In Vitro Selection of RNA Aptamer Specific to Salmonella Typhimurium

Han, Seung Ryul and Seong-Wook Lee*

Department of Molecular Biology, Institute of Nanosensor and Biotechnology, Dankook University, Yongin 448-701, Korea

Received: December 14, 2012 / Revised: January 25, 2013 / Accepted: January 26, 2013

Salmonella is one of the most commonly detected foodborne pathogens and a cause of salmonellosis. Salmonellosis rates increased significantly throughout the past decade. Sixteen million cases of typhoid fever, 1.3 billion cases of gastroenteritis, and 3 million deaths occur from Salmonella bacteria worldwide each year [16].

Salmonella (Sal.) Typhimurium is a Salmonella enteric serotype and a Gram-negative bacterium found in the intestinal lumen of animals and humans. Sal. Typhimurium is a major pathogen responsible for foodborne gastroenteritis. The traditional detection methods depend on culture methods that require a few days. Therefore, a number of new techniques for the quick and sensitive detection of Salmonella have been developed; for example, immunoassay [3], PCR-based methods [7], and surface plasmon resonance analysis [1]. Identification of ligands that can directly, avidly, and specifically recognize the pathogenic strain will be needed for the rapid, sensitive, and accurate detection of the bacteria.

Outer membrane protein C (OmpC) is one of the family of outer membrane pore-forming proteins called porins and expressed by most Gram-negative bacteria [13]. Cell surface epitopes of outer membrane protein including OmpC could be shielded by lipopolysaccharide, hampering exact detection of the bacterial cell when the OmpC-specific ligand is used. However, the outer membrane protein epitopes could be transiently exposed at the cell surface through natural turnover of cell wall materials during the life cycle of bacteria [2]. Moreover, Sal. Typhimurium OmpC has a different amino acid sequence, compared with other Gram-negative bacteria including Escherichia coli O157:H7 [14]. Therefore, OmpC is a candidate target molecule for the specific detection of Sal. Typhimurium.

Aptamers are single-stranded nucleic acids that can fit and bind to specific target molecules through formation of specific three-dimensional structures. Aptamers can be selected by the systematic evolution of ligand by exponential enrichment (SELEX) technique [15]. Aptamers could be alternates to the antibodies with regard to diagnostic ligands because of their target specificity and affinity, least batch-to-batch variations due to chemical synthesizability, easy optimizability, and wide target range from small chemicals and proteins to cells and even tissues [4, 5, 11, 12].

In this report, we identified and characterized an RNA aptamer specific to the OmpC protein of Sal. Typhimurium. In order to apply to a diagnostic tool, we generated a stable and RNase-resistant modifying RNA aptamer using 2’-fluoro-2’-deoxyribonucleotide. Importantly, the selectivity and specificity of the selected RNA aptamer to the target pathogen were determined.

Materials and Methods

Microorganisms and Preparation of Cells
Sal. Typhimurium (ATCC 15277), Staphylococcus aureus (S. aureus) (ATCC 6538), and E. coli O157:H7 (ATCC 43895) were purchased from
RT-PCR of SELEX binding buffer. Bound RNA was recovered, amplified by OmpC protein (6 pmole). The OmpC library or selected RNA pool (60 fmole) was incubated with OmpC protein (75 pmole) in 200 µl of Ni-NTA agarose beads, followed by phenol/chloroform extraction, and reverse-transcribed with MMLV reverse transcriptase recovered by Ni-NTA agarose beads, followed by phenol/chloroform extraction. The amount of bound RNA was assessed using quantitative RT-PCR analysis by real-time PCR. Saturation curves were plotted and the dissociation constant was calculated through nonlinear regression analysis by GraphPad Prism.

**Selection Procedure**

The initial RNA library was generated by in vitro transcription of synthetic DNA template with 2-deoxy-2-fluoro pyrimidine nucleotides (Epigenic Technologies, Madison, WI, USA), 2'-hydroxy normal purine nucleotides, and T7 RNA polymerase (Epigenic Technologies). The sequence of the RNA library was 5’-GGG AGAGGGGAAGGUGCGGCC-N40-CAUAACCCAGAGGUC GAUGOAUCCC-3’, where the N40 represents equimolar incorporation of A, G, C, and U. The RNA library (150 pmole) was first pre-cleared by incubation with 20 µl of Ni-NTA agarose beads in SELEX binding buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 2 mM dithiothreitol, and 1% BSA) for removing RNAs binding nonspecifically to the bead. The pre-cleared RNA pool was then incubated with OmpC protein (75 pmole) in 200 µl of SELEX binding buffer. Bound RNA was recovered, amplified by RT-PCR, in vitro transcribed, and used for the next SELEX round, as previously described [8]. After the fifth selection round, the amplified cDNA pool was cloned and sequenced.

**Real-Time PCR Analysis**

The library or selected RNA pool (60 fmole) was incubated with OmpC protein (6 pmole). The OmpC-RNA complex was then recovered by Ni-NTA agarose beads, followed by phenol/chloroform extraction, and reverse-transcribed with MMLV reverse transcriptase (Finnzyme, Vantaa, Finland) using a reverse primer (5’-GGGGGG ATCCATCGACCTCTGGGTAAG-3’) as a detection probe, and forward primer (5’-GGGAAGGAAACACACCCACAGGUGCC-N40-CAUAACCCAGAGGUC GAUGOAUCCC-3’), where the N40 represents equimolar incorporation of A, G, C, and U. The RNA library was amplified by the same method.

**Gel Shift Assay and Northern Blot Analysis**

RNA (60 fmole) was then incubated with OmpC protein (6 pmole) in a total volume of 20 µl of binding buffer (30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1.5 mM MgCl₂, 2 mM dithiothreitol). Assay mixtures were loaded on an 8% non-denaturing polyacrylamide gel (1× TBE, 2% glycerol, 10 mM MgCl₂) at 4°C. After gel electrophoresis, RNAs were transferred to a nylon membrane in 0.25× TBE. Northern blot was then performed with 5'-biotin-labeled oligonucleotide (5’-biotin-GGGGGGATCCATCGACCTCTGGGTAAG-3’) as a detection probe, according to the BrightStar BioDetect kit (Ambion, Huntington, UK) protocol. Alternatively, RNA was 5’-end labeled with biotin by in vitro protocol with 4 mM 5’-biotin-G-5-monophosphate (Trilink Biotechnologies, San Diego, CA, USA), 1 mM GTP, 5 mM ATP, 2′-deoxy-2′-fluoro CTP, 2′-deoxy-2′-fluoro UTP, and T7 RNA polymerase. The 5′-end biotin-labeled RNA was incubated with OmpC, and gel shift and northern analysis were employed with the mixtures as described above.

**Dissociation Constant (Kₐ) Analysis**

RNA aptamers (0.3 nM) were incubated with increasing concentration of OmpC protein (0.3–614.4 nM). RNA bound to OmpC was then recovered by Ni-NTA agarose beads, followed by phenol/chloroform extraction. The amount of bound RNA was assessed using quantitative RT-PCR analysis by real-time PCR. Saturation curves were plotted and the dissociation constant was calculated through nonlinear regression analysis by GraphPad Prism.

**Aptamer Truncation**

We constructed a truncated aptamer with 43 mer by PCR and in vitro transcription. Primers for the PCR were as follows; forward 5’-GGGAATACCTACATGAAGGGTGAGACCGTGAGTG-3’, reverse 5′-GGGTCCGATTTTGTGGCCGCTTTTCACTACGGCCT-3’, and reverse primer 5′-biotin-AGAGCGGAAGCGUGCUGGGCC-N40-CAUAACCCAGAGGUC GAUGOAUCCC-3’. The recombinant OmpC protein was tagged with 6× histidine at the N-terminal and purified with Ni-NTA agarose beads (Qiagen, Hilden, Germany).

**Aptamer immobilized ELISA**

5′-Biotin-labeled RNA (40 pmole) was immobilized in streptavidin-coated 96-well plates (Thermo Fisher Scientific, South Logan, UT, USA). Blocking reagent (0.05% Tween-20, 1.5 mM MgCl₂, 1× PBS) was treated for 1 h and incubated further with 1 × 10⁷ bacteria per well for 1 h. Unbound bacteria were removed, and each well was treated with 100 ng of bacteria specific primary antibody (Novus Biologicals, Littleton, CO, USA) for 1 h, washed 3 times, and then incubated with 100 ng of HRP-conjugated secondary anti-mouse antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA). QuantaBlu peroxidase substrate solution was added and the level of bound bacteria was measured using a fluorometer (Thermo Fisher Scientific, Excitation/Emission: 325 nm/420 nm).

**Aptamer-linked precipitation assay**

The 3′ terminal extended RNA aptamer (A16 tail, 10 pmole) was hybridized with biotin-conjugated oligo-dT at room temperature for 30 min. Hybrid RNA aptamer was incubated with S. aureus or Sal. Typhimurium (1 × 10⁶ cells each) at room temperature for 30 min and centrifuged. Precipitated RNA-bacteria complexes were resuspended in streptavidin-HPR solution (Millipore, Bedford, MA, USA; 1:1000), incubated at room temperature for 30 min, and centrifuged. The pellet was then resuspended in 100 µl of QuantaBlu peroxidase substrate solution (Thermo Fisher Scientific). The binding level of aptamer was measured using a fluorometer (Thermo Fisher Scientific, excitation/emission: 325 nm/420 nm).

Alternatively, the RNA aptamer was dephosphorylated at its 5′-end with calf intestinal phosphatase (New England Biolabs, Ipswich, MA, USA). Dephosphorylated RNA aptamer was then phosphorylated using γ-[32P]ATP (Perkin Elmer, Waltham, MA, USA) and T4 polynucleotide kinase (Roche, Basel, Switzerland), and purified from 8% polyacrylamide gel with 7 M urea. The 5′-end 32P-labeled RNA aptamer (50 fmole) was incubated with bacteria at room temperature.
for 30 min and centrifuged. The precipitated $^{32}$P-labeled RNA aptamer–bacteria complex was then resuspended in 100 µl of dH$_2$O. The amount (cpm) of binding aptamer was measured using a liquid scintillation counter (Beckman Coulter, Brea, CA, USA).

RESULTS AND DISCUSSION

Selection of RNA Aptamer Against *Sal. Typhimurium* OmpC Protein

We used the conventional SELEX method for the selection of RNA aptamers specifically targeting the OmpC protein of *Sal. Typhimurium*. The RNA library was incubated with the recombinant OmpC protein and bound RNA was amplified using RT-PCR and *in vitro* transcription. After 5 iterative cycles, enrichment of RNAs that bound to the OmpC protein was confirmed (Fig. 1A). RNA pools bound to OmpC proteins approximately 4-fold more than to Ni-NTA agarose beads. Of note, the amount of RNAs that bound to OmpC was markedly increased more than 23-fold in the selected RNA pools, compared with that of library RNA bound to the protein. The 5th round of RNA pools were amplified and cloned, and 22 different clones were sequenced (Fig. 1B).

Identification of Selected RNA Aptamer Clone That Can Bind to Both OmpC Protein and *Sal. Typhimurium*

We screened and identified RNA aptamer clones that could bind to OmpC proteins (Fig. 2A). Each tested RNA aptamer clone was found to bind the OmpC protein. In contrast, the library RNA was found to poorly bind the protein. We selected two clones, I-2 and II-1, for the test of their binding ability to *Sal. Typhimurium* (Fig 2B). The control aptamer specific to the carcinoembryonic antigen [10] and II-1 aptamer clone showed no preferential binding capacity to the bacterial strain, compared with library RNA. In sharp contrast, the I-2 aptamer clone bound to the *Sal. Typhimurium* strain 6-fold better than the control library RNA. The reason OmpC-binding II-1 and I-2 clones showed different binding ability to the bacteria is not clear from this study. The difference could be mainly due to the different binding epitopes between the I-2 and II-1 aptamer clones. Only the I-2 aptamer clone can bind to the bacteria, suggesting that the I-2 aptamer could bind cell surface-exposed epitopes of the OmpC protein, but the II-1 could not.

Characterization of the Selected RNA Aptamer and Binding Affinity for OmpC Protein

The I-2 aptamer clone was characterized further for its biochemical features. The secondary structure of the aptamer clone was predicted using the Mulfold program [17]. The region selected from the randomized sequence of the library RNA was anticipated to fold into a long stem-loop

![Fig. 1. Enrichment of the selected aptamer pool and sequence of the aptamer clones.](image)
Fig. 2. Binding ability of selected RNA aptamer clones to OmpC protein and bacteria.
(A) Library RNA or each RNA aptamer clone was incubated in the presence of OmpC. The amount of RNA bound to the protein was determined by gel shift analysis and represented as percentage of input RNA. (B) The 5'-biotin-labeled library or RNA aptamer clone was immobilized on streptavidin-coated 96-well plates, incubated with Salmonella Typhimurium, and treated with bacteria-specific antibody. The binding ability of each aptamer clone to the bacteria is presented as the relative fluorescence unit to the sample with library RNA.

Fig. 3. Truncation and binding affinity of RNA aptamer.
(A) Sequence and predicted secondary structure of the I-2 RNA aptamer and its 43-mer truncated aptamer (box). Nucleotides 23–64 represent the sequences selected from a randomized region of the RNA library. (B) Binding ability of the truncated aptamer to OmpC protein. Library RNA, full length I-2 aptamer, or truncated 43-mer aptamer RNA was incubated in the presence of OmpC protein. The amount of RNA bound to the protein was determined by gel shift analysis and represented as percentage of input RNA. (C) Determination of the dissociation constant ($K_d$) for the aptamer to the OmpC protein. The I-2 full-length or truncated RNA aptamer was incubated with increasing concentrations of OmpC protein. The amount of bound RNA was determined by quantitative real-time PCR. Each value represents the average of 3 independent analyses with standard deviation.
configuration with intra loops. To determine the critical region of the aptamer for its binding to OmpC, we truncated the full-length I-2 aptamer to generate a 43-mer derivative consisting of the apical stem-loop structure (Fig. 3A). The 43-mer truncated aptamer was found to bind the OmpC protein with a binding efficacy comparable to that associated with the full-length aptamer (Fig. 3B). We next determined and compared the binding affinity of the full-length aptamer I-2 and its 43-mer truncated aptamer (Fig. 3C). Both full-length and truncated aptamers showed high binding affinity to the target protein ($K_d$; 20.27 nM and 27.64 nM, respectively). Therefore, the I-2 aptamer could be truncated up to 43-mer without compromising its binding efficacy and avidity to *Sal. Typhimurium* OmpC protein.

**Specificity and Selectivity of the Selected RNA Aptamer to *Sal. Typhimurium***

In order to determine the binding specificity of the selected RNA aptamer to *Sal. Typhimurium*, a direct binding assay through precipitation of aptamer–cell complexes was performed. Selected I-2 RNA aptamer bound only to *Sal. Typhimurium*, but not to *S. aureus*, which is a Gram-positive bacterium and does not express OmpC protein (Fig. 4A). In contrast, the control aptamer could negligibly bind either *S. aureus* or *Sal. Typhimurium*. These suggest that the binding of I-2 RNA aptamer to *Sal. Typhimurium* was not due to nonspecific RNA interaction with bacteria surface. Next, we determined the bacteria cell number-dependent binding ability of the I-2 RNA aptamer (Fig. 4B). The binding efficacy of the I-2 RNA aptamer increased dependently on the cell number of *Sal. Typhimurium*. In contrast, *Sal. Typhimurium* did not bind to control aptamer even in the presence of highest cell number. This result indicates that *Sal. Typhimurium* specifically binds to the I-2 RNA aptamer. We next further determined the selectivity of the I-2 aptamer to *Sal. Typhimurium* by comparing the binding ability of the aptamer with another pathogenic Gram-negative bacterium, *E. coli* O157:H7 strain, which expresses OmpC protein (Fig. 4C). Both the full-length I-2 RNA aptamer and truncated 43-mer RNA aptamer bound only to *Sal. Typhimurium*, but not to *E. coli* O157:H7.

*E. coli* and *Salmonella* OmpC proteins contain a different amino acid composition, and monoclonal antibodies reacting with surface-exposed loops of OmpC of *Salmonella* did not cross-react with that of *E. coli* [14]. This epitope region contains an amino acid sequence extensively diverged from that of *E. coli*. Sequence comparison of amino acids of OmpC between *Salmonella* and other Gram-negative bacteria showed distinct differences at the externally exposed loops area (especially at L2, L4, L5, L6, and L7) (Fig. 5). In addition, several amino acid sequences at L5 to L8 of the OmpC protein differ even between related species of *Salmonella*. The aptamer could bind this diverged and unique epitope of OmpC of *Salmonella*, and hence be
useful for highly species-specific detection of *Sal.* Typhimurium. Further structural analyses will be needed to determine the epitope regions of the aptamer in detail.

Recently, other groups isolated DNA aptamer [9] or RNA aptamer [6] that specifically bound to *Salmonella* bacteria. However, target molecules to both aptamers were not clearly defined. For example, the identified DNA aptamer was reported to bind three target candidate molecules (OmpA, OmpD, ABC transpoter) without validation of its binding ability to each target molecule. Target molecules specific to the RNA aptamer isolated by the other group were also not identified. Moreover, the RNA aptamer by the others consisted of RNase-sensitive 2’-hydroxyl group ribonucleotides, and hence harbors potential limitation in its diagnostic application. Here, we generated an RNase-resistant RNA aptamer using 2’-fluoro-2’-deoxyribonucleotide substituted pyrimidine nucleotides, which will be more applicable as a diagnostic ligand.

In conclusion, we identified an RNase-resistant RNA aptamer that specifically and avidly bound to OmpC protein of *Sal.* Typhimurium. Importantly, the aptamer was observed to specifically bind and recognize *Sal.* Typhimurium bacteria, but not with other bacteria including *OmpC*-free Gram-positive and *OmpC*-expressing Gram-negative bacteria. Therefore, the *OmpC*-specific RNA aptamer would be a species-specific ligand for the direct detection and diagnosis of *Sal.* Typhimurium. It will be next needed to develop methods to specifically and directly diagnose and detect the pathogenic bacteria in complex samples, such as food or clinical specimens, using the aptamer.

Acknowledgments

We thank Kyu-Ho Lee (Sogang University, Korea), Youngkwon Lee (Sungkyunkwan University, Korea), and Jin-Soo Maeng and Yong-Jin Cho (Korea Food Research Institute, Korea) for supplying reagents, facilities, and useful discussions. We are grateful to Young Ju Lee, Min Joong Kim, and Won Il Lee (Dankook University, Korea) for their technical assistances.

References


