

한국에서 분리된 *Streptococcus mutans*의 분자 역학 및 특성김세연¹, 배일권², 이정하¹, 신정환³, 김진범¹¹부산대학교 치의학전문대학원 예방과사회치학교실, ²신라대학교 보건복지대학 치위생학과, ³인제대학교 의과대학 진단검사의학과Molecular epidemiology and characterization of *Streptococcus mutans* strains in KoreaSe-Yeon Kim¹, Il Kwon Bae², Jung-Ha Lee¹, Jeong Hwan Shin³, Jin-Bom Kim¹¹Department of Preventive and Community Dentistry, School of Dentistry, Pusan National University, Yangsan,²Department of Dental Hygiene, College of Health and Welfare, Silla University,³Department of Laboratory Medicine, Inje University College of Medicine, Busan, Korea

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Objectives: We investigated the characteristics of *Streptococcus mutans* in the national culture collection from Korea. Twenty-nine (dental plaque, n=27; endodontic infections, n=1; blood, n=1) isolates were included in this study.

Methods: Antimicrobial susceptibilities were tested using the disk diffusion test. Multilocus sequence typing (MLST), serotyping, and collagen-binding genes were used for polymerase chain reaction (PCR) and direct sequencing. A collagen-binding (to assess the adhesion properties) assay was performed. *S. mutans* demonstrated high susceptibility to antimicrobial agents. Differences in collagen-binding abilities of the *cnm*-positive and -negative groups were compared using the Mann-Whitney U test ($P<0.05$).

Results: MLST analyses revealed 25 sequence types (STs), 17 of which (ST213-ST229) contained new alleles. The strains were classified into four serotypes with the *c* type encompassing 79.3% of all strains, while the *e*, *f*, and *k* types representing 6.9% each. Analysis of the *cnm* and *cbm* genes, which encode the two surface adhesion components of *S. mutans*, revealed three *cnm*-positive strains, each displaying greater adhesion ability than those of the *cnm*-negative strains.

Conclusions: This study highlights the presence of a wide variety of *S. mutans* genotypes in Korea. These findings may provide useful information regarding the pathogenesis of infectious diseases, such as dental caries.

Key Words: Adhesion, Bacteremia, Collagen binding, MLST, Infection, Inflammation, *Streptococcus mutans*

Introduction

Streptococcus mutans is a gram-positive facultative anaerobic oral bacterium that is considered the major causative agent of dental caries¹⁾. There are numerous reports in which *S. mutans* isolates have been found to mediate dental caries and dental plaque on the tooth surface in human^{2,3)}. *S. mutans* gen-

erates an extracellular polysaccharide from sucrose which helps in the attachment of the bacterium. Furthermore, *S. mutans* also adhere to the external enamel thus assisting the progress of colonization⁴⁾. The high cariogenicity of *S. mutans* is mainly due to its ability to adhere to tooth surfaces. *S. mutans* comprises four serotypes (*c*, *e*, *f*, and *k*), according to the different polysaccharide composition of the cell wall, consisting of rhamnose-

glucose polymers^{5,6}). The distribution frequency of these serotypes among clinical oral isolates has been investigated^{7,8}) and the majority of them, approximately 70-80%, were classified as serotype c, followed by e (20%), while serotypes f and k were found to be extremely rare, accounting for less than 5%.

Several bacterial surface proteins have been shown to have collagen-binding capability. In this regard, the collagen-binding proteins Cnm and Cbm are important cell surface antigens involved in the adhesion of *S. mutans* to collagenous tissues^{9,10}). Cnm has been shown to bind to type I collagen¹¹), a major organic component of dentin. The distribution frequencies of *cnm* and *cbm* in *S. mutans* oral strains are approximately 10-20% and 2%, respectively¹²). Recent studies on *cnm* and *cbm* have linked the encoded adhesins to extraoral conditions, including infective endocarditis, hemorrhagic stroke, and atherosclerotic plaque development¹³⁻¹⁵). Endodontic infection is considered an important cause of bacteremia; however, little is known regarding the presence of *S. mutans* in dental pulp tissue¹⁶).

Multilocus sequence typing (MLST) is an unambiguous procedure for characterising isolates of bacterial species using the sequences of internal fragments of each house-keeping genes. For each house-keeping gene, the different sequences present within a bacterial species are assigned as distinct alleles and, it is a way to define for each isolate, the alleles at each of the loci define the allelic profile or sequence type. It is a high-resolution genetic typing approach to identify species and strains of pathogens impacting human health¹⁷).

Few reports have described the molecular characteristics of *S. mutans* isolated from various specimens, in Korea.

The aim of this study was to investigate the distribution, antimicrobial susceptibilities, adhesion properties, and molecular characteristics of *S. mutans* isolated from carious lesions and other specimens. Genotyping of *S. mutans* for epidemiological studies is important for an in-depth understanding of dental caries initiation and progression.

Materials and Methods

1. Bacterial strains and antimicrobial susceptibilities

A total of 29 isolates of *S. mutans* were obtained from the Korean Collection for Oral Microbiology (KCOM) and the National Culture Collection for Pathogens (NCCP). They included one blood isolate (NCCP PO 1463), one specimen isolate from an endodontic infection (KCOM 2084), and twenty-seven isolates from dental plaques. The control strains used in this study were ATCC 25175 and UA159, which is isolated from caries lesion. Identification of the strains was confirmed by 16S rRNA

sequencing and matrix-assisted laser desorption/ionization mass spectrometry. *S. mutans* was grown in Brain Heart Infusion medium (BHI, Bacto™ BHI, BD, NJ, USA) containing 1% dextrose¹⁸), at 37°C in an aerobic chamber. An optical density (OD) of 1.0 was determined to correspond to 2×10^9 colony forming units/mL at 620 nm wavelength. For bacterial preparation, an overnight culture was diluted to an OD₆₂₀ of 1.0 in BHI broth. *S. mutans* strains were tested for susceptibility to antimicrobials by the disc diffusion method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines¹⁹). The antimicrobial agents used were penicillin G (10 µg), vancomycin (30 µg), erythromycin (15 µg), tetracycline (30 µg), rifampicin (5 µg), trimethoprim-sulfamethoxazole (1.25 µg+23.75 µg), chloramphenicol (30 µg), clindamycin (2 µg), and levofloxacin (5 µg), respectively (Rosco, Tehran, IRI).

2. Amplification of bacterial serotyping and adhesion (*cnm*, *cbm*) genes

Primers used in this study are listed in Table 1. The primers for PCR amplification and sequencing were designed by selecting consensus sequences in multiple-nucleotide alignment collagen-binding gene (*cnm* and *cbm*) by using the Primer3 program (<http://bioinfo.ut.ee/primer3-0.4.0/>)^{9,10}). The serotype of each *S. mutans* strain was determined by PCR using serotype-specific sets of primers for serotypes c, e, f, and k²⁰).

3. Multilocus sequence typing (MLST)

MLST is a highly discriminatory method for characterizing bacterial isolates on the basis of the sequence type (ST)²¹⁻²³). Internal fragments of eight housekeeping gene loci were amplified, namely *tkt* (transketolase), *glnA* (glutamine synthetase subunit 1a), *gltA* (glutamate synthase), *glk* (glucose kinase), *gyrA* (DNA gyrase subunit A), *aroE* (shikimate-5 dehydrogenase), *murI* (glutamate racemase), and *lepC* (signal peptidase 1), and their nucleotide sequences were determined. The sequence of each housekeeping gene fragment was compared with the allele sequences on the MLST site (<http://pubmlst.org/oralstrep/>) in order to assign allele numbers using a non-redundant database. For each strain, eight allele numbers defined the allelic profile and, consequently, the ST of that strain²²⁻²⁴). We defined as groups of two or more independent isolates sharing identical alleles at five or more loci.

4. Collagen-binding assay of *S. mutans* strains

A collagen-binding assay was performed to confirm the adhesion ability of *S. mutans*. Briefly, 2 mg of type I collagen in 0.25 M acetic acid (Katayama Chemical Industries Co., Ltd., Osaka,

Table 1. PCR primers for *S. mutans* used in this study

Name	Purpose	Sequence (5' to 3')	Product size (bp)	Reference
<i>cnm</i> -F	Collagen-binding detection	TGCTGTTGATGGTAAGGAATTTAAC	717	This study
<i>cnm</i> -R		CTTCTTCGCCTGTGCTTG		
<i>cbm</i> -F	Serotyping	CGCGGACAAAGTGGTATCAG	847	Shibata et al. (2003)
<i>cbm</i> -R		TGTTTGAAGTTGAGCCTGCC		
<i>SC</i> -F		CGGAGTGCCTTTTACAAGTGCTGG	727	
<i>SC</i> -R		AACCACGGCCAGCAAACCCCTTAT		
<i>SE</i> -F		CCTGCTTTTCAAGTACCTTTCGCC	517	
<i>SE</i> -R		CTGCTTGCCAAGCCCTACTAGAAA		
<i>SF</i> -F		CCCACAATTGGCTTCAAGAGGAGA	316	
<i>SF</i> -R		TGCGAAACCATAAGCATAGCGAGG		
<i>CEFK</i> -F		ATTCCCGCGTTGGACCATTC	294	
<i>CEFK</i> -R		CCAATGTGATTTCATCCCATACC		
<i>tkl</i> -F*	MLST analysis	ACCGGGGTGTGTGTCATGGGCGTGC	432	Nakano et al. (2007)
<i>tkl</i> -R*		CATAGGATGACGCTTCGCCAGAAAC		
<i>glnA</i> -F*		CCTTGGGGAGATGAAAACGGAGCCG	462	
<i>glnA</i> -R*		TGGCCATAAAGTTGCATACAAACC		
<i>gltA</i> -F*		TGCCTTAACGATGTTAGAGAGAATG	387	
<i>gltA</i> -R*		AAAGACTATCTTCAAAAGCACACCC		
<i>glk</i> -F*		GACAAGTTCAGGAGAAATGGGCTAT	402	
<i>glk</i> -R*		CAGCAACTCCATGAATAAGATTGCC		
<i>aroE</i> -F*		GATGAAGTAACGAAAGCAGCAGATT	396	
<i>aroE</i> -R*		TGCCATCCATACCAACATTGGTCGC		
<i>murI</i> -F*		TCCGGAGTGGCGGTTTAAACGGTCG	432	
<i>murI</i> -R*		TCAACAATAGGAACAAATTTGGGGC		
<i>lepC</i> -F*		CCGCGTCTCTTTATCTGGTTTCTTG	423	
<i>lepC</i> -R*		GACAATGCGATCATCACCTAAAAGC		
<i>gyrA</i> -F*		TACAGGTGATGTCATGGGTAAATAC	417	
<i>gyrA</i> -R*		CCGGGTAGTACTTCCATTAGGTCAC		

*The asterisks indicate 8 housekeeping genes, *tkl* (transketolase), *glnA* (glutamine synthetase subunit 1a), *gltA* (glutamate synthase), *glk* (glucose kinase), *aroE* (shikimate-5 dehydrogenase), *murI* (glutamate racemase), *lepC* (signal peptidase 1) and *gyrA* (DNA gyrase subunit A).

JPN) were used for coating the wells of 96-well tissue culture plates (Corning Inc., NY, USA) and incubated overnight at 4°C. The plates were washed three times with phosphate buffered saline (PBS) and 5% bovine serum albumin (Bio Basic, NY, USA) in PBS was used as a blocking agent at 37°C for 1.5 h. Next, the wells were washed again with PBS containing 0.01% Tween 20 (Sigma, Darmstadt, DEU). Cells from the overnight cultures of *S. mutans* grown in BHI broth were collected by centrifugation, resuspended, and an aliquot of 2×10^9 cells was added to each well. After a 3-h incubation at 37°C, adherent cells were washed three times with PBS and then fixed with 200 µl 25% formaldehyde at room temperature for 30 min. After three additional washes with PBS, the adherent cells were stained with 200 µl 0.05% crystal violet for 1 minute and washed three times with PBS. Next, the dye was dissolved by adding 200 µl 7% acetic acid and the A620 values were determined (Elisa Reader, Tecan, AUT). Data were expressed as the mean \pm standard deviation of triplicate experiments^{20,25)}.

5. Statistics

The statistical analysis was performed with Statistical Package for the Social Sciences (SPSS Statistics for Windows, version 23, IBM Co., Armonk, NY, USA). The collagen-binding abilities of the *cnm*-positive and *cnm*-negative strains were compared using a Mann-Whitney's U test. *P*-values below 0.05 were considered to be significant.

Results

1. Antimicrobial susceptibilities

Control strains of *S. mutans* (ATCC 25175, UA159) were isolated from carious lesions and twenty-nine *S. mutans* strains were isolated from a variety of specimens, such as blood, endodontic infection, and dental plaque. The isolated *S. mutans* strains were not resistant to antibiotics, as assessed by the antimicrobial susceptibility test.

2. Bacterial serotypes and adhesion genes

The serotypes of 29 *S. mutans* strains were classified into four types, namely *c*, *e*, *f*, and *k*. Twenty-three isolates belonged to the control strain serotype, type *c*, whereas two isolates represented each of the *e*, *f*, and *k* serotypes (Table 2). In the analysis of *cnm* and *cbm* genes known to be associated with the adhesion properties of *S. mutans*, three *cnm*-positive strains (KCOM1225, KCOM1219, KCOM1195) were detected.

3. MLST analysis

MLST analysis of 31 *S. mutans* strains, including the control strains, revealed 25 STs, 17 of which (ST213 to ST229) contained new alleles. The allelic gene of *tkt* (#25), *gltA* (#36 to 38), *aroE* (#32), and *lepC* (#35) were defined the new type of alleles among the eight housekeeping gene. The existing sequence type was

almost identified in the dental plaque. Among the newly registered sequence type was identified in 14 of dental plaque, 1 of endodontic infection and blood. The control strain of *S. mutans* (ATCC25175) isolated from active caries subject was also registered with new sequence type. All new alleles defined in the present study are shown in bold in Table 2.

4. Collagen-binding activity

As a result of triplicate evaluation, the 29 *S. mutans* isolates showed collagen-binding activities in the range from 0.051 to 0.183 OD620 values. The control strains *S. mutans* ATCC 25175 and *S. mutans* UA 159 showed collagen-binding activities of 0.052, whereas the values corresponding to the activities of the *cnm*-containing strains were 0.139, 0.159, and 0.183, with a mean value of 0.160 ± 0.022 . However, the collagen-binding ac-

Table 2. Characteristics of *S. mutans* strains analyzed by PCR and sequencing

Species	<i>cnm</i>	<i>cbm</i>	Allelic profiles								ST	Serotype
			<i>tkt</i>	<i>glnA</i>	<i>gltA</i>	<i>glk</i>	<i>aroE</i>	<i>murI</i>	<i>lepC</i>	<i>gyrA</i>		
KCOM2966	—	—	6	5	9	3	17	4	1	3	221	<i>e</i>
KCOM2084	—	—	2	2	15	1	14	3	1	10	213	<i>c</i>
KCOM1225	+	—	1	3	8	1	25	3	1	1	123	<i>f</i>
KCOM1223	—	—	2	5	9	1	4	3	3	1	93	<i>c</i>
KCOM1220	—	—	2	5	9	1	4	3	3	1	93	<i>c</i>
KCOM1219	+	—	25	2	1	1	9	10	3	1	222	<i>k</i>
KCOM1216	—	—	1	14	36	3	32	2	11	1	223	<i>c</i>
KCOM1212	—	—	1	2	8	1	14	18	22	15	214	<i>c</i>
KCOM1209	—	—	1	14	15	4	4	3	11	1	215	<i>c</i>
KCOM1203	—	—	1	14	15	4	4	3	11	1	215	<i>c</i>
KCOM1201	—	—	2	3	15	3	4	5	35	1	224	<i>c</i>
KCOM1200	—	—	1	14	15	4	4	3	11	1	215	<i>c</i>
KCOM1195	+	—	1	1	1	1	18	5	3	1	216	<i>e</i>
KCOM1194	—	—	1	1	1	1	17	17	1	9	217	<i>c</i>
KCOM1188	—	—	1	14	15	4	4	3	1	1	225	<i>c</i>
KCOM1187	—	—	1	14	5	3	7	19	11	1	218	<i>c</i>
KCOM1186	—	—	2	2	4	3	4	25	1	4	209	<i>c</i>
KCOM1183	—	—	1	1	1	13	1	1	1	1	2	<i>c</i>
KCOM1179	—	—	3	2	37	3	4	3	1	1	226	<i>k</i>
KCOM1178	—	—	2	3	15	3	4	5	35	1	224	<i>c</i>
KCOM1147	—	—	1	5	5	1	23	2	1	1	121	<i>f</i>
KCOM1146	—	—	3	8	38	1	7	3	11	1	227	<i>c</i>
KCOM1145	—	—	1	14	15	1	2	19	11	9	219	<i>c</i>
KCOM1143	—	—	2	2	4	5	4	22	30	1	181	<i>c</i>
KCOM1142	—	—	1	1	1	13	1	1	1	1	2	<i>c</i>
KCOM1141	—	—	6	1	1	3	2	4	1	3	72	<i>c</i>
KCOM1140	—	—	6	1	1	3	2	4	1	3	72	<i>c</i>
KCOM1139	—	—	2	2	15	8	4	3	30	1	220	<i>c</i>
PO1463	—	—	4	1	1	2	2	5	1	1	228	<i>c</i>
ATCC25175	—	—	1	1	1	8	11	3	19	1	229	<i>c</i>
UA159	—	—	1	1	1	1	1	1	1	1	1	<i>c</i>

Bold letters indicate new alleles and sequence type (ST) registered with PubMLST for *S. mutans* MLST scheme in this study.

tkt (#25), GenBank accession no. AB281747; *gltA* (#36), GenBank accession no. AB281970; *gltA* (#37), GenBank accession no. AB281943; *gltA* (#38), GenBank accession no. AB282001; *aroE* (#32), GenBank accession no. AB282285; *lepC* (#35), GenBank accession no. AB282484.

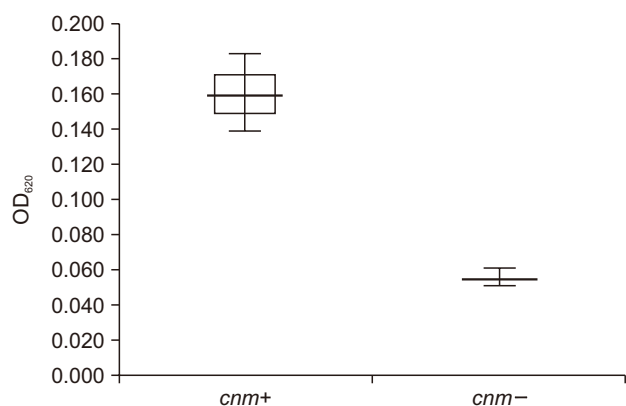


Fig. 1. Comparison of collagen-binding activities between *cnm*-positive (*cnm*+) and *cnm*-negative (*cnm*-) strains.

tivity of *cnm*-negative strains not identified by ranged from 0.051 to 0.061, with a mean of 0.054 ± 0.002 . The difference observed between *cnm*-positive and -negative strains was statistically significant (Fig. 1, $P < 0.001$).

Discussion

Streptococcus mutans is generally known as a major pathogen responsible for dental caries and, occasionally, as a possible causative agent of bacteremia and infective endocarditis^{5,6}. Several *S. mutans* strains were shown to possess collagen-binding activities^{26,27}. Most of them display cell-surface collagen-binding proteins and the genes encoding them (*cnm*, *cbm*) have been cloned and sequenced^{9,10}. This study was conducted to investigate the resistance status of *S. mutans* isolated from various specimens, including dental plaque, endodontic infection, and blood, as well as the mechanism of adhesion of different *S. mutans* strains. All the 29 strains, isolated from clinical settings, were found to be susceptible to the antimicrobial agents tested, indicating that treatment failure due to acquired resistance was unlikely. However, biofilms were reported to be resistant to antimicrobial agents in the oral environment^{28,29}. Although MLST analysis has been widely applied in recent study, but this is the first report of the MLST based epidemiology study of *Streptococcus mutans* in Korea.

The *cnm* and *cbm* gene fragments were used as primers to evaluate the adhesion genes in strains of *S. mutans*, including control strain of *S. mutans* (UA159 and ATCC 25175)^{20,30}. In *S. mutans*, the collagen-binding adhesin protein encoded by *cnm* has been described as a strain-specific collagen-binding molecule¹¹. The frequency of *cnm* gene in previously identified *cnm*-positive strains isolated from clinical specimens was approximately 20%¹², which differs from the approximate 10% value

observed in the present study. Evaluation of the adhesion ability of *S. mutans* showed that the three strains expressing *cnm* displayed higher collagen-adhesion ability when compared to other strains. The collagen-binding activity of the *cnm*-positive strains corresponded to 0.139, 0.159, and 0.183 OD₆₂₀ values, respectively. The mean collagen-binding activity of the *cnm*-positive strains was approximately three times higher compared to that of the *cnm*-negative strains.

S. mutans is classified into serotypes *c*, *e*, *f*, and *k* based on the chemical composition of serotype polysaccharides; 70-80% of the species are classified as serotype *c*; 20%, serotype *e*; less than 5%, serotypes *f* and serotype *k* in the mouth²⁵. The isolation of single serotypes was displayed the following distribution among the four serotypes: *c* type, 79.3%; *e*, *f* and *k* type, 6.9%, respectively. The *cbm*-positive strain has been associated with serotype *k*¹⁰, but the isolates identified by this serotype in our study did not express a *cbm* gene. In a previous study focusing on *cnm*-positive *S. mutans*, serotypes *f*, *k*, *c*, and *e* were found in 81.3%, 41.7%, 7.0%, and 3.2% cases, respectively^{10,24}. The serotypes of *cnm*-positive *S. mutans* were confirmed as *e*, *f*, and *k* (6.2%, respectively). However, one strain isolated from an endocarditis infection did not carry the *cnm* gene. Differences were observed in these studies between cardiovascular patients (single serotype, 41.0%; multiple serotypes, 53.8%), healthy mothers (single serotype, 75.0%; multiple serotypes, 25.0%) and healthy children (single serotype, 78.3%; multiple serotypes, 21.7%)^{12,24}. The isolation of single serotypes was displayed the following distribution among the four serotypes: *c* type, 79.3%; *e*, *f* and *k* type, 6.9%, respectively.

Although MLST analysis is a commonly used genotyping method, little is known about its application in the dental field. In this study, eight housekeeping loci were used because constitutive genes and their products are typically needed for maintenance of cell. Among the 17 newly identified STs, ST216 could be classified, by matching 5 or more genes, into the same clonal complex as ST1, ST150, ST165, ST169, and ST192 isolated from the USA. Although, based on the same criterium, ST217 could be classified into the same clonal complex as ST1, the other newly identified STs were different from those reported in previous studies. In general, serotypes are related to the entire genotype defined by the ST, and closely related STs constitute similar clonal complexes. Therefore we confirmed that in *S. mutans* the same ST may associate to different serotype or gene expression. The control strain *S. mutans* UA 159 belonged to ST1, but the other control strain (*S. mutans* ATCC 25175) was registered as ST229 and assigned to serotype *c*. Similar characteristics were observed in ST121, ST123, ST181, and ST209²⁰. The strains

belonging to ST121 and 123 with same in four alleles were assigned to serotype *f*; ST181 and 209 with same in four alleles were assigned to serotype *c*. The strains identified by ST2, ST93 and ST72 were also assigned to serotype *c*.

The discrepancies observed in various studies based on MLST might be accounted for by differences in recombination, in *S. mutans*, resulting in heterogeneous virulence²³. Among the 17 newly analyzed STs (ST213–228), ST216 could be classified into the same clonal complex by matching 5 or more genes with ST1, ST150, ST165, ST169, and ST192 isolated from the USA. Although the isolates Thailand ST126 and ST217 could be classified into the same clonal complex by matching 5 or more genes with ST1, the other newly discovered STs were different from those reported in previous studies. The strain classified as ST229 was the control strain ATCC 25175 isolated from caries active subject. This study has been used to investigate characteristics of *S. mutans* strains isolated from Korea, there is the limit that could not be verified for non-isolated *S. mutans* strains. Therefore, it is necessary to subsequent analysis for later isolated *S. mutans* strains.

In conclusions, the analysis of 29 *S. mutans* strain isolates from various specimens revealed 17 STs with new alleles. In the evaluation of *cnm* and *cbm*, i.e., genes associated with the adhesion ability of *S. mutans*, three *cnm*-positive strains were detected, all displaying adhesion abilities 3 times higher than those of *cnm*-negative strains. These findings may provide potentially useful information on the pathogenesis of infectious diseases including dental caries.

Conclusions

This study was to investigate the distribution, antimicrobial susceptibilities, adhesion properties, and molecular characteristics of *S. mutans* isolated from carious lesions and other specimens.

1. The analysis of 29 *S. mutans* strain isolates from various specimens revealed 17 STs with new alleles in Korea.

2. The serotypes of 29 *S. mutans* strains were classified into four types (*c*, *e*, *f*, and *k*).

3. In the evaluation of genes associated with the adhesion ability of *S. mutans*, three *cnm*-positive strains were detected. Moreover, the adhesion properties of *cnm*-positive strains were significantly 3 times greater than those of *cnm*-negative strains.

These findings may provide potentially useful information on the pathogenesis of infectious diseases including dental caries.

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