Detection of *Streptococcus mutans* in human saliva and plaque using selective media, polymerase chain reaction, and monoclonal antibodies

Yong Jin Lee¹, Mi-Ah Kim², Jae-Gon Kim^{2*}, Jae-Hwan Kim^{1*}

¹Department of Pediatric Dentistry, School of Dentistry, Chonnam National University, Gwangju, Korea

²Department of Pediatric Dentistry, School of Dentistry, Chonbuk National University, Jeonju, Korea

The objective of this study was to evaluate the distribution of dental caries-associated *Streptococcus mutans* in human saliva and plaque. The samples for this study were collected from 90 subjects (30 children, 30 adolescents, and 30 adults). The decayed, missing, and filled teeth (DMFT) and significant caries indices were evaluated. We applied polymerase chain reaction (PCR) analysis to detect *S. mutans* in each sample. Enumeration of *S. mutans* was conducted through culturing on Dentocult-SM and mitis salivarius agar medium. For saliva samples enzyme-linked immunosorbent assay (ELISA) analysis using monoclonal antibodies specific to antigen I/II and glucosyltransferase was done. We detected *S. mutans* in 79.7% and 56.8% of all saliva and plaque samples, respectively using PCR analyses. *S. mutans* were detected in 59.1%, 88.0%, and 88.9%, and in 86.4%, 56.0%, and 33.3% of saliva and plaque samples from children, adolescents, and adults, respectively. There were significantly higher levels of *S. mutans* in adolescents' saliva more than any groups. There was a positive correlation between DMFT and the level of *S. mutans* reactivity measured using ELISA. Our results suggest that there should be more emphasis on adolescents' oral hygiene more than in children or adults early prevention and research of dental caries.

Key Words: Antibodies, Dental plaque, Saliva, *Streptococcus mutans*

© This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/4.0>) which permits unrestricted noncommercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

INTRODUCTION

Dental caries is a chronic disease that is common worldwide, and which occurs most frequently in children and adolescents [1,2]. Dental caries develop due to the bacterial plaque that covers the teeth [3]. However, it has been shown that caries do not develop when the bacteria that cause the disease are not present in the plaque [4]. Car-

iogenic bacteria are typically acquired by children via the mother's saliva [5], and while there are greater than 1,000 bacterial species that exist within the oral cavity [6], mutans streptococci are the bacteria primarily associated with dental caries [7,8]. The mutans streptococci species *Streptococcus mutans* and *Streptococcus sobrinus* usually exist separately in an individual, with *S. mutans* occurring more frequently in regions around the caries and exhibiting a

Received November 27, 2018; **Revised** December 11, 2018; **Accepted** December 11, 2018

Co-corresponding author: Jae-Hwan Kim, Department of Pediatric Dentistry, School of Dentistry, Chonnam National University, 33 Yongbong-ro, Buk-gu, Gwangju 61186, Korea.

Tel: +82-62-530-5668, **Fax:** +82-62-530-5559, **E-mail:** jhbcss@hanmail.net

Co-corresponding author: Jae-Gon Kim, Department of Pediatric Dentistry, School of Dentistry, Chonbuk National University, 20 Geonji-ro, Deokjin-gu, Jeonju 54907, Korea.

Tel: +82-63-250-2223, **Fax:** +82-63-250-2131, **E-mail:** pedokjg@jbnu.ac.kr

Copyright © 2019, Oral Biology Research Institute

close relationship with the disease [9,10].

Colonization of young children by caries-causing bacteria results in earlier onset and more severe cases of dental caries. As a result, there have been efforts to prevent the disease by performing caries activity tests to detect the caries-associated bacteria [11,12]. In particular, passive immunization studies targeting *S. mutans* virulence factors, such as antigen I/II (Ag I/II) and glucosyltransferases (Gtfs), were conducted using polyclonal and monoclonal antibodies [13-16]. As a cell surface adhesion molecule, Ag I/II is involved in the adhesion of oral streptococci to the teeth via interaction with various proteins in the saliva. Meanwhile, *S. mutans* encodes at least three types of Gtfs (GtfB, GtfC, and GtfD). These proteins have been shown to utilize sucrose for the synthesis of glucan, which serves as a binding site for *S. mutans* and other oral bacteria, and are known to play a key role in the formation of virulent dental plaques [16,17].

The purpose of this study was 1) to compare the prevalence of *S. mutans*, the primary causative agent of dental caries, in the saliva of children and adolescents with that of adults, 2) to examine the correlation between indices related to dental caries and the differences between each group, and 3) to examine the efficacy of monoclonal antibodies against Ag I/II and the Gtfs for detection of *S. mutans* in saliva samples.

MATERIALS AND METHODS

Study population

A total of 90 individuals lacking any special medical history were selected from three population groups residing in Jeonju, South Korea. Group 1 consisted of 30 second grade elementary school children (8 years old, 1:1 male-female ratio); Group 2 consisted of 30 second year high school adolescents (17 years old, 1:1 male-female ratio); and Group 3 consisted of 30 adults in their middle and late twenties who were admitted to a dental clinic (average of 28.4 years old, 1:1 male-female ratio). All participants provided informed, written consent. For study participants that were minors, consent was obtained from both the participant and their guardian after explaining the purpose

of the research and the experimental methods in advance. The present study was performed following the guidelines of the Institutional Review Board of Chonbuk National University Hospital, and passed the review procedure process (IRB no: 2014-08-004).

Clinical examination

All clinical examinations were performed by the same researcher. This dentist performed oral examinations under natural daylight using a dental mirror and dental explorer. Teeth were evaluated and the decayed, missing, and filled teeth (DMFT) index was determined based on World Health Organization standard methods and criteria [18]. In addition, we determined the significant caries index (SiC index), a recently described evaluation index used for assessing the risk of dental caries, for each patient. For these analyses, the DMFT value for each patient was compared to the average value derived from the third of the population that exhibited the highest DMFT values [19,20].

Plaque and saliva sampling

Plaque and saliva samples were collected (between 9 to 10 AM) from individuals who had brushed their teeth after breakfast and did not consume any food or drinks for at least an hour prior to the examination. Plaque samples were taken by applying gentle pressure to the buccal of the first molar with a sterilized toothpick. After obtaining the plaque, saliva samples were obtained and collected using paraffin tablets and stored in 5 to 10 mL plastic tubes. The saliva and plaque samples were immediately placed in an ice box set below 4°C, and experiments were promptly conducted on the day the samples were obtained.

Culture methods

Examination of *S. mutans* levels using mitis salivarius (MS) agar medium (log₁₀ colony forming units [CFU]/mL)

Saliva samples were mixed by vortexing, and 100 µL of each sample was diluted 1:10 with double-distilled water in 1.5 mL tubes. Samples were then serially diluted, and 100 µL aliquots were spread onto MS agar, a selective

medium used for detection of *S. mutans*. Plates were incubated at 37°C with 5% CO₂ for more than 48 hours, the resulting *S. mutans* colonies were quantified, and the log₁₀ CFU/mL of each sample was calculated.

Examination of *S. mutans* levels using the Dentocult-SM kit

Enumeration of *S. mutans* using the Dentocult-SM kit (Orion Diagnostica, Espoo, Finland) was performed according to the manufacturer instructions. Bacitracin discs were placed into test tubes containing liquid cultures. After approximately 15 minutes, the screening strips from the kit were immersed into the test tubes containing the saliva and then transferred to clean test tubes. The caps were loosely sealed and tubes were cultured at 37°C for 48 hours. The caries activity was then characterized as level 0, 1, 2, or 3 using the evaluation chart provided by the manufacturer, which is based on the number of *S. mutans* colonies present in the sample.

Detection of *S. mutans* by polymerase chain reaction (PCR)

PCR analysis of saliva and plaque samples was performed using the oligonucleotide primers GtfB-F (5'-AGC CAT GCG CAA TCA ACA GGT T-3') and GtfB-R (5'-CGC AAC GCG AAC ATC TTG ATC AG-3'), which were designed to amplify a fragment of the *gtfB* gene of *S. mutans*. Reactions were carried out in 20 µL volumes containing 1 µL of genomic DNA, 4 µL 5× PCR mixture (Elpis Biotech, Daejeon, Korea), 1 µL of each primer (5 pM stocks), and 14 µL of distilled water. The reaction conditions were as follows: 95°C for 1 minute, followed by 30 cycles of 95°C for 10 seconds, 58°C for 10 seconds, and 72°C for 10 seconds, and a final extension at 72°C for 5 minutes. PCR products were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining.

Detection of *S. mutans* virulence factors in saliva by enzyme-linked immunosorbent assay (ELISA) analysis

Monoclonal antibodies specific to Ag I/II, GtfB, GtfC,

and GtfD, which were developed previously in our laboratory [21-24], were utilized for ELISA-based detection of *S. mutans* virulence determinants within saliva samples. For these experiments, 100 µL of each saliva sample was pipetted into 96-well plates coated with 20 µL of recombinant Ag I/II, GtfB, GtfC, or GtfD protein (Nunc A/S, Roskilde, Denmark), and incubated at 4°C overnight. After blocking with 3% skim milk at room temperature for 30 minutes, plates were washed three times with phosphate-buffered saline (PBS). Corresponding monoclonal antibodies were then added and incubated at 37°C for 1 hour. After washing with PBS to remove the antibodies, samples were incubated with alkaline phosphatase-tagged goat anti-mouse immunoglobulin G (Sigma Chemical Co., St. Louis, MO, USA) at 37°C for 1 hour. Plates were again washed four times with PBS, and alkaline phosphatase substrate was added. Plates were inserted into the ELISA reader (Packard Instrument Co., Downers Grove, IL, USA) and the optical density at 405 nm (OD₄₀₅) was measured. Reactions with each monoclonal antibody were performed three times, and data are presented as average values.

Statistical analysis

One-way analysis of variance (ANOVA) and Student's *t*-tests were utilized to evaluate differences in the results obtained for each group, and Spearman's correlation analysis was conducted to examine correlations between groups. All analyses were performed using SPSS version 19.0 software (IBM Corp., Armonk, NY, USA). *p* < 0.05 was considered statistically significant for all tests.

RESULTS

Analysis of the DMFT and SiC indices

Not surprisingly, the group 3 subjects (adults) exhibited the highest average DMFT value (average, 7.10 [female, 7.47; male, 6.73]), indicating a tendency towards an increased risk of dental caries with age. Notably, the average DMFT value was slightly higher in the female population than in the male population of each of the three age groups. Meanwhile, although group 2 exhibited the highest

SiC index value, there was no statistically significant difference between this value and those obtained for groups 1 and 3 (Table 1).

Salivary levels of *S. mutans*

The highest average Dentocult-SM score (1.17) was obtained from the group 2 samples (female, 1.27; male, 1.07). Similarly, the group 2 samples yielded significantly higher levels of *S. mutans* ($\log_{10}$ CFU/mL) on MS agar (average, 5.34 [female, 5.38; male, 5.30]) than the group 1 and 3 samples. When compared by gender, the Dentocult-SM score and the levels of *S. mutans* on MS agar were slightly higher in females than in males (Table 2).

Detection of *S. mutans* in saliva and plaque by PCR

PCR analysis detected *S. mutans* in 79.7% and 56.8% of saliva and plaque samples, respectively. Notably, the percentage of saliva samples from children that contained *S. mutans* (59.1%) was significantly lower than those from adolescents (88.0%) and adults (88.9%). Conversely, *S. mutans* was detected significantly more frequently in the plaque samples harvested from children (86.4%) than in those harvested from adolescents (56.0%) and adults (33.3%) (Table 3).

Detection of *S. mutans* virulence factors in saliva by ELISA analysis

The saliva samples were subjected to ELISA analysis using the following monoclonal antibodies: anti-AgI/II, anti-

Table 1. DMFT and SiC index according to gender and groups

Index	Total (n=90)	Group 1 (n=30)	Group 2 (n=30)	Group 3 (n=30)	p-value
DMFT					
Female	7.11±3.95	6.80±4.21	7.07±4.10	7.47±3.78	0.902
Male	5.89±4.84	4.27±3.26	6.67±6.15	6.73±4.57	0.288
Total	6.50±4.43	5.53±3.92	6.87±5.14	7.10±4.14	0.340
p-value	0.193	0.076	0.835	0.636	
SiC					
Total	11.40±3.36	9.90±2.33	12.40±4.65	11.90±2.33	0.217

Values are presented as mean±standard deviation.

Group 1, children (age: 8 years); Group 2, adolescents (age: 17 years); Group 3, adults (mean age: 28.4 years); DMFT, decayed, missing, and filled teeth; SiC, significant caries index.

Group 1= DMFT+dmft.

Table 2. Dentocult-SM scores and MS agar *Streptococcus mutans* levels ($\log_{10}$ CFU/mL) according to gender and groups

	Total (n=90)	Group 1 (n=30)	Group 2 (n=30)	Group 3 (n=30)	p-value
Dentocult-SM scores					
Female	1.00±0.83	0.87±0.83	1.27±0.88	0.87±0.74	0.316
Male	0.89±0.71	0.80±0.86	1.07±0.80	0.80±0.41	0.509
Total	0.94±0.77	0.83±0.83	1.17±0.83	0.83±0.59	0.154
p-value	0.497	0.831	0.521	0.764	
MS agar <i>S. mutans</i> levels ($\log_{10}$ [CFU/mL])					
Female	5.07±0.60	4.95±0.41	5.38±0.70	4.89±0.54	0.044 ^a
Male	4.84±0.66	4.74±0.60	5.30±0.54	4.48±0.57	0.001 ^b
Total	4.96±0.64	4.84±0.52	5.34±0.62	4.68±0.59	<0.001 ^c
p-value	0.082	0.299	0.711	0.055	

Values are presented as mean±standard deviation.

MS, mitis salivarius; CFU, colony-forming units; Group 1, children (age: 8 years); Group 2, adolescents (age: 17 years); Group 3, adults (mean age: 28.4 years).

^a $p<0.05$, ^b $p<0.01$, ^c $p<0.001$.

Table 3. Comparison between saliva and plaque detection of *Streptococcus mutans* using polymerase chain reaction (PCR) (%)

	Group 1		Group 2		Group 3		Total		p-value
	O	X	O	X	O	X	O	X	
PCR-saliva	59.1	40.9	88.0	12.0	88.9	11.1	79.7	20.3	0.012 ^a
PCR-plaque	86.4	13.6	56.0	44.0	33.3	66.7	56.8	43.2	

Analysis was done only on individuals with *S. mutans*.

Group 1, children (age: 8 years); Group 2, adolescents (age: 17 years); Group 3, adults (mean age: 28.4 years); O, total percent rate of detection individuals with *S. mutans*; PCR-saliva X, the detected percent rate of individuals with *S. mutans* in plaque who did not show *S. mutans* in saliva; PCR-plaque X, the detected percent rate of individuals with *S. mutans* in saliva who did not show *S. mutans* in plaque.

^a $p < 0.05$.

Table 4. Levels of monoclonal antibodies reacted to *Streptococcus mutans* in saliva according to groups*

Monoclonal antibody	Total	Group 1	Group 2	Group 3	p-value
Anti-AgI/II	1.34±0.17	1.20±0.20	1.40±0.17	1.42±0.14	0.601
Anti-GtfB	1.20±0.19	1.15±0.16	1.03±0.16	1.44±0.16	0.188
Anti-GtfC	1.52±0.27	1.09±0.16	1.26±0.17	2.23±0.45	<0.001
Anti-GtfD	1.42±0.25	1.06±0.21	1.10±0.12	2.09±0.30	<0.001

Values are presented as mean±standard deviation.

Group 1, children (age: 8 years); Group 2, adolescents (age: 17 years); Group 3, adults (mean age: 28.4 years); Ag, antigen; Gtf, glucosyltransferase.

*Measured at optical density=405 nm.

Table 5. Correlation analysis between variables of all subjects*

	Dentocult-SM	MS agar	PCR-saliva	PCR-plaque	Anti-AgI/II	Anti-GtfB	Anti-GtfC	Anti-GtfD
DMFT	0.022	0.038	0.080	-0.227 ^a	0.144	0.307 ^b	0.248 ^a	0.282 ^b

MS, mitis salivarius; PCR, polymerase chain reaction; Ag, antigen; Gtf, glucosyltransferase; DMFT, decayed, missing, and filled teeth.

*Spearman's correlation coefficient.

^a $p < 0.05$, ^b $p < 0.01$.

GtfB, anti-GtfC, and anti-GtfD. The OD₄₀₅ values obtained for each monoclonal antibody are depicted in Table 4.

Correlation analysis between variables

Spearman's correlation analysis was performed to examine whether there were associations between the DMFT values and caries variables for all participants. There was a positive correlation between DMFT values and the level of anti-GtfB ($r=0.307$, $p < 0.01$), anti-GtfC ($r=0.248$, $p < 0.05$), and anti-GtfD ($r=0.282$, $p < 0.01$) reactivity, as detected by ELISA analysis. Conversely, there was no correlation between the results obtained by Dentocult-SM analysis and by cultivation of samples on MS agar (Table 5).

DISCUSSION

Dental caries is an infectious oral disease that is prevalent in children and adolescents, and *S. mutans* is a primary causative agent of the disease [1,3,25]. The acidogenic bacteria that cause dental caries do not attach directly to the surface of teeth, but adhere to teeth via the formation of a pellicle. In particular, *S. mutans* adheres to teeth through both sucrose-independent and sucrose-dependent processes. In the absence of sucrose, expression of the combination of Ag I/II protein and salivary agglutinin glycoprotein promotes adherence to the oral biofilm. Meanwhile, in the presence of sucrose, the Gtfs (GtfB, GtfC, and GtfD) synthesize glucan, which is bound by bacterial cells (both streptococcal and non-streptococcal species) expressing

glucan-binding proteins, thereby promoting bacterial clustering and increasing the number of *S. mutans* within the biofilm [8,17].

In this study, the highest DMFT values for both males and females were observed in the group 3 subjects, and there was a general trend towards an increase in DMFT values with age. Meanwhile, the average SiC index, Dentocult-SM score, and log₁₀ CFU/mL of *S. mutans* were higher in the group 2 subjects than in the other groups. In particular, the *S. mutans* levels were significantly higher in the adolescent group, for both males and females, than in the other two groups.

Studies have shown that there are differences between males and females in the prevalence of dental caries, and that these differences are due to physiological, environmental, and behavioral factors, including sex hormones, early eruption of teeth, snacking frequency, and saliva secretion. Specifically, most studies have detected a higher prevalence of dental caries in females [26–28]. Consistent with these findings, we detected slightly higher DMFT values, Dentocult-SM scores, and *S. mutans* levels on MS agar in the females of each age group compared to the males.

To date, there have been conflicting reports regarding the presence of a correlation between DMFT values and the levels of *S. mutans* present in the saliva. Indeed, while several studies have detected a significant relationship between these factors [29–33], other studies have failed to detect such a correlation [34–37]. Consistent with this latter group, we did not observe a statistically significant relationship between these factors. Notably, according to one report, the development of dental caries is not dependent on the number of *S. mutans* present, but on the presence of particular *S. mutans* strains that exhibit higher levels of toxicity and acid production [35]. This factor could explain the inconsistent detection of a statistically significant relationship between DMFT and *S. mutans* levels.

A recently developed saliva-based diagnostic test allows for convenient, painless sample collection. This test can simultaneously detect host immune factors and markers of the bacterial species responsible for dental caries and periodontal disease within the oral cavity. In addition, it can detect systemic diseases such as human immunodeficiency virus and hepatitis. As a result, in recent years, this ap-

proach has been considered a more useful diagnostic tool than the collection of plaque [11]. To assess the efficacy of this method, we compared the sensitivity of a PCR-based test for the detection of *S. mutans* in saliva and plaque samples. While *S. mutans* was detected in significantly fewer saliva samples from children than from adolescents or adults, the organism was detected in a significantly larger number of plaque samples from children than from the other groups. Previous studies have demonstrated that *S. mutans* is detected with greater accuracy in plaque samples than in saliva samples [11,33,38]. Notably, however, while the results of the present study indicate that *S. mutans* is detected with greater accuracy in children using plaque samples, greater accuracy is obtained in both adolescents and adults via the analysis of saliva samples. Therefore, different methods for the detection of *S. mutans* should be used in accordance with age. Meanwhile, a possible explanation for the increased Dentocult-SM scores and *S. mutans* levels on MS agar observed in adolescents is that this age comprises a transitional period in the development of an individual from a child to an adult. As children gain more independence during this period, oral hygiene may become neglected due to reduced parental control over eating habits and oral hygiene. Therefore, stricter management of oral hygiene is needed in adolescents, as microbial factors combined with lifestyle changes in this age group can result in an increased risk for the development of oral disease.

Many researchers have attempted to effectively prevent dental caries using Ag I/II and Gtfs, which are virulence factors of *S. mutans* [39–42]. In the present study, Ag I/II-, GtfB-, GtfC-, and GtfD-specific monoclonal antibodies developed in our laboratory were used to measure the levels of these virulence factors in saliva samples [21–24]. Similar to the observed age-dependent increase in DMFT values, there was a trend towards an increase in the levels of these proteins with increasing age. Furthermore, there was a statistically significant positive correlation between DMFT values and the signals obtained using the anti-GtfB, anti-GtfC, and anti-GtfD antibodies. Indeed, our results indicate that there was a better correlation between DMFT values and the results obtained by ELISA analysis using our monoclonal antibodies than between DMFT values and the levels

of *S. mutans* observed on MS agar. As such, these findings suggest that ELISA analysis using our monoclonal antibodies could be utilized as a tool to rapidly measure caries activity in the clinical setting.

Dental caries not only induce pain but also hinder nutrient intake, thereby adversely affecting proper physical growth. Hence, early diagnosis and treatment of dental caries during childhood and adolescence is very important. Therefore, we examined the prevalence of *S. mutans* among three distinct age groups and analyzed correlations between indices related to dental caries. Higher numbers of *S. mutans* were detected in adolescents with permanent dentition than in children with mixed dentition, demonstrating the need to further reinforce oral hygiene in adolescents. Moreover, the present study provides valuable data that can be utilized for the early prevention of dental caries in children and adolescents as well as for relevant future studies.

In conclusion, this study, we evaluated the distribution of dental caries-associated *S. mutans* in the saliva and plaque of humans. Notably, there were significantly higher levels of *S. mutans* in the saliva of female and male adolescents than in the other groups, and females exhibited higher levels than males in all groups. Analyses of correlations between caries-related indices and age detected a positive correlation between DMFT index and the level of *S. mutans* reactivity measured by ELISA, but not between DMFT index and the salivary levels of *S. mutans*, as detected by cultivation methods. Our results suggest that oral hygiene should be emphasized in adolescents more than in children or adults, and that this group should be targeted for early prevention and research of dental caries.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

ORCID

Yong Jin Lee

<https://orcid.org/0000-0003-3146-4441>

Mi-Ah Kim

<https://orcid.org/0000-0002-5475-7477>

Jae-Gon Kim

<https://orcid.org/0000-0002-8789-6756>

Jae-Hwan Kim

<https://orcid.org/0000-0001-8088-6216>

REFERENCES

1. Petersen PE. The World Oral Health Report 2003: continuous improvement of oral health in the 21st century--the approach of the WHO Global Oral Health Programme. *Community Dent Oral Epidemiol* 2003;31 Suppl 1:3-23. doi: 10.1046/j..2003.com122.x.
2. Filstrup SL, Briskie D, da Fonseca M, Lawrence L, Wandera A, Inglehart MR. Early childhood caries and quality of life: child and parent perspectives. *Pediatr Dent* 2003;25:431-440.
3. Loesche WJ. Role of *Streptococcus mutans* in human dental decay. *Microbiol Rev* 1986;50:353-380.
4. Marsh PD. Microbiological aspects of the chemical control of plaque and gingivitis. *J Dent Res* 1992;71:1431-1438. doi: 10.1177/00220345920710071501.
5. Berkowitz RJ, Jones P. Mouth-to-mouth transmission of the bacterium *Streptococcus mutans* between mother and child. *Arch Oral Biol* 1985;30:377-379. doi: 10.1016/0003-9969(85)90014-7.
6. Wade WG. The oral microbiome in health and disease. *Pharmacol Res* 2013;69:137-143. doi: 10.1016/j.phrs.2012.11.006.
7. Bimstein E, Ram D, Irshied J, Naor R, Sela MN. Periodontal diseases, caries, and microbial composition of the subgingival plaque in children: a longitudinal study. *ASDC J Dent Child* 2002;69:133-137.
8. Mitchell TJ. The pathogenesis of streptococcal infections: from tooth decay to meningitis. *Nat Rev Microbiol* 2003;1:219-230. doi: 10.1038/nrmicro771.
9. Caufield PW, Cutter GR, Dasanayake AP. Initial acquisition of mutans streptococci by infants: evidence for a discrete window of infectivity. *J Dent Res* 1993;72:37-45. doi: 10.1177/00220345930720010501.
10. Raadal M, Espelid I. Caries prevalence in primary teeth as a predictor of early fissure caries in permanent first molars. *Community Dent Oral Epidemiol* 1992;20:30-34. doi: 10.1111/j.1600-0528.1992.tb00669.x.
11. Alaluusua S, Renkonen OV. *Streptococcus mutans* establishment and dental caries experience in children from 2 to 4 years old. *Scand J Dent Res* 1983;91:453-457. doi: 10.1111/j.1600-0722.1983.tb00845.x.
12. Köhler B, Andréen I, Jonsson B. The earlier the colonization

- tion by mutans streptococci, the higher the caries prevalence at 4 years of age. *Oral Microbiol Immunol* 1988;3:14-17. doi: 10.1111/j.1399-302X.1988.tb00598.x.
13. Koga T, Oho T, Shimazaki Y, Nakano Y. Immunization against dental caries. *Vaccine* 2002;20:2027-2044. doi: 10.1016/S0264-410X(02)00047-6.
14. Smith DJ. Caries vaccines for the twenty-first century. *J Dent Educ* 2003;67:1130-1139.
15. Guo JH, Jia R, Fan MW, Bian Z, Chen Z, Peng B. Construction and immunogenic characterization of a fusion anti-carries DNA vaccine against PAc and glucosyltransferase I of *Streptococcus mutans*. *J Dent Res* 2004;83:266-270. doi: 10.1177/154405910408300316.
16. Taubman MA, Nash DA. The scientific and public-health imperative for a vaccine against dental caries. *Nat Rev Immunol* 2006;6:555-563. doi: 10.1038/nri1857.
17. Bowen WH, Koo H. Biology of *Streptococcus mutans*-derived glucosyltransferases: role in extracellular matrix formation of cariogenic biofilms. *Caries Res* 2011;45:69-86. doi: 10.1159/000324598.
18. World Health Organization. Oral health surveys: basic methods. Geneva: World Health Organization; 2013:42-47.
19. Bratthall D. Introducing the significant caries index together with a proposal for a new global oral health goal for 12-year-olds. *Int Dent J* 2000;50:378-384. doi: 10.1111/j.1875-595X.2000.tb00572.x.
20. Marthaler TM. Changes in dental caries 1953-2003. *Caries Res* 2004;38:173-181. doi: 10.1159/000077752.
21. Kim MA, Yang YM, So YR, Ko YH, Lim SM, Lee KY, Kim JG. Development of a monoclonal antibody against glucosyltransferase D of *Streptococcus mutans* GS 5. *Hybridoma (Larchmt)* 2011;30:375-380. doi: 10.1089/hyb.2011.0011.
22. Kim MA, Lee MJ, Jeong HK, Song HJ, Jeon HJ, Lee KY, Kim JG. A monoclonal antibody specific to glucosyltransferase B of *Streptococcus mutans* GS-5 and its glucosyltransferase inhibitory efficiency. *Hybridoma (Larchmt)* 2012;31:430-435. doi: 10.1089/hyb.2012.0047.
23. Kim MA, Lee KY, Kim JG. Monoclonal antibodies specific to *Streptococcus mutans* GS-5 glucosyltransferase-C inhibit bacterial glucosyltransferase. *Monoclon Antib Immunodiagn Immunother* 2013;32:330-335. doi: 10.1089/mab.2013.0028.
24. Kim MA, Jeon HS, Shin SY, Baik BJ, Yang YM, Lee KY, Kim JG. Rapid detection of *S. mutans* surface antigen I/II using a sensitive monoclonal anti-Ag I/II antibody by ELISA. *Monoclon Antib Immunodiagn Immunother* 2013;32:336-340. doi: 10.1089/mab.2013.0017.
25. Nishikawara F, Nomura Y, Imai S, Senda A, Hanada N. Evaluation of cariogenic bacteria. *Eur J Dent* 2007;1:31-39.
26. Lukacs JR, Largaespa LL. Explaining sex differences in dental caries prevalence: saliva, hormones, and "life-history" etiologies. *Am J Hum Biol* 2006;18:540-555. doi: 10.1002/ajhb.20530.
27. Ferraro M, Vieira AR. Explaining gender differences in caries: a multifactorial approach to a multifactorial disease. *Int J Dent* 2010;2010:649643. doi: 10.1155/2010/649643.
28. Lukacs JR. Sex differences in dental caries experience: clinical evidence, complex etiology. *Clin Oral Investig* 2011;15:649-656. doi: 10.1007/s00784-010-0445-3.
29. Carlsson P, Gandour IA, Olsson B, Rickardsson B, Abbas K. High prevalence of mutans streptococci in a population with extremely low prevalence of dental caries. *Oral Microbiol Immunol* 1987;2:121-124. doi: 10.1111/j.1399-302X.1987.tb00274.x.
30. Beighton D, Manji F, Baelum V, Fejerskov O, Johnson NW, Wilton JM. Associations between salivary levels of *Streptococcus mutans*, *Streptococcus sobrinus*, lactobacilli, and caries experience in Kenyan adolescents. *J Dent Res* 1989;68:1242-1246. doi: 10.1177/00220345890680080601.
31. Matee MI, Mikx FH, Maselle SY, Van Palenstein Helderman WH. Mutans streptococci and lactobacilli in breast-fed children with rampant caries. *Caries Res* 1992;26:183-187. doi: 10.1159/000261440.
32. Gábris K, Nagy G, Madlén M, Dénes Z, Márton S, Keszthelyi G, Bánóczy J. Associations between microbiological and salivary caries activity tests and caries experience in Hungarian adolescents. *Caries Res* 1999;33:191-195. doi: 10.1159/000016516.
33. Sánchez-Pérez L, Acosta-Gío AE. Caries risk assessment from dental plaque and salivary *Streptococcus mutans* counts on two culture media. *Arch Oral Biol* 2001;46:49-55. doi: 10.1016/S0003-9969(00)00095-9.
34. Macpherson LM, MacFarlane TW, Geddes DA, Stephen KW. Assessment of the cariogenic potential of *Streptococcus mutans* strains and its relationship to in vivo caries experience. *Oral Microbiol Immunol* 1992;7:142-147. doi: 10.1111/j.1399-302X.1992.tb00527.x.
35. Toi CS, Cleaton-Jones PE, Daya NP. Mutans streptococci and other caries-associated acidogenic bacteria in five-year-old children in South Africa. *Oral Microbiol Immunol* 1999;14:238-243. doi: 10.1034/j.1399-302X.1999.140407.x.
36. Karaoğlu S, Akgül N, Akgül HM. The association between the DMFS index and levels of salivary *Streptococcus mutans* and lactobacilli of subjects living in Erzurum, Turkey. *J Dent Sci* 2010;5:70-74. doi: 10.1016/S1991-7902(10)60011-6.
37. Zainab J, Yasameen AA, Ghada I. Correlation between caries related bacteria in plaque and saliva in different age group children. *J Bagh Coll Dent* 2012;24:140-144.
38. Seki M, Karakama F, Terajima T, Ichikawa Y, Ozaki T, Yoshida S, Yamashita Y. Evaluation of mutans streptococci in plaque and saliva: correlation with caries development in preschool children. *J Dent* 2003;31:283-290. doi: 10.1016/S0300-5712(03)00033-2.

39. Lehner T, Russell MW, Wilton JM, Challacombe SJ, Scully CM, Hawkes JE. Passive immunization with antisera to *Streptococcus mutans* in the prevention of caries in rhesus monkeys. *Adv Exp Med Biol* 1978;107:303-315. doi: 10.1007/978-1-4684-3369-2_35.
40. Ma JK, Smith R, Lehner T. Use of monoclonal antibodies in local passive immunization to prevent colonization of human teeth by *Streptococcus mutans*. *Infect Immun* 1987;55:1274-1278.
41. Shi W, Jewett A, Hume WR. Rapid and quantitative detection of *Streptococcus mutans* with species-specific monoclonal antibodies. *Hybridoma* 1998;17:365-371. doi: 10.1089/hyb.1998.17.365.
42. Gu F, Lux R, Anderson MH, del Aguila MA, Wolinsky L, Hume WR, Shi W. Analyses of *Streptococcus mutans* in saliva with species-specific monoclonal antibodies. *Hybrid Hybridomics* 2002;21:225-232. doi: 10.1089/153685902760213822.