Screening and Development of DNA Aptamers Specific to Several Oral Pathogens

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Introduction

Aptamers are composed of single-stranded oligonucleotides that can selectively bind desired molecules. It has been reported that RNA or DNA could act as not only a genetic messenger but also a catalyst in metabolic pathways. RNA aptamers (average sizes 40–50 bp) are smaller than antibodies and have strong binding capacities to target molecules, similar to antigen-antibody interactions. Once an aptamer was selected, it can be readily produced in large quantities at low cost. The objectives of this study are to screen and develop aptamers specific to oral pathogens such as Porphyromonas gingivalis, Treponema denticola, and Streptococcus mutans. The bacterial cell pellet was fixed with formaldehyde as a target molecule for the screening of aptamers. The SELEX method was used for the screening of aptamers and a modified western blot analysis was used to verify their specificities. Through SELEX, 40 kinds of aptamers were selected and the specificity of the aptamers to the bacterial cells was confirmed by modified western blot analysis. Through the SELEX method, 40 aptamers that specifically bind to oral pathogens were screened and isolated. The aptamers showed possibility as effective candidates for the detection agents of oral infections.

Keywords: Aptamer, Porphyromonas gingivalis, Treponema denticola, Streptococcus mutans, Streptococcus oralis, Streptococcus sanguis
found that *T. denticola* is located in the surface layers of subgingival plaque, whereas *P. gingivalis* is predominantly observed beneath the *T. denticola* [9]. In addition, in vitro or in vivo studies have shown that *T. denticola* and *P. gingivalis* not only have a symbiotic relationship for nutrient utilization but also have synergistic virulence in a murine periodontitis model [19].

In this study, aptamers specific for several oral pathogens such as *P. gingivalis*, *T. denticola*, *S. mutans*, *S. oralis*, and *S. sanguis* were screened and isolated for the development of biosensors of oral infections. We believe that by combining antibody and aptamers together in a system, there is a greater possibility of increasing the sensitivity of fiber-optic-based sensors, since aptamers are very small and close proximity to target proteins on the surface of bacteria allows for an enhanced signal [12].

**Materials and Methods**

**Bacterial Strains**

*Porphyromonas gingivalis* ATCC 33277, *Treponema denticola* ATCC 33251, *Streptococcus mutans* ATCC 25175, *Streptococcus oralis* ATCC 10557, and *Streptococcus sanguis* ATCC 10556 were used in this study. BHI broth containing 0.1% Vitamin K₁ (Sigma Co. Ltd., MO, USA) and 1% Hemin (Sigma) was used for the cultivation of *P. gingivalis* [5]. *T. denticola* was cultivated in TYGVS medium [4]. *P. gingivalis* and *T. denticola* were grown at 37°C in an anaerobic chamber (Forma Scientific Co., OH, USA) with 85% N₂, 10% H₂, and 5% CO₂ mixed gas. The other strains were grown in BHI medium (Becton Dickinson, USA). *pCR2.1-TOPO TA cloning vector was as a host strain of the vector.*

**SELEX**

SELEX was carried out by the method of Tuerk and Gold [20] with slight modification. The DNA library was prepared with 44mer and 38mer of primer sequences attached to the 5' end and 3' end of randomly synthesized 39 nucleotides. The forward primer consists of 44mer and has the 17 promoter sequence and *BamHI* restriction endonuclease site, whereas the 38mer of the reverse primer has a *BamHI* site only. The primer sequences are listed in Table 1. After denaturation of the aptamer library at 100°C for 5 min and quick cooling on ice, 10 µl of bacterial cell suspension was added to the aptamer library and kept at 4°C for 1 h with slow agitation for the aptamer to bind to the cell surfaces. The cell and aptamer mixture was then centrifuged at 13,000 × g, 4°C, for 10 min, and washed with 50 µl of PBS twice. Two hundred microliters of 500 mM NaCl solution was added to the cell pellet, and kept on ice for 10 min to detach the aptamers bound to the cell surfaces. This solution was used as an aptamer solution for the further procedures of cloning aptamers.

**Cloning of Aptamers**

Aptamers bound to the cell pellet were eluted with 500 mM NaCl solution and ligated into *pCR2.1-TOPO cloning vector* (Invitrogen, USA) for further analysis. Recombinant plasmid vectors harboring each aptamer were isolated and digested with *BamHI* restriction enzyme to verify the inserts. Vectors harboring corresponding aptamers were designated as APG followed by a sequential number for *P. gingivalis* such as APG-1, APG-2, and so on. In the same way, aptamers for *T. denticola* were named as ATD, ASM for *S. mutans*, ASO for *S. oralis*, and ASS for *S. sanguis*.

Several transformants were selected and inoculated into LB medium containing 100 µg/ml of ampicillin and incubated overnight to prepare the plasmid harboring aptamer fragments. Plasmids isolated were digested with restriction endonuclease and run in 1.5% agarose gel electrophoresis to confirm the insert of aptamers. Nucleotide sequences of the aptamers cloned to the vector were analyzed.

**Modified Western Blot Assay**

Binding of aptamers to the bacterial cell pellet and cell lysate was tested by “modified” Western blot analysis. In this modified western blot analysis, DIG-labeled aptamers were used as antibody substitutes. First, bacterial cell pellet or cell lysates were blotted to a PVDF membrane and blocked with 3% skim milk in TTBS (0.2 M Tris-HCl, pH 7.6, 1.37 M NaCl, 0.1% Tween 20). The DIG-labeled aptamers were diluted in 10 ml of blocking buffer to make 10 pmol of final concentration and hybridized to the membrane. NBT/BCIP solution (Roche, IN, USA) was used as a substrate according to the method described in the manufacturer’s instruction.

**Results**

**Cloning of Aptamer**

Aptamers bound to the cell pellet were eluted with

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**Table 1. Random DNA library and primers used for SELEX.**

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
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<tbody>
<tr>
<td>APT-N40 (86mer)</td>
<td>5'CGGATCCATGGGCACACTATTTATATCAGAATGAAAAATGATGATGTCGTTGGTGGCCC-3'</td>
</tr>
<tr>
<td>APT-F (44mer)</td>
<td>5'CCGCGGATCCATGGGCACACTATTTATATCAGAATGAAAAATGATGATGTCGTTGGTGGCCC-3'</td>
</tr>
<tr>
<td>APT-R (38mer)</td>
<td>5'CCGCGGATCCATGGGCACACTATTTATATCAGAATGAAAAATGATGATGTCGTTGGTGGCCC-3'</td>
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</tbody>
</table>
500 mM NaCl solution and ligated into pCR2.1-TOPO cloning vector for further analysis. Recombinant plasmid vectors harboring each aptamer were isolated and digested with BamHI restriction enzyme to verify the inserts (Fig. 1).

Vectors harboring corresponding aptamers were designated as APG followed by a sequential number for P. gingivalis such as APG-1, APG-2, and so on. In the same way, aptamers for T. denticola were named as ATD, ASM for S. mutans, ASO for S. oralis, and ASS for S. sanguis.

Nucleotide Sequences of Aptamers

Aptamers intended to bind to P. gingivalis were designated as APG-1 to APG-8. Likewise, the others were designated as ATD-1 to ATD-8, ASM-1 to ASM-7, ASO-1 to ASO-9, and ASS-1 to ASS-8 for T. denticola, S. mutans, S. oralis, and S. sanguis, respectively. The nucleotide sequences of all 40 aptamers bound to each bacterial strain were analyzed and are listed in Tables 2 to 6. The GC contents of the APGs ranged from 46% to 59%, and the average GC content was 52.7%. The GC contents of 4 aptamers out of 9 were about 56%, whereas those of ATDs vary from 54% to 64%. The GC contents of ATDs and ASMs were slightly higher than those of ASOs and ASSs.

Binding of Aptamers to Cell Lysate

To verify the specificity of the aptamers, bacterial cell lysates of P. gingivalis, T. denticola, S. mutans, S. oralis, and S. sanguis were immobilized on PVDF membrane and modified with 3 Kb.
western blotting was carried out with the corresponding aptamers as secondary antibody substitutes (Fig. 2). Cell lysate of \textit{E. coli} M15 was used as a negative control. For the APG aptamers, all 8 aptamers showed binding to the cell lysates of \textit{P. gingivalis}. However, 3 out of 8 aptamers showed slight binding to the lysates of \textit{T. denticola}. Even those aptamers showed binding to the lysates of \textit{E. coli} used as a negative control. For ATD, all 8 aptamers bound to the cell lysates of \textit{T. denticola}. Several of those aptamers also showed cross-binding to \textit{P. gingivalis} and \textit{E. coli}. However, none of those aptamers showed any cross-reaction to \textit{S. mutans} cell lysates. Likewise, aptamers of ASM and ASO showed selective binding to \textit{S. mutans} or \textit{S. oralis} cell lysates without cross-reaction to the cell lysates of \textit{P. gingivalis} or \textit{T. denticola}. However, several ASS aptamers showed cross-reactions to the lysates of \textit{P. gingivalis} and/or \textit{T. denticola}.

**Discussion**

Early detection of oral microorganisms is an important process in preventing oral disease and in oral hygiene. Oral plaque is a microbial biofilm formed by organisms tightly bound to each other and to the solid surface of teeth [16].

\begin{table}[h]
\centering
\caption{Nucleotide sequences of aptamers specific to \textit{Streptococcus mutans}.}
\begin{tabular}{|l|l|l|}
\hline
Name & Sequences & GC contents (\%) \\
\hline
ASM-1 & 5'-GCATCGGTCCTGAAGTTGCTCTAGTGCCCGTGTGCTCAA-3' & 59 \\
ASM-2 & 5'-GGCTAAGGCCGGATACACCCATTTCCGCTTGAGCTGCAA-3' & 59 \\
ASM-3 & 5'-TGTAAACGGTGAGCTGTGTTTGGAGGAGGCCTGCATGCATAA-3' & 54 \\
ASM-4 & 5'-TGGCAACCGGAGTGGCTTTGTTGAGAGGCGCAATGCCTAA-3' & 59 \\
ASM-5 & 5'-CCCTACATGGTTGAGCTTTCTATGTCACCGTCCATGCATAA-3' & 56 \\
ASM-6 & 5'-CGGTCTCGCGCTATTGTCGCTGAAACCCGACCGCTCAAA-3' & 56 \\
ASM-7 & 5'-GTGTTCGCCGCTATTTCGCCATCAAACCGCGGACCCCAAA-3' & 54 \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
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\caption{Nucleotide sequences of aptamers specific to \textit{Streptococcus oralis}.}
\begin{tabular}{|l|l|l|}
\hline
Name & Sequences & GC contents (\%) \\
\hline
ASO-1 & 5'-CCCTACATGGTGAGCTTTCTATGTCACCGTCCATGCATAA-3' & 46 \\
ASO-2 & 5'-CCGTGGATGAGCAATTAACCCGACACCTAGCCTACCAA-3' & 49 \\
ASO-3 & 5'-GGCACTATCGTCCCGAGTACCCCGCGGCTGTGAGCTCAA-3' & 59 \\
ASO-4 & 5'-GCTTACTACAGGTATGAGCTTTCTATGTCACCGTCCATGCATAA-3' & 46 \\
ASO-5 & 5'-CCCATAACCTTTTCGATGTAATGCGCAGTCCCATCTCAAA-3' & 44 \\
ASO-6 & 5'-GCTTAGAACCCCGCATGCAATGCGCAGTCCCATCTCAAA-3' & 54 \\
ASO-7 & 5'-GCTTACCTCGCTATACCCGCTTGGTCTGACCTAGCCTACCAA-3' & 51 \\
ASO-8 & 5'-TGCCCGGATCTGATGCGCAGTCCCATGCTGTCGCTCAA-3' & 49 \\
ASO-9 & 5'-CGGTAGTACTACTGAGAACAACCTCCGATACCTTCAATAA-3' & 41 \\
\hline
\end{tabular}
\end{table}

\begin{table}[h]
\centering
\caption{Nucleotide sequences of aptamers specific to \textit{Streptococcus sanguis}.}
\begin{tabular}{|l|l|l|}
\hline
Name & Sequences & GC contents (\%) \\
\hline
ASS-1 & 5'-TTGACTACAGTGATGAGCTTTCTATGTCACCGTCCATGCATAA-3' & 49 \\
ASS-2 & 5'-CGGTAGTACGAGAAGACACCTCCGACTATTTAGTAA-3' & 41 \\
ASS-3 & 5'-GCTGGGATCTGAGGAGAAATGACTTGACCTAGTAA-3' & 46 \\
ASS-4 & 5'-GCCCGTGACGACTATTTAGTAA-3' & 46 \\
ASS-5 & 5'-GCTTACCTCGCTATGCAATGCGCAGTCCCATCTCAAA-3' & 59 \\
ASS-6 & 5'-CCGTGACTTTACAGCGGCTTGCTGATGCGCATAATCAA-3' & 54 \\
ASS-7 & 5'-TGCAGCGGATCTGATGAGCTTTCTATGTCACCGTCCATGCATAA-3' & 49 \\
ASS-8 & 5'-CGAGGAGTGGTGTTGTTCTGCTGCTGCGACCGCAAAA-3' & 41 \\
\hline
\end{tabular}
\end{table}
Characteristics of this biofilm suggest that oral bacteria may have developed some sophisticated interspecies communication mechanisms [17]. Since numerous kinds of microorganisms inhabit the human mouth, naturally not only early detection but also accurate identification is needed. For this purpose, aptamers might be an effective tool in the oral health care system. Therefore, development of aptamers that specifically bind to oral infectious bacteria will provide a novel way to diagnosis oral diseases.

For the development of aptamers, several important steps should be considered. The first step is immobilizing the target molecule. Since the bacterial cell pellet was used as a target for the screening of aptamers in this study, fixing the cell pellet was considered as the most critical step. Failure to fix the cell pellet results in the leakage of cell compartments, which might influence the specificity of the aptamers. The second important step was detaching the aptamers from the cell pellet. We have applied a high concentration of NaCl solution in this study. Otherwise, a direct PCR procedure with cell-bound aptamer might amplify the aptamers in an enough amount to clone into the appropriate cloning vector. More high-throughput procedures might be achieved by packing the cell pellet into a small syringe column and letting the aptamer library solution flow through the column.

The binding specificity of the aptamers isolated in this study was confirmed by modified western blot analysis. This analysis also demonstrates the efficacy of the aptamer as an antibody substitute. In conventional western blot analysis, usually two kinds of antibodies are used that are known as the primary and secondary antibodies. However, by using the aptamer as an antibody substitute, the later step that uses a secondary antibody could be skipped by directly labeling the aptamer with DIG, horseradish peroxidase, or other fluorescent agents for visualization. This advantage of the aptamer will effectively shorten the detection process. Moreover, the comparatively smaller molecular size of aptamers than antibody enables more opportunity to bind to the target molecules. This characteristic might provide higher sensitivity to the system.

Among 8 aptamers specific to \textit{P. gingivalis}, it was confirmed that all aptamers were effectively bound to \textit{P. gingivalis} cell pellet. However, 5 out of 8 aptamers for \textit{T. denticola} were revealed to be able to bind \textit{P. gingivalis} also. These aptamers did not bind to the \textit{S. mutans} cell pellet. In contrast, only 2 out of 7 aptamers specific to \textit{S. mutans} cross-reacted to \textit{P. gingivalis}. This phenomenon might imply that some of the surface molecules of \textit{P. gingivalis} and \textit{T. denticola} have similar chemical characteristics. Since \textit{P. gingivalis} and \textit{T. denticola} are related to periodontitis simultaneously, this might be reasonable. Although gram-positive \textit{S. mutans} is also one of the major oral pathogens, it is not directly related to periodontitis. It is instead related to dental plaque.

Although several aptamers isolated in this study have cross-reacted to other species, these aptamers might be categorized as broad-range aptamers. Since oral infections are usually arising in complex infection, these aptamers...
might be used as a primary agent for the rough diagnosis of oral infection with one aptamer simultaneously.

Since aptamers are known to be highly specific to the target molecules, it is useful for the development of novel substances in various fields. Furthermore, aptamers are easy to synthesize artificially and amplify. Specifically, their ability to bind to a certain protein expressed on the surfaces of the cell or tissues in a certain disease is a useful tool to develop a biosensor or a diagnostic chip for the early detection of the infection or disease. Conclusively, these results presented in this study confirm the possibility of aptamers as a useful tool to detect and diagnosis oral infections. Further studies about the mechanical devices that could make them easily attachable to the human mouth and process the electrical signal to visualize the data should be followed for a complete oral diagnostic system.

Acknowledgments

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References