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# Development of ssDNA Aptamers for the Sensitive Detection of *Salmonella typhimurium* and *Salmonella enteritidis*

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**Abstract** *Salmonella enterica* subsp. *enterica* ser. *enteritidis* and *Salmonella enterica* subsp. *enterica* ser. *typhimurium* are the most common and severe food-borne pathogens responsible for causing salmonellosis in humans and animals. The development of an early and ultra-sensitive detection system is the first critical step in controlling this disease. To accomplish this, we used the cell systematic evolution of ligands by exponential enrichment (Cell-SELEX) technique to identify single-stranded DNA (ssDNA) aptamers to be used as detection probes that can specifically bind to *S. enteritidis* and *S. typhimurium*. A total of 12 target-specific ssDNA aptamers were obtained through ten rounds of Cell-SELEX under stringent selection conditions, and negative selection further enhanced the selectivity among these aptamers. Aptamer specificity was investigated using the gram-negative bacteria *E. coli* and *P. aeruginosa* and was found to be much higher towards *S. enteritidis* and *S. typhimurium*. Importantly, three candidate aptamers demonstrated higher binding affinities and the dissociation constants (Kd) were found to be in the range of nanomolar to submicromolar levels. Furthermore, individual aptamers were conjugated onto polyvalent directed aptamer polymer, which led to 100-fold increase in binding affinity compared to the individual aptamers alone. Taken together, this study reports the identification of higher affinity and specificity ssDNA

**Highlights** Identified ssDNA aptamers that selectively bind to *S. enteritidis* and *S. typhimurium*.
The candidate aptamers showed high affinity in the nanomolar to sub micromolar range.
Development of conjugated aptamers (PDAP) showed enhanced affinity and specificity.
The identified short 30-mer DNA aptamers can be useful as sensitive detection probes.

Hae-Chul Park and Irshad Ahmed Baig has contributed equally.

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aptamers (30mer), which may be useful as capture and detection probes in biosensor-based detection systems for salmonellosis.

**Keywords** DNA aptamer · Cell-SELEX · *Salmonella* · PDAP · Biosensor · Salmonellosis

## Introduction

*Salmonella* is one of the most common foodborne pathogens and is the leading cause of the foodborne illness salmonellosis [1]. *Salmonella* infections can be fatal if not detected and treated at early stages. Salmonellosis outbreaks in humans are mostly associated with the ingestion of *Salmonella*-contaminated foods mainly originating from animal sources, such as poultry egg, milk, beef, and raw food, and also from non-animal sources, including fruits, vegetables, and spices. The person infected with *Salmonella* develops diarrhea, fever, and abdominal cramps 12 to 72 h post-infection [2, 3]. Despite several disease control and prevention strategies, *Salmonella* outbreaks have significantly increased in the past decade [4].

The World Health Organization (WHO) reported that approximately 16 million cases of typhoid fever, 1.3 billion cases of gastroenteritis, and 3 million deaths occur due to *Salmonella* bacterial infections worldwide each year [5]. The Centers for Disease Control and Prevention (CDC) estimated that approximately 40,000 cases of salmonellosis are reported in the USA and 400 persons die each year from acute salmonellosis [6]. According to a recent survey, the rate of *Salmonella* infection in Korea has also increased [7]. Several *Salmonella* species were identified as food pathogens, but among them, all *Salmonella typhimurium* and *Salmonella enteritidis* are currently the most widely spread critical pathogens causing foodborne illnesses in humans and animals. Both *S. enteritidis* and *S. typhimurium* are typical serovars belonging to the *S. enterica* subspecies [8]. The *Salmonella* serotype infection not only causes foodborne illness but also causes severe losses to microbial food spoilage and contamination of food products, leading to significant negative impacts on the economy. Thus, the identification of a novel and specific probe for the rapid and sensitive detection of *Salmonella* spp. is critical to ensure human health and food safety.

So far, various detection strategies have been developed for *Salmonella* detection. These include conventional microbiological culture methods, PCR, enzyme-linked immunosorbent assays (ELISAs), and biosensors [9–15]. Although the conventional microbiological culture method has proved useful, it is tedious and requires days for pathogen identification and confirmation and cannot provide real-time monitoring information. Recently, biosensor-based systems have demonstrated great potential for the rapid and sensitive detection of bacterial pathogens [16–18]. Importantly, the main advantage of biosensor-based detection systems is that they allow for the monitoring of bioreactions in real time for on-site diagnosis because they allow for the immobilization of various ligands on the surface of a physical sensor [19]. Thus, many antibody-based biosensor systems were developed for the early diagnosis of *Salmonella* infections. However, low stability, high cost of synthesis, and a limited shelf life restricts their wide use as a detection probe. Alternatively, nucleic acid aptamers hold great potential as novel detection probes and offer various advantages over antibody-based detection systems. The aptamers provide several feasible advantages such as higher stability, lower cost of synthesis, high affinity, and specificity towards their detection target. Furthermore, aptamers can be easily chemically modified, fluorescently labeled, and conjugated with other nanomaterials to develop novel biosensor-based detection system [19].

In this study, we used the Cell-SELEX method to select high affinity ssDNA aptamers towards live *Salmonella* cells as a target [20–23]. Cell-SELEX has several advantages compared with general purified molecule targeted SELEX [24].

Briefly, this study focused on the screening and identification of specific single-stranded DNA (ssDNA) aptamers against *S. enteritidis* and *S. typhimurium* using Cell-SELEX. In

addition, the binding affinity and specificity of candidate aptamers were further improved using a polyvalent directed aptamer polymer (PDAP) system.

## Methods and Materials

### Chemicals and Bacteria

Lyophilized *S. typhimurium* and *S. enteritidis* were commercially purchased from the Korean Culture Center of Microorganisms (KCCM, South Korea). Cells were incubated at 35 °C overnight in brain-heart infusion medium (Difco, NJ, USA). Whole broth was harvested by centrifugation, washed three times with phosphate-buffered saline (PBS), and resuspended in buffer each round prior to performing Cell-SELEX. All other chemicals and reagents were of analytical grade and purchased from commercial sources.

### Amplification of a Single-Stranded DNA (ssDNA) Library

The ssDNA template was designed with specific primers at each end and 30 random nucleotide sequences. The ssDNA template sequence was 5′-GCGCGGATCCCGCGC (N30) CGCGCGAA GCTTGCG-3′. Specific primers were desk90igned as follows: the forward primer and reverse primer were 5′-GCGCGGATCCCGCGC-3′ and 5′-GCGCAAGCTTCGCGC-3′, respectively (the BamHI and HindIII restriction sites were underlined, respectively.) In order to amplify the ssDNA template, asymmetric polymerase chain reaction (PCR) was performed and subsequently analyzed using 2.5 % agarose electrophoresis. PCR conditions for 45 cycles were as follows: 30 s at 98 °C for denaturation, 30 s at 72 °C for extension, and 15 s at 55 °C for annealing, followed by 5 min at 72 °C for a final extension. The PCR conditions were the same under all selection processes.

### In Vitro Cell-SELEX

After preparation of the ssDNA library, the first selection was performed under specific SELEX conditions (Table 1). Briefly, various cell culture pellet concentrations were resuspended at each round with selection buffer and incubated at 30 °C for 90 min. The selection of *S. typhimurium* and *S. enteritidis* bound ssDNA aptamers was carried out using a high-speed chilled centrifugation method. After incubation, cells and the ssDNA nucleotide complex were pelleted in a centrifuge (Micro 17, Hanil Science, Seoul, Korea) at 12,000 rpm for 15 min in order to remove unbound ssDNA nucleotides. The supernatant was removed, and the pellets were washed with PBS. This step was repeated three times every round during the washing step. To elute the bound ssDNA aptamers on the bacterial surface, the bacteria-ssDNA complex in PBS was boiled for 10 min at 95 °C, which was followed by chilling on ice for 10 min to denature the bound ssDNA pool. After chilling, the mixture was centrifuged for 10 min at 12,000 rpm in a chilled rotor. The supernatant was harvested and used for amplification during the next selection round. For the counter selection against each serovar type, the bound ssDNA pool was incubated with *S. enteritidis*, *S. typhimurium*, *Pseudomonas aeruginosa*, and *Escherichia coli* after termination of the 7th round of selection.

### Aptamer Sequencing and Secondary Structure Analysis

After completion of the selection steps, the final PCR products were cloned into the pET-28a (Novagen, Madison, WI) vector for DNA sequencing. Briefly, after 10 rounds of Cell-SELEX, the final ssDNA pool was amplified by PCR, and the products were purified using the crush

**Table 1** Buffer conditions for each round of SELEX

| Condition | SELEX Round | | | | | |
|---|---|---|---|---|---|---|
| | 1st–3rd | 4th–7th | Counter SELEX | 8th | 9th | 10th |
| Tris (mM) | 30 | 30 | 30 | 30 | 30 | 30 |
| NaCl (mM) | 150 | 200 | 200 | 200 | 250 | 300 |
| Cells (CFU/ml) | $10^8$ | $10^8$ | $10^8$ | $10^4$ | $10^4$ | $10^3$ |
| ssDNA (μg) | 2.0 | 2.0 | 2.0 | 1.0 | 1.0 | 0.5 |
| Incubation (min) | 60 | 60 | 60 | 60 | 40 | 30 |

and soak method [25]. After DNA purification, the products were digested with BamHI and HindIII and ligated into the pET-28a vector. Subsequently, the ligation mixture was used to transform *E. coli* DH5α and several colonies were picked and sequenced using a T7 terminator primer (Macrogen, Daejeon, Korea). The secondary structure of the ssDNA aptamers was predicted using Mfold free software [26, 27].

## Analysis of Binding Affinity

To evaluate the dissociation constant (Kd) value of the aptamers, the aptamers were synthesized in vitro at a 30-mer length and chemically modified with 6-FAM phosphoramidite (Bioneer, Daejeon, Korea). The FAM-aptamers were incubated with $10^8$ CFU/ml *Salmonella* at room temperature for 1 h in a microfuge tube, and the mixture was centrifuged at 12,000 rpm for 15 min to remove unbound aptamers. The pellet-containing cells and FAM-aptamers were washed three times with PBS followed by a final resuspension in PBS. The mixture was then transferred to black colored 96-well plates (SPL, Pocheon, Korea) to measure fluorescence (exCitation, 450 nm; emission, 550 nm) using a microplate fluorescence reader (Molecular Devices, Sunnyvale, CA).

## Conjugation of Polyvalent Directed Aptamer Polymer

A polyvalent directed aptamer polymer was synthesized according to our previous report [28]. In this study, 30-mer aptamer were conjugated to poly-D-Lysine (PDL) and N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP) was used as the molecular linker between the peptide and the PDL. To conjugate aptamers on PDL, aptamers were double modified with 5′ FAM and 3′ SH, where FAM acted as the signal probe and the SH group was used as the anchor molecule between the aptamer and SPDP by disulfide bond formation. A total of 100 mg (0.033–0.066 μmol) of PDL was dissolved in 1.2 mL of 75 mM sodium acetate buffer and brought to pH 8.5 with sodium bicarbonate. Then, 20 μL of 3.2 mM SPDP was added to the PDL solution and mixed vigorously. After a 1-h incubation at room temperature, the mixture was injected onto a Superdex75 10/300 GL gel filtration column (Amersham Biosciences), and the fractions were harvested based on the PDL length range of 150 to 300 kDa. The product yield was to be 0.017-0.033 μmol of PDL activated with 1.13 μmol dithiopyridine linker in a total volume of 5.0 mL. The SH-labeled aptamers were added to the SPDP-activated PDL solution and incubated for 2 h at room temperature. After incubation, the mixture was injected onto a 10/300 GL Superdex75 gel filtration column (Amersham Biosciences) and the conjugated aptamers were confirmed by measuring the fluorescence intensity of the eluted fractions (excitation, 450 nm; emission, 550 nm).

## Results

### Selection of ssDNA aptamers towards *S. enteritidis* and *S. typhimurium*

The high affinity, single-stranded DNA aptamers recognizing *S. enteritidis* and *S. typhimurium* were selected through 10 rounds of Cell-SELEX. The stringent conditions followed during each round of Cell-SELEX selection are presented in Table 1. The incorporation of BSA, *E. coli*, and *P. aeruginosa* during the negative selection (Counter-SELEX) resulted in considerable removal of non-specific aptamers. Consequently, the ssDNA aptamers that recognized the target with higher affinity and specificity were enriched. The unbound *Salmonella* aptamers were removed through the repeated selection process by centrifugation, leaving only bound ssDNA aptamers. The target bound ssDNA aptamers were heat eluted and PCR amplified. The amplified PCR products were extracted following the crush and soak method [25]. The presence of an ssDNA band on a native PAGE gel after each round of selection confirmed the binding of ssDNA aptamers to *S. enteritidis* and *S. typhimurium* (data not shown). Thus, the amplified bound ssDNA aptamers were subsequently used for the next successive rounds. After ten rounds of Cell-SELEX, the aptamers directed towards *S. enteritidis* and *S. typhimurium* were cloned and sequenced. Sequence analysis of all clones showed that some sequences dominated the pool and these sequences were further investigated as candidate aptamers. Briefly, 7 aptamers (Se1-Se7), 5 aptamers (St1-St5) recognizing *S. enteritidis* and *S. typhimurium*, respectively, were selected. Specifically, the performance of Se1, Se2, and St1 candidate aptamers in target recognition exceeded that of the other selected aptamers (Table 2). Hence, the Se1, Se2, and St1 aptamers were further investigated in detail.

### Analysis of Binding Affinity, Specificity, and Secondary Structure

The binding affinity of the candidate aptamers was measured using a fluorometric method with fluorescein (FAM)-labeled aptamers. The binding affinity of Se1 and Se2 towards *S. enteritidis* was measured to be 4.66 and 3.83 μM, respectively (Fig. 1). Interestingly, the binding affinity of the St1 aptamer was in the low nanomolar range at 530 nM. After the binding analysis of each aptamer, the specificity of aptamers was assessed towards other gram-negative bacteria belonging to other genera (*P. aeruginosa* and *E. coli*). It was observed that the fluorescence intensity of the Se1, Se2, and St1 aptamers was higher towards the respective *S. enteritidis* and *S. typhimurium* targets when compared to other control species, such as *P. aeruginosa* and *E. coli*, indicating the high target specificity of the selected aptamers (Fig. 2). It is noteworthy that the control aptamers, random DNA sequence library pool did not show any significant binding towards the target species (data not shown). This result suggested that the binding of identified aptamers were sequence specific and not due to non-specific polyanionic effect.

The secondary structure analysis of these candidate aptamers as assessed using the Mfold program revealed a typical stem–loop structure (Fig. 3). We anticipate that the stem–loop

**Table 2** ssDNA aptamer sequences obtained by Cell-SELEX

| Target | Aptamer | Sequence (5′-3′) | Length | Frequency |
|---|---|---|---|---|
| *S. enteritidis* | Se1 | CACACCGGAAGGGATGCCACCTAAACCCC | 30-mer | 3/20 |
| *S. enteritidis* | Se2 | CACAGATGACGTCTGGCACATAATTAACAC | 30-mer | 6/20 |
| *S. typhimurium* | St1 | CCGATGTCCGTTAGGGCTCCTCCATAGAT | 29-mer | 5/20 |

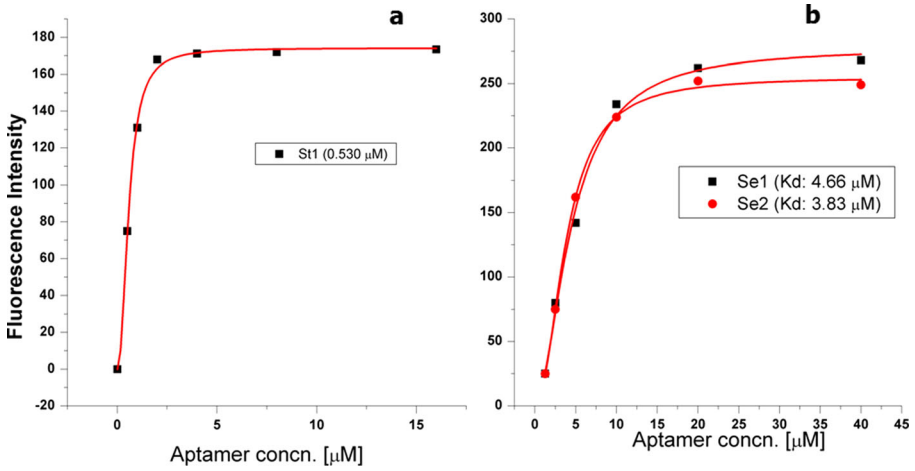


**Fig. 1** Binding affinities of selected aptamers **a** St1 and **b** Se1 and Se2 towards *S. enteritidis* and *S. typhimurium* were estimated in 96-well black plate containing cell-aptamer complex and were measured as 0.530±0.01, 4.66± 0.35, and 3.83±0.10 μM for St1, Se1, and Se2, respectively. All aptamers were fluorescently labeled with 5′-FAM

region might play a particularly crucial role during aptamer–cell epitope interactions, thus leading to higher selectivity in these aptamers. Overall, these results imply that the selected aptamers (Se1, Se2, and St1) should be ideal candidates as probes for the detection of *S. enteritidis* and *S. typhimurium*.

## Development of Polyvalent Directed Aptamer Polymer

In order to further enhance the binding efficiency of the candidate aptamers towards their target, Se1, Se2, and St1 aptamers were conjugated with poly-D-lysine (PDL), leading to the formation of a (Figure S1). Interestingly, the PDAPs of Se1, Se2, and St1 showed several-fold increase in their binding affinities towards *S. enteritidis* and *S. typhimurium* (Fig. 4). Importantly, the binding efficiency of the PDAPs of Se1 and Se2 was significantly increased by approximately 100-fold and 80-fold, respectively, compared to the single aptamer (Table 3). Furthermore, during cross-reaction tests, the PDAP of Se1 and Se2 aptamers showed higher specificity towards *S. enteritidis*, but very low affinity towards *P. aeruginosa*, *E. coli*, and *S. typhimurium* (Fig. 5a, b). Similarly, the PDAP of St1 aptamer demonstrated higher specificity towards the *S. typhimurium* target in comparison to that of *S. enteritidis* (Fig. 5c).

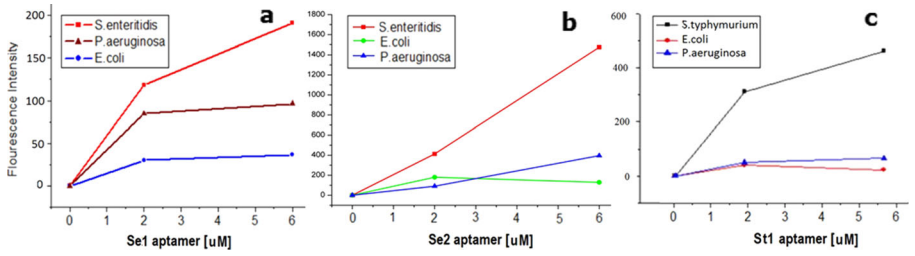


**Fig. 2** Binding specificity evaluation of **a** Se1, **b** Se2, and **c** St1 was performed for *E. coli*, *S. enteritidis*, *P. aeruginosa*, and *S. Typhimurium*. All aptamers were fluorescently labeled with 5′-FAM

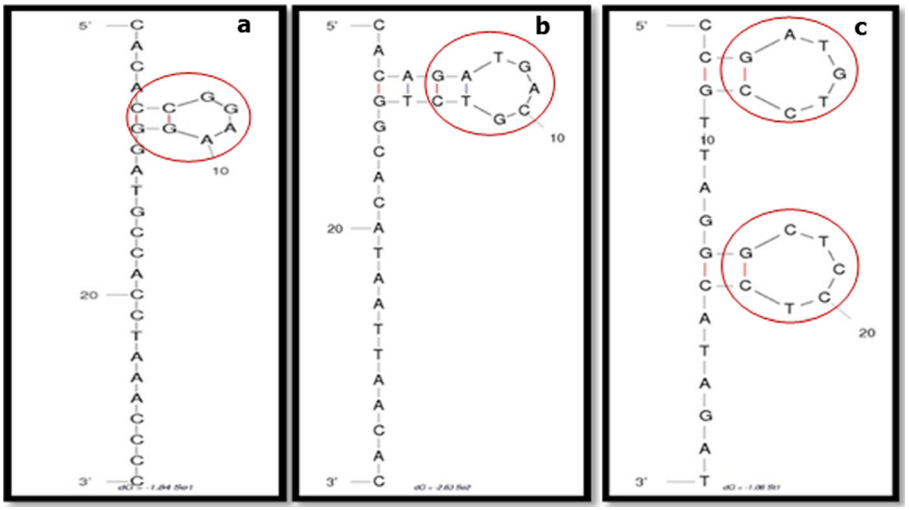


**Fig. 3** Predicted secondary structure of ssDNA aptamers (30-mer) by Mfold. **a** Se1, **b** Se2, and **c** St1. The indicated *red inner circle* represents the possible aptamer-binding region towards their respective target cell epitope

## Discussion

Single-stranded aptamers are considered to be novel ligands for the detection of many pathogenic bacteria [29–31]. Recent studies have emphasized the significance of nucleic acid aptamers as detection probes for the detection of various pathogens [32, 33]. The unique three-dimensional structural folding of these short oligonucleotides gives them a unique feature of higher affinity and specificity towards the target. Thus, identification of aptamers with higher selectivity is of key importance for the development of novel biosensor detection systems towards various pathogenic bacteria. Previous studies have reported DNA/RNA aptamers

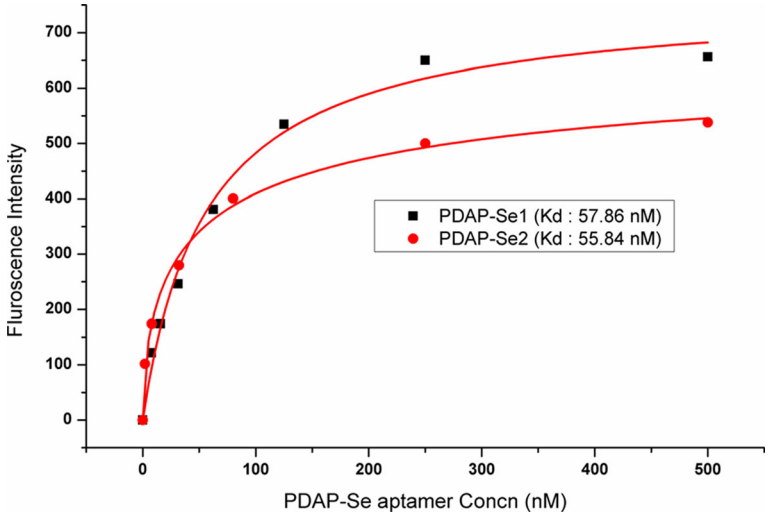


**Fig. 4** Binding affinities of PDAP **a** Se1 and **b** Se2 towards *S. enteritidis* were measured as 57.86±14.5 and 55.84±24.7 nM, respectively. All aptamers were fluorescently labeled with 5′-FAM

**Table 3** Binding affinity analysis of single aptamer and PDAP towards *S. enteritidis* and *S. typhimurium*

| Aptamer | Single aptamer (Kd) | PDAP (Kd) | Fold increase |
|---|---|---|---|
| Se1 | 4.66±0.35 μM | 57.86±14.5 nM | ~100 |
| Se2 | 3.83±0.10 μM | 55.84±24.7 nM | ~80 |
| St1 | 0.530±0.01 μM | 25±5.6 nM | ~20 |

using conventional protein-SELEX towards bacterial pathogens, targeting mainly purified extracellular surface proteins [32]. However, unlike protein-SELEX, which uses isolated, purified protein, Cell-SELEX provides a platform to select the aptamers that interact directly with the cellular epitope in its native conformation leading to the selection of aptamers with higher target affinity and specificity [33, 34].

In this study, we focused on the identification of higher affinity and specificity ssDNA aptamers (30-mer) towards *S. enteritidis* and *S. typhimurium*, through Cell-SELEX. The DNA sequence analysis of obtained clones identified 12 unique aptamer sequences with higher frequency. Importantly, the Se1, Se2, and St1 aptamers directed towards *S. enteritidis* and *S. typhimurium*, respectively, demonstrated higher binding affinities and were observed to be in the sub-micromolar to nanomolar range (Table 2). The binding affinities of the Se1, Se2, and St1 aptamers are consistent with those previously reported for aptamers generated by whole-cell SELEX [14]. Furthermore, the Se1, Se2, and St1 aptamers directed towards *S. enteritidis* and *S. typhimurium*, respectively, demonstrated higher target specificity (Fig. 2). In comparison to *E. coli* and *P. aeruginosa*, the selectivity of Se1, Se2, and St1 DNA aptamers was much higher towards *S. enteritidis* and *S. typhimurium*, respectively. Furthermore, conjugation of the Se1 and Se2 aptamers to PDAP led to a significant increase in their binding affinity (~100-fold and 80-fold respectively) than the individual aptamers.

The secondary structure analysis of nucleic acid aptamers by Mfold can be a useful tool to determine the binding motif for the aptamers [26, 27]. Many studies have suggested that the stem–loop region of the aptamers acts as the binding motif or the region responsible for its biological activity [35, 36]. Moreover, mutational analyses of various aptamers have emphasized the functional significance of the aptamer stem–loop structure [37]. Interestingly, Se1, Se2, and St1 aptamers have a typical stem–loop structure, as shown in (Fig. 3). Thus, we anticipate that the stem–loop region might play a particularly crucial role during aptamer–cell epitope interactions, thus leading to higher selectivity in these aptamers. It is noteworthy that the length of the stem–loop in these aptamers is small, which is unlike larger stem–loop structures seen in other previously reported aptamers. Thus, we anticipate that the unique shorter loops in these aptamers could minimize steric hindrances and thus provide exquisite specificity.

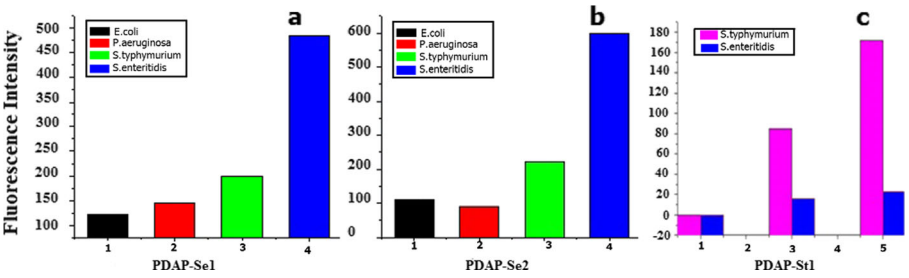


**Fig. 5** Binding specificity evaluation of PDAP-Se1 and PDAP-Se2 against *E. coli*, *S.enteritidis*, *P. aeruginosa*, and *S. typhimurium*. Specificity analysis of PDAP-St1 was performed against *S. typhimurium* and *S. enteritidis*. All aptamers were fluorescently labeled with 5′-FAM

## Conclusion

In conclusion, we report the identification of ssDNA aptamers that demonstrate higher affinity (nanomolar) and specificity towards *S. enteritidis* and *S. typhimurium*, as identified through a cell-SELEX screen. Furthermore, the conjugation of individual aptamers to a PDAP confirmed the improvement of binding affinity up to 100-fold relative to the individual aptamer. Therefore, we suggest that the identified ssDNA aptamers towards *S. enteritidis* and *S. typhimurium* should be useful as ultra-sensitive capture and detection probes for the development of an aptamer-based biosensor detection system for salmonellosis.

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