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Original Article

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Identification of ssDNA aptamers specific to clinical isolates of *Streptococcus mutans* strains with different cariogenicity

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Abstract

Streptococcus mutans, a Gram-positive facultative anaerobic bacterium, is considered to be a major etiological factor for dental caries. In this study, plaques from dental enamel surfaces of caries-active and caries-free individuals were obtained and cultivated for *S. mutans* isolation. Morphology examination, biochemical characterization, and polymerase chain reaction were performed to identify *S. mutans*. The cariogenicity of *S. mutans* strains isolated from clinical specimens was evaluated by testing the acidogenicity, aciduricity, extracellular polysaccharide production, and adhesion ability of the bacteria. Finally, subtractive SELEX (systematic evolution of ligands by exponential enrichment) technology targeting whole intact cells was used to screen for ssDNA aptamers specific to the strains with high cariogenicity. After nine rounds of subtractive SELEX, sufficient pool enrichment was achieved as shown by radioactive isotope analysis. The enriched pool was cloned and sequenced randomly, followed by MEME online and RNA structure software analysis of the sequences. Results from the flow cytometry indicated that aptamers H1, H16, H4, L1, L10, and H19 could discriminate highly cariogenic *S. mutans* strains from poorly cariogenic strains. Among these, Aptamer H19 had the strongest binding capacity with cariogenic *S. mutans* strains with a dissociation constant of 69.45 $\pm$ 38.53 nM. In conclusion, ssDNA aptamers specific to highly cariogenic clinical *S. mutans* strains were successfully obtained. These ssDNA aptamers might be used for the early diagnosis and treatment of dental caries.

Key words: *Streptococcus mutans*, aptamers, selection, SELEX

Introduction

The relationship of *Streptococcus mutans* with the formation of caries is complex. The traditional view is that adhesion to enamel surfaces, acidogenicity (ability to produce acid), aciduricity (ability to live in acidic conditions), and the ability to produce extracellular polysaccharides are major factors in *S. mutans*, facilitating the progress of dental caries [1–4]. *Streptococcus mutans* is able to synthesize extracellular polysaccharides in the presence of sucrose [5,6], fructose, and glucose [7,8]. Glucose homopolysaccharides are also known as glucans, while fructose homopolysaccharides are called fructans [9,10]. Glucans are synthesized by glucosyltransferases (GTF) and fructans are synthesized by fructosyltransferases (FTF) [11]. Both glucans and fructans mediate *S. mutans* adhesion on the tooth enamel surface. Large-scale production of extracellular polysaccharides from sucrose is an important factor in *S. mutans* cariogenicity. Other presumed cariogenic agents of *S. mutans* are unknown, but evidence for their existence is that traditional prevention strategies against caries disease do not have a significant effect for *S. mutans*. There may be unidentified factors or interactions between factors that are not yet understood. Thus, studying *S. mutans*, especially clinical isolates with different cariogenicity and further identifying the difference between them are necessary for pathogenicity research and prevention of dental caries. At the same time, it is also necessary to study *S. mutans* to reveal the cariogenic mechanism. Thus, we need a high-throughput screening method to find new caries-related factors. SELEX (systematic evolution of ligands by exponential enrichment) is a useful tool for this purpose. SELEX is a combinatorial chemistry methodology for *in vitro* selection of specific ligands/oligonucleotide ligands [12,13]. With polymerase chain reaction (PCR) amplification techniques *in vitro* and specific combinations of target molecules, through dozens of rounds of screening, combinatorial oligonucleotide ligands with high specificity and high affinity can be obtained.

Traditional SELEX technology is used to screen pure and soluble proteins. Morris *et al.* [14] applied SELEX to human red blood cell ghosts as a model to develop the methodologies for obtaining and manipulating a pool of ligands which could recognize many targets in a complex mixture. This method was called 'complex targets SELEX' [15,16]. Based on complex targets SELEX, Wang *et al.* [15] developed a new SELEX procedure incorporating the principle of subtraction. Differentiated PC12 cells were used to select targets. Undifferentiated PC12 cells were used as subtraction targets. They specifically identified ssDNA aptamers that could recognize differentiated PC12 cells from undifferentiated PC12 cells. Whole cell SELEX enables the selection and identification of ssDNA aptamers specific to clinical isolates of *S. mutans* with different levels of cariogenicity.

In this study, plaques from enamel surfaces of caries-active (CA) and caries-free (CF) individuals were obtained and cultivated for *S. mutans* isolation. Morphology examination, biochemical characterization, and PCR were performed to identify *S. mutans*. The cariogenicity of *S. mutans* strains isolated from clinical specimens was evaluated by testing the acidogenicity, aciduricity, extracellular polysaccharide production, and adhesion ability of the bacteria. Finally, subtractive SELEX technology targeting intact whole cells was used to screen for ssDNA aptamers specific to strains with high cariogenicity.

Materials and Methods

Ethical statement of the study protocol

All experimental procedures were approved by the Ethical Committee for Research in Humans of the Chinese PLA General Hospital and written informed consent was obtained from all subjects before sample collection.

Patient selection and grouping by the decayed, missing, and filled teeth index

Forty adults of both sexes aged between 23 and 53 years (33.64 ± 10.03) were included in this study. Patients were selected from those seeking treatment at the Department of Stomatology, Chinese PLA General Hospital (Beijing, China). Antibiotic therapy, topical application of fluoride, and the use of antiseptic mouthwashes in the previous 3 months were the exclusion criteria. The extent of caries was measured by the decayed, missing, and filled teeth index (DMFTI) according to guidelines set by the World Health Organization [17]. CA patients were characterized by one or more cavitated lesions (DMFTI = 7.40 ± 2.07) with >6 manifest or restored caries lesions. CF patients were characterized by no clinically observable new caries in the last 5 years (DMFTI = 0) with no dental caries. Clinical examinations were carried out by the same examiner. The intra-examiner agreement was high ($\kappa = 0.92$).

Isolation of *S. mutans*

Patients (30 CA and 10 CF) were instructed not to brush their teeth during the preceding 12 h, and not to drink or eat anything for 2 h before sampling. Samples of dental plaques were taken from healthy enamel surfaces with sterile dental probes. A total of 40 pooled plaque samples were transferred to Cary–Blair transport medium and then transferred to a microbiological laboratory on ice and processed within 2 h. Samples were vortexed for 1 min, and underwent serial 10-fold dilution (10^{-1} to 10^{-7}) with physiological (0.9%) saline solution. Aliquots (100 µl) of appropriate dilutions of suspensions were spread on tryptone-yeast extract-cysteine plates supplemented with 20% sucrose and 0.2 U/ml bacitracin (TYCSB) for *S. mutans* isolation [18]. Plates were incubated in an anaerobic workstation (MART, Amsterdam, Netherlands) under 10% H_2, 10% CO_2, and 80% N_2 at 37°C for 48 h. After 2 days of growth, tiny white colonies were observed within a clear outer zone [19,20]. After growth on Mitis Salivarius with Bacitracin (MSB) agar plates, colonies that were most representative of the morphological patterns of *S. mutans* were selected and examined by light microscopy [21]. Strains with characteristics of Gram-positive, non-motile bacteria with short-chain alignment were selected for further identification. Only one isolate was obtained from each sample of the CF patients, and two or three isolates were obtained from each sample of CA patients.

Preparation of *S. mutans*

All the 46 clinical isolates of *S. mutans* from CA and CF individuals were cultured on brain heart infusion (BHI) broth. *Streptococcus mutans* Ingbritt and a clinical isolate of *Staphylococcus aureus* Strain 188 (*S. mutans* Ingbritt from School of Stomatology, Capital Medical University, Beijing, China; *Staphylococcus aureus* Strain 188 from Beijing Institute of Basic Medical Sciences, Beijing, China) [22] were also cultured for control purpose. All strains were stored at −80°C in 25% glycerol. For assay of cariogenic virulence, aliquots (100 µl) of each isolate were transferred to 20-ml fresh BHI broth and grown under 10% H_2, 10% CO_2, and 80% N_2 at 37°C until late-log growth phase with an OD_{600} of 0.5. Bacteria were then harvested by centrifugation at 664 *g* for 15 min at 4°C. The pellet was washed twice with sterile neutral phosphate-buffered saline (PBS) and then diluted to 5×10^8 colony-forming units (CFU)/ml.

Table 1. Sequence of primers used in PCR

Gene	Sequence
gtfB	Forward: 5′-ACTACACTTTCGGGTGGCTTGG-3′
	Reverse: 5′-CAGTATAAGCGCCAGTTTCATC-3′
spaP	Forward: 5′-AACGACCGCTCTTCAGCAGATACC-3′
	Reverse: 5′-AGAAAGAACATCTCTAATTTCTTG-3′
dexA	Forward: 5′-TATGCTGCTATTGGAGGTTC-3′
	Reverse: 5′-AAGGTTGAGCAATTGAATCG-3′

Identification of clinical isolates of *S. mutans* by PCR

Genomic DNA was extracted according to the methods described by Alam *et al.* [23]. All the clinical isolates of *S. mutans* were identified by PCR using primers designed to specifically amplify 192-bp, 517-bp, and 1272-bp fragments of *spaP* [24], *gtfB* [17], and *dexA* [25] DNA sequences. The primers used in this study were listed in **Table 1** and synthesized by Invitrogen (Carlsbad, USA). Each reaction mixture (total volume = 50 µl) contained 5 µl of 10 × *Taq* buffer, 0.2 mM of dNTPs, 0.5 µM of each primer, 1.5 µl of genomic DNA as template, and 1 U of *Taq* DNA polymerase. The reaction was conducted as follows: 95°C for 10 min; followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and finally, 10 min at 72°C for extension. PCR products were evaluated by 1.0% agarose gel electrophoresis in Tris-acetate (40 mM) plus ethylenediaminetetraacetic acid (2 mM) buffer (TAE; pH 8.5), stained with ethidium bromide, and then visualized under ultraviolet light. *Streptococcus mutans* Ingbritt and *S. aureus* 188 were used as the positive and negative controls, respectively.

Genotyping of clinical isolates of *S. mutans* by arbitrarily primed-PCR

Genotyping was undertaken by arbitrarily primed-PCR (AP-PCR) [26] using primer OPA5 (5′-AGGGGTCTTG-3′) [27]. Each reaction mixture (total volume = 50 µl) contained 5 µl of 10 × *Taq* buffer, 300 of µM dNTPs, 2 µM of primer, 5 µl of template, and 5 U of *Taq* DNA polymerase. The reaction was conducted as follows: 94°C for 5 min; followed by 35 cycles of 94°C for 1 min, 36°C for 2 min, and 72°C for 2 min; and finally 5 min at 72°C for extension. PCR products were separated by 2.0% agarose gel electrophoresis using TAE buffer and stained with ethidium bromide. Images were captured with Eagle Eye II (Stratagene, La Jolla, USA) and interpretation of the patterns was undertaken with BioNumerics software (Applied Maths, St-Martens-Latern, Belgium) using the Dice similarity coefficient. A tree indicating relative genetic similarity was constructed on the basis of the unweighted pair group method of averages with a position tolerance of 1%. Clusters were defined as DNA patterns sharing ≥70% similarity. Similarity that differed by <5% was considered as subtypes within the main group.

Adherence assay of clinical isolates of *S. mutans*

Adherence of *S. mutans* to saliva-coated hydroxyapatite (SHA) was assayed *in vitro* according to the methods of Clark *et al.* [28] and Kantorshi *et al.* [29] with some modifications. Spheroidal HA beads (4 mg, diameter 0.4–1 mm; Beijing YHJ, Beijing, China) were washed and equilibrated in 200 µl adherence buffer (50 mM KCl, 1 mM $CaCl_2$, 0.1 mM $MgCl_2$, and 1 mM KH_2PO_4, pH 6.8) [30] overnight at 37°C in polypropylene micro-tubes. HA beads were then incubated with 100 µl of clarified whole saliva, which was also prepared according to the methods described by Clark *et al.* [28] and Kantorshi *et al.* [29] for 2 h at room temperature with gentle agitation. HA beads were then washed twice with adherence buffer and incubated in 100 µl of adherence buffer containing 5 g/l of bovine serum albumin (BSA) for 30 min with gentle agitation. After coating, HA beads were washed three times with adherence buffer. Bacteria were centrifuged at 664 *g* for 15 min at 4°C. The pellete was washed twice with sterile neutral PBS and then diluted to 5×10^8 CFU/ml, before being added to respective tubes containing 4-mg SHA. Incubation was anaerobic with gentle agitation for 90 min at 37°C. Non-adherent bacteria were removed, and the SHA beads with adherent bacteria were washed three times with 500 µl of sterile adherence buffer. Beads were then resuspended following the addition of 200 µl sterile neutral PBS buffer and glass pearls by vortexing the tube for 2 min to detach bacteria adhered to the SHA beads. From this suspension, 10-fold serial dilutions were made. Aliquots of 100 µl from the 10^{-2} and 10^{-3} dilutions were cultured on MSB plates, which were incubated anaerobically at 37°C for 48 h. The formed colonies were counted to determine the mean number of CFU/ml for each isolate. All assays were carried out at least three times for each isolate, and there were triplicate samples of each isolate at any one time.

Assay for extracellular syntheses of polysaccharides

For polysaccharide extraction, aliquots of 500 µl suspensions of each isolate (or *S. mutans* Ingbritt) were inoculated into 5-ml BHI broth containing 1% sucrose and incubated anaerobically at 37°C for 24 h. Cultures were centrifuged at 1844 *g* for 30 min at 4°C and the supernatants were collected for extraction of water-soluble polysaccharides (WSPs). Pellets were washed twice with 5 ml deionized water and the supernatants were combined for WSP extraction. Pellets were then dissolved in 5 ml of 0.5 M NaOH and centrifuged. The dissolution was repeated one more time and the supernatants were combined for extraction of water-insoluble polysaccharides (WISPs). Three volumes of ethanol were added to the supernatants containing WSP and WISP, respectively, and then incubated at 4°C overnight to precipitate the polysaccharides. The precipitate was collected by centrifugation and dissolved in 5 ml deionized water for WSP and 0.5 M NaOH for WISP [31,32]. An anthrone-sulfuric acid colorimetric microassay was used to quantify the polysaccharides in the extracts according to the method of Laurentin and Edwards [33]. Each sample was analyzed in triplicate.

Acidogenicity and aciduricity assays of clinical isolates of *S. mutans*

To evaluate acidogenicity, aliquots of 400 µl suspensions of each isolate were inoculated into 4 ml BHI broth at pH values of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, or 7.0 containing 5% glucose and incubated anaerobically as described above. The final pH of each isolate was measured with a Microprocessor pH Meter (Beckman Coulter, Fullerton, USA). The pH drop between initial and final readings was defined as the acidogenicity measurement. To evaluate aciduricity, aliquots of 400-µl suspensions of each isolate were inoculated into 4-ml BHI broth at pH values of 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, or 7.0 and incubated anaerobically at 37°C for 48 h, followed by spectrophotometric measurement of suspended cells at 600 nm on a Hitachi Spectrophotometer (Hitachi, Tokyo, Japan). Aciduricity was assessed by the cell density [34]. *Streptococcus mutans* Ingbritt and sterile saline were used as positive and negative controls, respectively. All of these experiments were carried out in triplicate.

Random ssDNA library and primers

The ssDNA library GP45 [5™′-GCAATGGTACGGTACTTCC (45N) CAAAAGTGCACGCTACTTTGCTAA-3′] was amplified to dsDNA using primers Plong-1 (5′-GCAATGGTACGGTACTTCC-3′) and P11 (5′-TTAGCAAAGTAGCGTGCACTTTTG-3′). Primers Plong-1 and Pstem-loop3 (5′-GCTAAGCGGGTGGGACTTCCTAG TCCCACCCGCTTAGCAAAGTAGCGTGCACTTTTG-3′) were used for the amplification of ssDNA by unequal length PCR [35]. The library and primers were synthesized by Sangon Biotech Company (Shanghai, China). *Streptococcus mutans* clinical isolate Strain 17 was chosen as a highly cariogenic clinical isolate for screening; and Strain 5 (low cariogenicity) was chosen as the subtractive target.

Subtractive cell SELEX procedure

An ssDNA library dissolved in screening buffer [0.1 mg/ml salmon sperm DNA, 0.1 mg/ml tRNA, 1% BSA, 1 × PBS, and 0.05% (v/v) Tween-20] was denatured at 100°C for 5 min followed immediately by cooling on ice. The ssDNA library and subtractive target bacteria were mixed in blocking buffer [0.1 mg/ml salmon sperm DNA, 0.1 mg/ml tRNA, 1% BSA, 0.05% (v/v) Tween-20, 1 × PBS, and 5 mM $MgCl_2$] at 37°C for 2 h. The supernatant containing free ssDNA was collected as a subtractive library, which was further incubated with the target bacteria at 37°C for 2 h followed by washing with buffer, and the ssDNA-bacterium complex was collected by centrifugation at 664 *g*. The bound ssDNA was amplified by PCR using the primers Pstem-loop3 and Plong-1, and the ssDNA-bacterium complex as a template. Polypropylene acrylamide gel electrophoresis was used to separate and purify the ssDNA library for the next round of screening. After nine rounds of subtractive SELEX, the enriched library was amplified to dsDNA, cloned into vector pGEM-T (Promega, Madison, USA) and transformed into *Escherichia coli* DH5α. Forty-four positive clones were selected and subject to DNA sequencing. The sequences were analyzed using RNA structure 4.6 software and MEME online software at http://meme.sdsc.edu/meme/Cgi-bin/meme.

Radiolabeled pool binding assays

The original naive library and enriched pools from each SELEX round were radiolabeled by phosphorylation with T4 polynucleotide kinase and [γ-^{32}P]ATP (Furui Biology Engineer Co. Ltd., Beijing, China) using a DNA 5′ terminal labeling kit (TaKaRa, Dalian, China) as described previously [35]. Radiolabeled pools were precipitated in ethanol and incubated with 3×10^6 bacteria at 37°C for 2 h in 100 µl of screening buffer. After centrifugation, the bacterial pellet was washed with PBS until there was no radioactivity in the supernatant. The bacterial pellet was air-dried and the amount of radiolabeled ssDNA in each sample was measured by scintillation counting.

Flow cytometry analysis

The synthesized 5′ FAM-labeled aptamers (Sangon Biotech) were incubated with bacteria in 500 µl screening buffer at 37°C for 2 h in the dark. After being washed three times with PBS buffer, bacteria were resuspended in 300 µl PBS. The fluorescence intensity of bacteria was monitored in a FACSCalibur flow cytometer (BD, Franklin Lakes, USA) by counting 5000 events. The FAM-labeled naive library served as a negative control. The results were analyzed by FCS express V3 software [35].

Determination of the binding parameters of aptamers to *S. mutans*

Different concentrations of FAM-ssDNA were incubated with the target bacteria in the dark for 2 h and the mean fluorescence intensity of target bacteria was determined as described for the flow cytometry assay. The equilibrium dissociation constant K_d of the aptamer from target bacteria was calculated using the following equation: $Y = B_{max}X/(K_d + X)$ (OriginPro 8.1).

Results

Identification of *S. mutans*

Forty-six clinical isolates of *S. mutans* were identified by PCR amplification with bacterial genomic DNA as templates, and spaP-F (R) [24], gtfB-F (R) [17], and dexA-F (R) [25] as primers. *Streptococcus mutans* Ingbritt and *S. aureus* clinical isolate 188 were used as controls. Each of the three types of PCR product was subject to electrophoresis. Electrophoretic patterns showed the amplification products from the 47 *S. mutans* strains (including Ingbritt) to be single bands of 192, 517, and 1272 bp, respectively (**Fig. 1**). As expected, no target band was amplified from *S. aureus* 188, the negative control reaction. Genetic polymorphism analysis was carried out using OPA5 primers as random primers in PCR amplification of the genomic DNA of the 46 clinical isolates of *S. mutans*. Of the 46 clinical isolates, 5 had the same genotype as 1 of the other 41 isolates (**Table 2**) and were thus excluded. Hence, a total of 41 clinical isolates of *S. mutans* with different genotypes were obtained. **Figure 2** showed the results of genotyping analyses. The horizontal lines above the electrophoresis bands in **Fig. 3** indicate similarity of strains, whereas the vertical bars indicate each strain and strains which are similar.

Adhesion of clinical isolates of *S. mutans*

To identify *S. mutans* clinical isolates with high and low cariogenicity, the adhesion of clinical isolates of *S. mutans* to SHA was detected under sugar-free conditions and compared between strains. The adhesion capacity (in CFU/ml) was determined by counting the number of colonies on culture plates. As shown in **Fig. 3**, the adhesion capacity of the clinical isolates of *S. mutans* to HA generally varied from 1.5×10^5 to 3.5×10^5 CFU/ml. Among all the 41 *S. mutans* isolates, only 5 isolates (numbered 12, 16, 17, 33, and 38) showed adhesion above this range, and 5 isolates (numbered 1, 3, 4, 5, and 9) showed adhesion below this range.

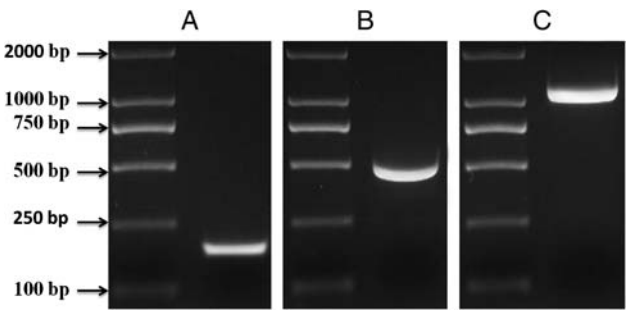

Figure 1. Identification of *spaP, gtfB,* and *dexA* from *Streptococcus mutans* strains from clinical samples PCR amplification of the gene fragments. (A) *spaP;* (B) *gtfB;* (C) *dexA.*

Table 2. Origin of *Streptococcus mutans* strains from clinical samples

Number of clinical isolates	Source	Genotypes
1–10	Healthy people with no caries, 1 strain of each sample	Different genotypes
11, 14, 15, 20, 21, 24, 28, 31, 37, 46	Highly cariogenic patients, 1 strain of each sample	Different genotypes
(18, 19), (22, 23), (29, 30), (38, 39), (40, 41), (44, 45)	Highly cariogenic patients, pairs in brackets are the 2 strains obtained from the same sample	Different genotypes
(32, 33, 34)	Highly cariogenic patients, 3 strains from the same sample	Different genotypes
(12, 13), (16, 17), (35, 36), (42, 43)	Highly cariogenic patients, pairs in brackets are 2 strains obtained from the same sample	Same genotype in brackets, strains numbered 13, 17, 36, and 43 were excluded
(25, 26, 27)	Highly cariogenic patients, 3 strains from the same sample.	Same genotype in 26 and 27, strain number 27 was excluded

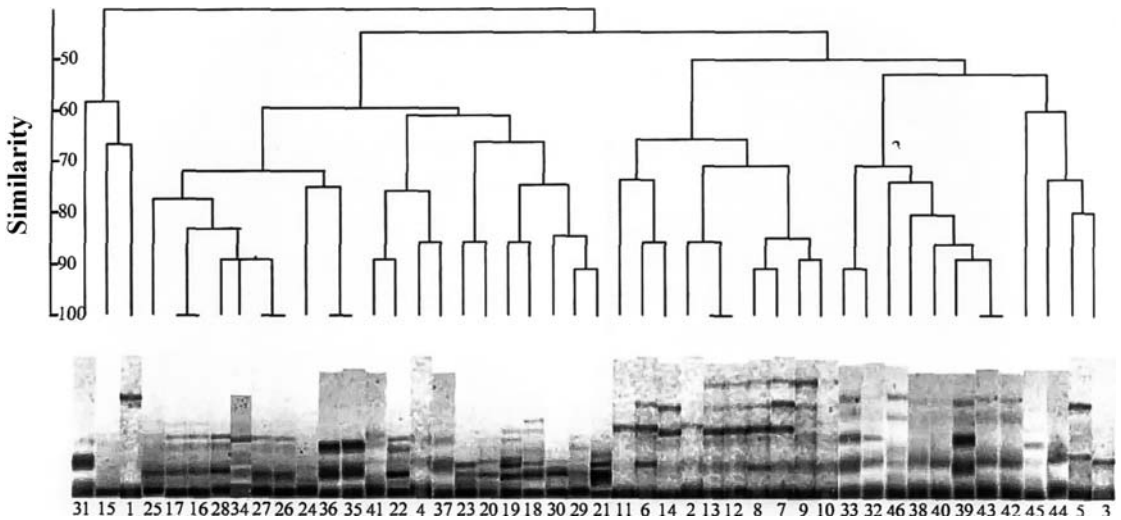

Figure 2. Genotypes of *Streptococcus mutans* strains from clinical samples

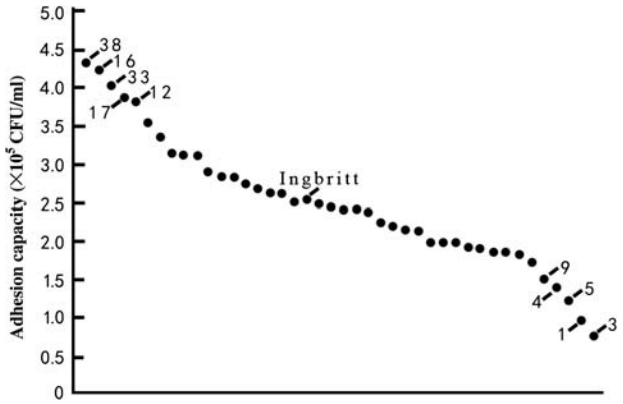

Figure 3. Adhesion features of 41 strains of *Streptococcus mutans* isolated from clinical specimens

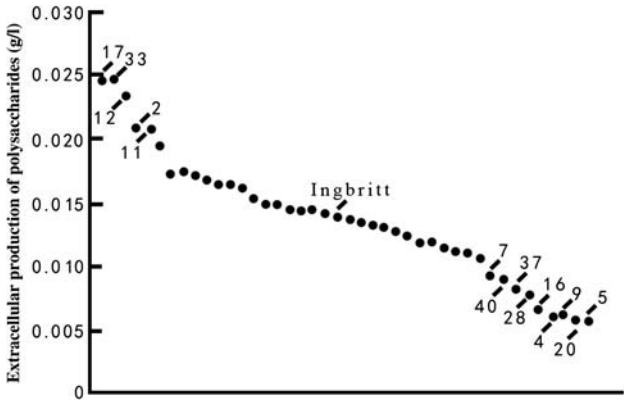

Figure 4. Extracellular production of polysaccharides by 41 strains of *Streptococcus mutans* isolated from clinical specimens

Extracellular production of polysaccharides by clinical isolates of *S. mutans*

Clinical isolates of *S. mutans* showed different capacities in the synthesis of WISPs. **Figure** 4 showed that the production of WISPs was usually in the range from 0.01 to 0.02 g/l. Among all the 41 *S. mutans* isolates, only 5 clinical isolates (numbered 2, 11, 12, 17, and 33) showed WISP production above this range, and 9 isolates (numbered 4, 5, 7, 9, 16, 20, 28, 37, and 40) showed WISP production below this range.

Acidogenicity of clinical isolates of *S. mutans*

The acidogenicity of each strain was decreased with decreasing pH. The lower the pH value, the stronger the inhibition. When the pH

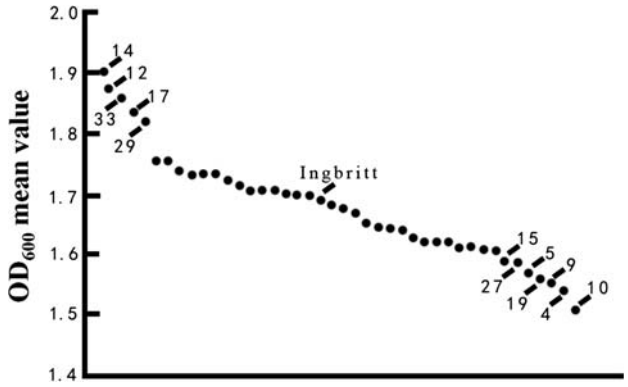

Figure 5. Acidogenicity features of 41 strains of *Streptococcus mutans* isolated from clinical specimens

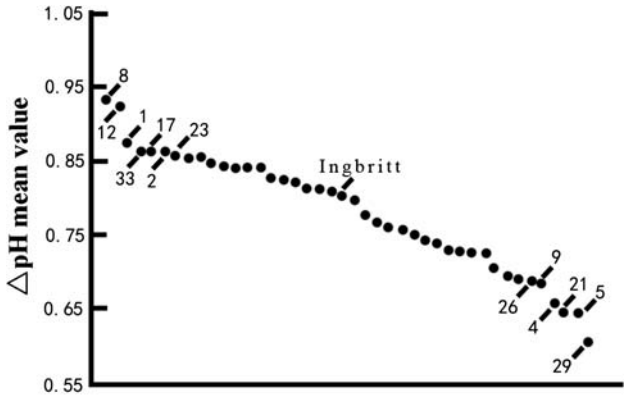

Figure 6. Aciduricity features of 41 strains of *Streptococcus mutans* isolated from clinical specimens

value reached 4.5 or lower, virtually none of the strains produced acid (data not shown). The greater the change in pH (ΔpH) caused by the strain, the stronger the acidogenicity. The mean ΔpH at different pH values was used to evaluate the acidogenicity of each clinical isolate of *S. mutans* using the following equation:

$$\begin{aligned}\Delta\text{pH (mean)} = [&\Delta\text{pH (pH 7.0)} + \Delta\text{pH (pH 6.5)} \\ &+ \Delta\text{pH (pH 6.0)} + \Delta\text{pH (pH 5.5)} \\ &+ \Delta\text{pH (pH 5.0)}]/5.\end{aligned}$$

Figure 5 showed that the vast majority of clinical isolates of *S. mutans* had the mean ΔpH values between 1.6 and 1.8; only isolates numbered 12, 14, 17, 33, and 29 showed the mean ΔpH values above this range; and only isolates numbered 4, 5, 9, 10, 15, 19, and 27 showed the mean ΔpH values below this range.

Aciduricity of clinical isolates of *S. mutans*

In the different pH conditions, the growth of different strains was significantly different. The growth of the strains was inhibited by decreasing pH; the lower the pH value, the stronger the inhibition. We used the mean OD_{600} values of strains at different pH values to evaluate the aciduricity of the various clinical isolates of *S. mutans* using the following equation:

$$\begin{aligned}OD_{600}(\text{mean}) = [&OD_{600}(\text{pH 7.0}) + OD_{600}(\text{pH 6.5}) \\ &+ OD_{600}(\text{pH 6.0}) + OD_{600}(\text{pH 5.5}) \\ &+ OD_{600}(\text{pH 5.0}) + OD_{600}(\text{pH 4.5}) \\ &+ OD_{600}(\text{pH 4.0})]/7.\end{aligned}$$

Figure 6 shows that most mean OD_{600} values of clinical isolates of *S. mutans* were between 0.70 and 0.85; only 7 *S. mutans* isolates (numbered 1, 2, 8, 12, 17, 23, and 33) showed mean OD_{600} values above this range; and 5 isolates (numbered 4, 5, 9, 21, and 26) showed mean OD_{600} values below this range.

It is reasonable to assume that clinical isolates of *S. mutans* with high capacities for adhesion, WISP production, acidogenicity, and aciduricity are highly cariogenic. Based on our data and this assumption, out of the 41 *S. mutans* isolates, only 3 isolates (numbered 12, 17, and 33) have high *in vitro* cariogenic capabilities, and another 3 isolates (numbered 4, 5, and 9) have low *in vitro* cariogenic capabilities.

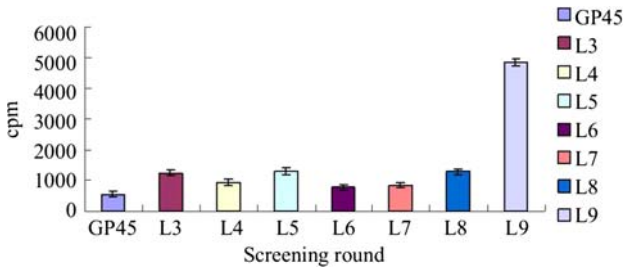

Figure 7. Determination of enrichment of aptamers that bind to *Streptococcus mutans* Strain 17 using a radioactive isotope GP45: initial round pools; L3–L9: third to ninth round pools.

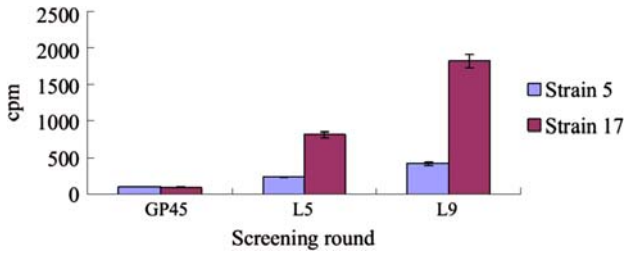

Figure 8. Determination of enrichment of the aptamers that bind to *Streptococcus mutans* Strains 17 and 5 by radioactive isotope GP45: initial round pools; L5: fifth round pools; L9: ninth round pools.

Radioactive isotope detection of ligand enrichment

Radioactive isotope detection results showed that with the increase in screening rounds, specific aptamers to Strain 17 gradually became enriched, and in the ninth round, the amount of enrichment of aptamers was significantly improved compared with previous rounds (**Fig.** 7). Ligands to Strain 17 were increased significantly after nine rounds of screening, whereas there was no significant increase in ligands to Strain 5 (**Fig.** 8). Thus, the enriched aptamers showed highly specific recognition of cariogenic *S. mutans* clinical isolate Strain 17, but not of non-cariogenic *S. mutans* clinical isolate Strain 5.

Aptamer H19 has the highest binding to the target strain among 12 candidates

By cloning, sequencing, and structural analysis, 12 candidate aptamers with higher structural stability according to RNA structure

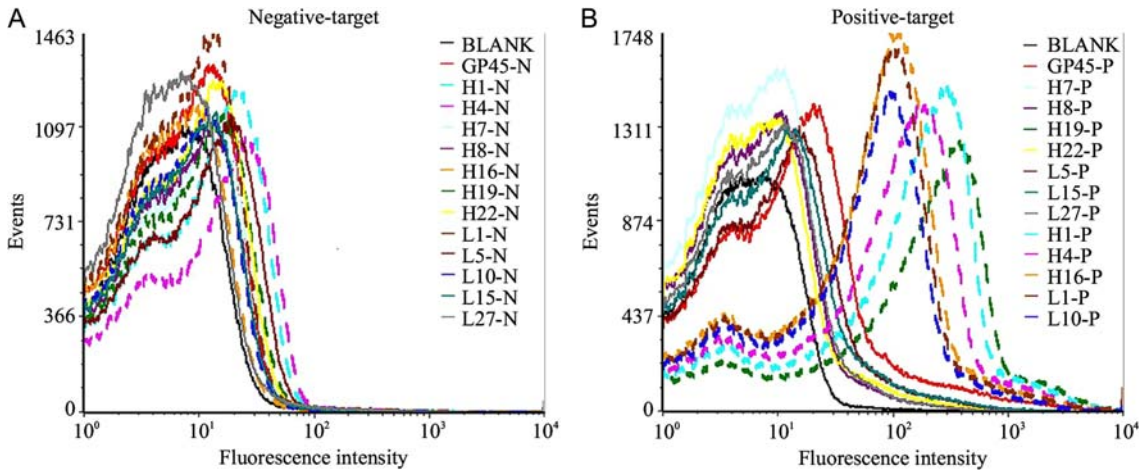

Figure 9. Determination of the aptamers binding with *Streptococcus mutans* by flow cytometry (A) *Streptococcus mutans* Strain 5. (B) *Streptococcus mutans* Strain 17.

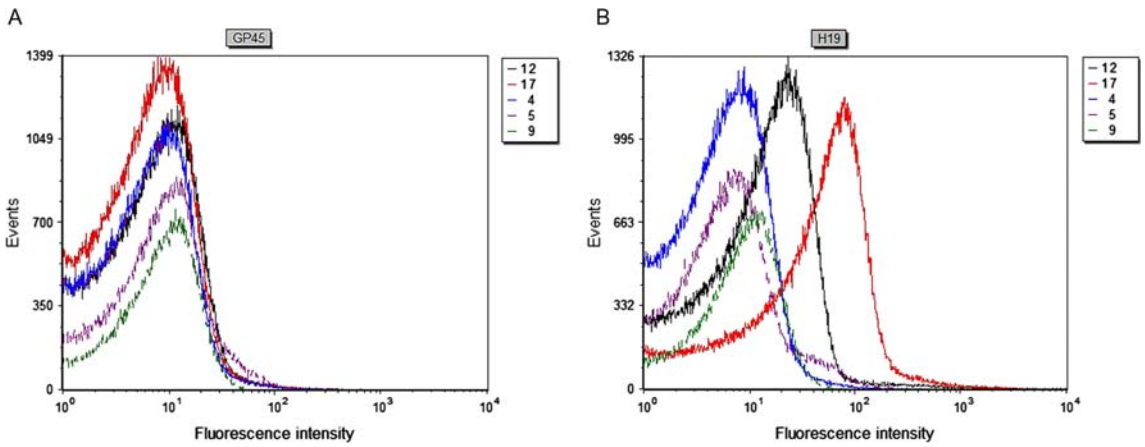

Figure 10. The combination of Ligand H19 and clinical isolates of *Streptococcus mutans* with different cariogenicity Original naive library (A) and Ligand H19 (B) that were incubated with Strains 12, 17, 4, 5, and 9 respectively. Strains 12 and 17 had high levels of cariogenicity compared to Strains 4, 5, and 9. Data for these five strains are shown in black, red, blue, purple, and green lines, respectively.

analysis were selected from 44 positive clones. The original naive library and these 12 aptamers were incubated with Strains 5 and 17, respectively. Flow cytometry was used to determine the fluorescence intensity of each combination. After incubation with Strain 5 (low cariogenicity), no significant difference in fluorescence intensity was observed (**Fig. 9A**). In contrast, after incubation with Strain 17 (high cariogenicity), the fluorescence obtained from 6 aptamers (H1, H16, H4, L1, L10, and H19) was markedly increased when compared with the original naive library. Among the 12 aptamers, the fluorescence was increased the most for Aptamer H19 (**Fig. 9B**).

The recognition of Aptamer H19 by clinical isolates of *S. mutans* with different cariogenicity

Aptamer H19 and the original naive library GP45 were incubated with methanol-fixed Strains 12, 17, 4, 5, and 9, of which Strains 12 and 17 had higher levels of cariogenicity. Flow cytometry analysis of the original naive library with clinical isolates of *S. mutans* revealed no difference in intensity. However, Aptamer H19 showed obvious binding with high levels of fluorescence intensity with Strains 12 and 17 compared with other strains (**Fig. 10**). These results suggest that Aptamer H19 may be specific for clinical isolates of *S. mutans* with high levels of cariogenicity.

K_d values for Aptamer H19 to Strain 17

The equilibrium dissociation constant (K_d) was determined by incubating different concentrations of FAM-H19 with Strain 17 and using average fluorescence intensity measurements. Data were analyzed by curve-fitting using OriginPro 8.1 software. The K_d was determined to be 69.45 ± 38.53 nM (**Fig. 11**).

Discussion

In this study, plaques from dental enamel surfaces of CA and CF individuals were obtained and cultivated for *S. mutans* isolation. Morphology examination, biochemical characterization, and PCR were used to identify *S. mutans* strains. To obtain pure cultures from dental plaques, we used specific culture media to select and isolate bacteria. It has been shown that the *S. mutans* detection rate in the human oral cavity can reach >90%, and the *S. sobrinus* detection rate can reach 10%–76% [8]. Previous studies have

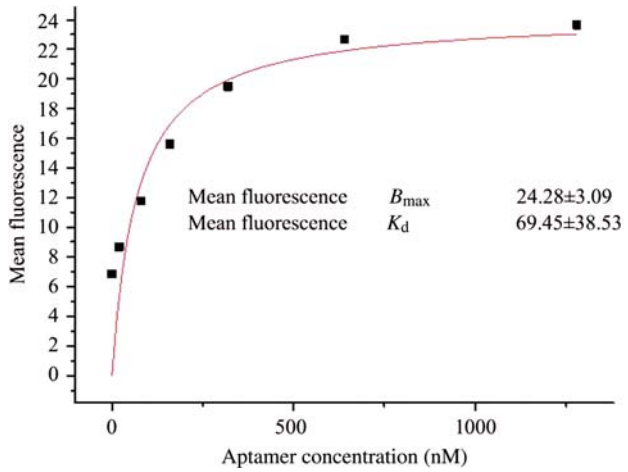

Figure 11. Determination of the dissociation constant (K_d) of the aptamer H19 binding with *Streptococcus mutans* Strain 17

confirmed that TYCSB media can, with high sensitivity and high specificity, rapidly distinguish between *S. mutans* and *S. sobrinus* through colony morphology difference [18]. The addition of high concentrations of sugar and bacitracin to the growth medium can effectively inhibit the growth of most other bacteria found in dental plaques.

We then used PCR technology to confirm the putative *S. mutans* clinical isolates that we obtained. The key to PCR identification of *S. mutans* is the strain-specific design of primers. Genes used for identification include *spaP* [24], *gtfB* [17], *dexA* [25], 16 S rRNA [36], and so on. Expression of *SpaP*, a *S. mutans* surface protein, can promote bacterial adhesion and engraftment on the tooth surface. The expression of *gtfB* gene (of GTF-I) is required for the use of sucrose as the substrate to synthesize water-insoluble glucan, which is involved in sucrose-dependent bacterial adhesion. The *dexA*-dependent expression of glucanase leads to the degradation of glucan and aids bacterial adherence, thus playing an important role in plaque formation. We obtained 46 isolates of *S. mutans* from 40 clinical specimens and confirmed by PCR.

Identification and classification is the basis of *S. mutans* cariogenicity research [37,38]. The existing classification methods include AP-PCR, random amplified polymorphic DNA, plasmid DNA, and chromosomal DNA fingerprinting, and karyotype analysis [39–41]. In this study, OPA5 primers and AP-PCR were used to genotype the 46 *S. mutans* clinical isolates. An AP-PCR map using BioNumerics database software was generated for processing, image strips, uniform molecular weight standard calibration and calibration strip position, with manual correction when necessary. By AP-PCR classification and analysis, five isolates were found to have a similar genotype to others, leaving 41 different types of strains to be assessed in the subsequent experiments.

Cariogenicity is the material basis for the formation of caries involving *S. mutans*. *Streptococcus mutans* induces caries disease depending on the following important virulence characteristics: (i) water-insoluble extracellular polysaccharides to promote bacterial adhesion and aggregation on tooth surfaces to form a plaque; (ii) production of bacteriocin to suppress other bacterial strains; (iii) efficient metabolism of carbohydrates to produce acid; and (iv) ability to survive in a low pH environment. These interrelated virulence factors make *S. mutans* dominant in dental plaques, which can lead to tooth decay. Numerous studies have demonstrated that there are differences between the cariogenicity of *S. mutans* isolated from CA and CF individuals [42,43].

We identified *S. mutans* strains with high cariogenicity from 41 genotypically distinct clinical isolates by assessing their acidogenicity, aciduricity, extracellular polysaccharide production, and adhesion ability. Three isolates (Nos. 12, 17, and 33) were found to be highly cariogenic, and another three isolates (Nos. 4, 5, and 9) were poorly cariogenic. Therefore, for screening using whole cell targets in SELEX technology, highly cariogenic Strain 17 was selected as the clinical screening target and poorly cariogenic Strain 5 was chosen as the subtraction target. Using the SELEX approach, we screened for ssDNA ligands with the differentially cariogenic clinical isolates and then studied the aptamers, respectively.

SELEX technology can screen a wide range of target molecules and ligands recognized by target molecules with high affinity and specificity. SELEX has been successfully applied to a variety of targets including proteins [44], small peptides [45], metal ions [46], and small organic molecules [47]. In basic medical research, this method has been used for drug screening [48–51], clinical diagnosis [52], and treatment [53]. Whether the library is enriched or not is one of the important indicators of SELEX selection success or failure. Radioactive isotopes are a common way to test for library enrichment [54]. In this study, after nine rounds of subtractive SELEX selection, radioactive isotope testing confirmed the enrichment of the library by >1 order of magnitude. After nine rounds of screening, the library could specifically bind to the screening target but rarely bind to the subtractive target.

After the enrichment, 44 positive clones were randomly selected for DNA sequencing. Using the online software MEME structure and RNA analysis software to analyze sequences, 12 representative aptamers, with high structural stability and low sequence similarity were picked out. By flow cytometry, we found that six aptamers were able to specifically identify the screening target that rarely combined with subtractive targets. The combination of each sequence and screening target is slightly different; H19 was more specific and bound more tightly than other sequences. The six specific sequences identified were able to discriminate *S. mutans* clinical isolates to different degrees with different cariogenicity. The specificity of the strongest ligand, H19, may be used to distinguish clinical isolates of *S. mutans* with high and low cariogenicity [55]. The dissociation constant we determined for Aptamer H19 and the cariogenic Strain 17 was of nM(nmol) order, similar to that reported in a previous study in whole cell targets using SELEX [50].

In summary, we have successfully screened for specific identification of clinical isolates of *S. mutans* with high cariogenicity using ssDNA ligands. We found that Aptamer H19 is specific to clinical isolates with high cariogenicity. Using Ligand H19 as a target, we distinguished high cariogenicity strains from the complex mixture of clinical isolates of *S. mutans*, which will be helpful for future researches in caries disease pathogenicity.

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