladder; Lanes R1 - R10: rounds 1 - 10.

Table 1. Representative unique sequences from the two most frequent enriched single-stranded DNA aptamers targeting *S. agalactiae*.

Sequence ID	Frequency	Sequences
SA1	231	F: TAATACGACTCACTATAGGGCCAGGCAGCGAGTAGTCCAGATTAT CCCCAAGCCCCCCACACCTGTGGACCTCCGACCACACGCGTCCGAGA R: TCTCGGACGCGTGTGGTCGGAGGTCCACAGGTGTGGGGGGCTTGGG GATAATCTGGACTACTCGCTGCCTGGCCCTATAGTGAGTCGTATTA
SA2	16	F: TAATACGACTCACTATAGGGCCAGGCAGCGAGAACTTTAAGCCTGT ATCCCCAAGCCCACCCACGCGCCGACCCGACCACACGCGTCCGAGA R: TCTCGGACGCGTGTGGTCGGGTCGGCGCGTGGGTGGGCTTGGGGAT ACAGGCTTAAAGTTCTCGCTGCCTGGCCCTATAGTGAGTCGTATTA

Underlined sequences correspond to the fixed regions of the forward and reverse primers.

Discussion

Whole-cell SELEX is an effective approach for developing DNA aptamers with high affinity and specificity toward bacterial targets including pathogens of concern in aquaculture. Comparable strategies have produced aptamers for other aquaculture pathogens such as *Renibacterium salmoninarum*, the agent of bacterial kidney disease in salmonids.[29] The cell-SELEX workflow can be further refined through fluorescence-activated cell sorting, counter-SELEX to boost selectivity.[30] Aptamers derived in this way offer a rapid, antibody-free alternative for detecting pathogens directly in production systems.

Recent advances in high-throughput sequencing have made the SELEX process even more efficient by providing round-by-round, deep profiling of enriched aptamer pools. High-throughput sequencing pinpoints dominant candidates early, tracks enrichment dynamics and often reduces the number of selection rounds required. It also reveals sequence diversity and structural convergence, information that is essential for choosing high-performance aptamers. Coupled with bioinformatics analysis, high-throughput sequencing enables the rational design and confirmation of aptamers with optimal binding characteristics, thereby, accelerating the development of diagnostic and therapeutic tools for aquaculture.[31] Taking advantage of these developments, this study successfully identified high-affinity and high-specificity ssDNA aptamers for *S. agalactiae* using whole-cell SELEX. This approach facilitated aptamer selection without requiring prior knowledge of the molecular surface epitopes of the target, which was demonstrated to be a powerful tool for identifying molecules that recognized complex bacterial surfaces in native conformations.[11]

After ten iterative rounds of selection and amplification, the enrichment of aptamers specific to *S. agalactiae* was confirmed. High-throughput sequencing technology was then employed to sequence the enriched DNA aptamer, resulting in a comprehensive dataset conmprising 180,987 reads. While analysis using VSEARCH showed that the aptamer library was highly diverse, some unique sequences appeared with remarkable frequency, indicating strong selection pressure and successful enrichment of high-affinity aptamers. Notably, the most common sequence, designated SA1, was observed in 231 reads, while the remaining sequences exhibited low-frequency repeats. Moreover, the capacity of SELEX to yield highly specific aptamers is further substantiated by secondary structures predicted via mFold for the most frequently occurring sequences. These structures often exhibit stable hairpin or stem-loop conformations, which are structural motifs recognized for their important role in target recognition and binding, as observed in enriched aptamer libraries.[32,33] Among the top two analyzed aptamers, SA2_reverse strand indicated the highest thermodynamic stability of predicted secondary structure with the most negative ΔG value.[34] Conversely, SA1_Forward strand suggested the lowest structural stability with least negative ΔG value (Fig. 4). Despite this, SA1 represented a unique sequence with relatively high read frequency and potential binding capability to surface proteins on target cells. These characteristics suggested that SA1 remained a strong candidate for further development as a functional aptamer. Further experimental validation is required to determine and compare the binding affinities of these candidate aptamers.

These findings underscored the effectiveness of whole-cell SELEX for aptamer selection across diverse bacterial species, even without specific surface marker knowledge. Cell-SELEX has been successfully employed to select aptamers for *Mycobacterium tuberculosis*, *Staphylococcus aureus*, and *Escherichia coli*, demonstrating the broad applicability of this method.[30,35,36] The exact molecular targets of the selected aptamers on the bacterial surface have not yet been identified. However, since whole-cell SELEX was performed without prior disruption of the bacterial cell wall, it is likely that the aptamers recognize naturally exposed surface structures, such as polysaccharides, membrane proteins, or other cell wall components. A previous study demonstrated that aptamers selected against *E. coli* specifically bound to lipopolysaccharide and were able to inhibit lipopolysaccharide -induced macrophage activation.[37] Future studies using techniques such as pull-down assays or aptamer-based imaging may help clarify the exact binding epitopes. The key advantage lies in capturing the comprehensive surface architecture of intact bacterial cells, potentially revolutionizing diagnostic tool development.[38] However, a crucial next step involves rigorously evaluating aptamer performance in complex biological matrices like serum and tissue fluids.[39]

In conclusion, the integration of whole-cell SELEX with high-throughput sequencing enabled the identification of high-affinity, high-specificity ssDNA aptamers targeting *S. agalactiae*. These findings provided a solid foundation for the development of aptamer-based diagnostic and therapeutic tools in aquaculture. The notable affinity and selectivity exhibited by the aptamers as well as their structural integrity, underscored the potential as promising candidates for further investigation and practical implementation.

Acknowledgments

The authors would like to acknowledge the valuable support of Center for Life Science Research, Ho Chi Minh City Open University, Ho Chi Minh City, Vietnam, Department of Pharmaceutical and Medical Biotechnology,

Faculty of Biotechnology, Ho Chi Minh City Open University, Ho Chi Minh City, Vietnam, the Faculty of Veterinary Science, Rajamangala University of Technology, Srivijaya, Nakhon Si Thammarat, Thailand, and Department of research and development Queen Saovabha Memorial Institute, Thai Red Cross Society, Bangkok, Thailand.

Conflicts of interest

The authors declare no conflict of interest.

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