

Direct detection of cariogenic streptococci in metal brackets *in vivo* using polymerase chain reaction

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Streptococcus mutans and *Streptococcus sobrinus* are major etiological agents in enamel demineralization around orthodontic appliances. This study was designed to examine the prevalence of these streptococci on orthodontic brackets *in vivo* using polymerase chain reaction. Four incisor brackets in the upper and lower arches were removed and collected from 80 patients at the time of debonding. The genomic DNA of adhered bacteria was extracted and each dextranase gene of *S. mutans* and *S. sobrinus* was amplified using the specific oligonucleotide primers. The results showed that the maxillary incisor brackets were colonized by both cariogenic streptococci to a somewhat higher degree than that taken from the mandible. The prevalence of *S. mutans* was 50.0% on the maxillary incisor brackets and 33.8% on the mandibular incisor brackets, and that of *S. sobrinus* was 17.5% and 15.0%, respectively. Both species were detected on the maxillary incisor brackets of 7 patients (8.8%) and the mandibular incisor brackets of 5 patients (6.3%). These results suggest that cariogenic streptococci can adhere to the incisor brackets and may be resident species on the incisor brackets.

(Key words: Cariogenic streptococci, Detection, Metal bracket, Polymerase chain reaction)

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INTRODUCTION

Enamel demineralization or white spot formation is a common side effect of orthodontic treatment with fixed appliances. The incidence of enamel demineralization after using fixed orthodontic appliances can occur in up to 50% of patients.^{1,2} Visible plaque around the orthodontic appliance is a risk for the development of white spots during orthodontic treatment.³ The enamel

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demineralization is caused by organic acids produced mainly by mutans streptococci (MS), which are known to be the prime causative organisms of dental caries.^{4,5} MS are now classified into seven species: *Streptococci cricetus* (serotype a), *S. rattii* (serotype b), *S. mutans* (serotypes c, e, and f), *S. sobrinus* (serotype d and g), *S. downei* (serotype h), *S. ferus* (serotype c), and *S. macacae* (serotype c).⁶ Of these species, *S. mutans* and *S. sobrinus* are most frequently isolated from the human oral cavity and have been implicated as the main causative organisms of human dental caries.^{7,8}

Recently, several epidemiologic studies have shown that *S. sobrinus* is more closely associated with a high caries activity than *S. mutans*.^{9,10} In addition, *S. sobrinus* has been reported to be associated with smooth-surface caries and enamel demineralization in orthodontically treated children.^{11,12} However, *S. mutans* was the predominant species and was often found alone, while *S. sobrinus* was usually detected in the teeth harboring *S. mutans*.¹³ This indicates that information on *S. mutans* and *S. sobrinus* is helpful for identifying patients at risk of developing enamel demineralization and planning dental caries prevention programs in the orthodontic patients.

Among the many orthodontic appliances, orthodontic brackets may play a significant role in enamel demineralization because they are attached continuously to the dentition during almost all the orthodontic treatment period and their complex design provides a unique environment, which impedes access to the tooth surfaces for cleaning. Previous report showed extensive patterns of plaque accumulation associated with bonded orthodontic brackets.¹⁴ In particular, metallic brackets have been found to cause specific changes in the oral environment, such as a decrease in pH and an increase in plaque accumulation.¹⁵ This indicates that orthodontic brackets impose a potential risk for enamel demineralization. Therefore, isolation frequency of *S. mutans* and *S. sobrinus* from orthodontic brackets is helpful for identifying patients at risk of developing enamel demineralization and planning dental caries prevention programs during orthodontic treatment.

The isolation and identification of cariogenic streptococci was usually based on the colonial morphology on

mitis-salivarius-bacitracin agar. The isolated colonies are then identified by biochemical, immunological, and genetic tests.^{16,17} However, these procedures are sometimes inaccurate, time-consuming, and laborious. In recent years, new tools such as polymerase chain reaction (PCR) have been used to overcome these limitations.^{16,17} PCR is a simple, rapid, and highly specific method for the detection and identification of microorganisms in a sample. The purpose of this study was to examine the prevalence of *S. sobrinus* and *S. mutans* on metal brackets collected from orthodontic patients by the *S. sobrinus*- and *S. mutans*-specific PCR methods.

MATERIAL AND METHODS

Bacterial strains

Eleven strains of mutans streptococci as well as other gram-positive streptococci (5 strains of 3 species) and gram-negative species (11 strains of 9 species) were used as positive and negative controls in PCR (Table 1). Gram-positive bacteria were grown anaerobically at 37°C on brain heart infusion (BHI) (Difco Laboratories, Detroit, MI, USA) agar plates. Gram-negative bacteria were grown anaerobically at 37°C BHI agar plates supplemented with 5% horse blood, hemin (5 µg/ml), and menadione (50 µg/ml).

PCR primers

Known specific primers that amplify dextranase gene of *S. mutans* and *S. sobrinus* were selected.^{18,19} For *S. mutans*, the upper primer, 5'-TAT GCT GCT ATT GGA GGT TC-3' is complementary to the sequence 973 to 992, and the lower primer, 5'-AAG GTT GAG CAA TTG AAT CG-3' is complementary to the sequence 2225 to 2244. The size of expected PCR product was 1215 base pairs (bp). The upper primer, 5'-TAC TAT CTT TCC CTA GCA TG-3' is complementary to the sequence 134-153, and the lower primer, 5'-GGT ATT CGG TTT GAC TGC-3' is complementary to the sequence 1743-1726 for *S. sobrinus*. The size of expected PCR product was 1610 bp. All primers were commercially synthesized by

Table 1. Bacterial strains used

<i>Mutans streptococci</i>	<i>Other gram-positive bacteria</i>	<i>Gram-negative bacteria</i>
<i>S. cricetus</i> E49	<i>Streptococcus gordonii</i> DL1	<i>Escheritia coli</i> DH5a
<i>S. ratti</i> BHT 9	<i>Streptococcus gordonii</i> M5	<i>Actinobacillus acetemcomitans</i> 33385
<i>S. ratti</i> FA-1F	<i>Streptococcus mitis</i> 9811	<i>Actinomyces naslundii</i> 12104
<i>S. mutans</i> Ingbritt	<i>Streptococcus sanguis</i> 10558	<i>Actinomyces viscosus</i> 19226
<i>S. mutans</i> LM7	<i>Streptococcus sanguis</i> MPC1	<i>Fusobacterium nucleatum</i> 10953
<i>S. mutans</i> KPSK-2		<i>Fusobacterium nucleatum</i> 27067
<i>S. mutans</i> OMZ175		<i>Lactobacillus acidophilis</i> 5906
<i>S. mutans</i> GH5IS		<i>Prevotella intrmedia</i> 25611
<i>S. sobrinus</i> B13		<i>Porphyromomas gingivalis</i> 381
<i>S. sobrinus</i> SL1		<i>Porphyromomas gingivalis</i> W50
<i>S. sobrinus</i> 6715		<i>Bacteroides intermedius</i> 532-70A

Takara Korea Co., Seoul, Korea.

Preparation of orthodontic brackets

A total of 80 patients, who just completed fixed orthodontic treatment, were selected for this study. They were treated with the same metal brackets (Victory series, 3M/Unitek, Monrovia, CA, USA). The subjects had neither received professional cleaning nor antibiotic medication for the 3 months prior to the study. The 4 upper and lower incisor brackets were carefully removed and collected at the time of debonding and a total of 320 brackets were used in both the maxilla and mandible. The brackets were gently washed 3 times by the addition of 3 mL sterile phosphate-buffered saline (PBS, pH 7.4) to remove unbound bacteria. The brackets were then immediately used for extraction of chromosomal DNA from adhered bacteria.

Extraction of chromosomal DNA

Chromosomal DNAs of the bacteria were extracted by a standard miniprep procedure,²⁰ to which we performed a lysozyme treatment. Briefly, bacterial cells were incubated at 37°C by 300 μ l of PBS with 1,000 units of lysozyme with agitation for 1 hr. After centrifugation at 12,000 *g* for 10 min, the supernatant was removed and the cells were suspended with 300 μ l of lysis

solution (10 mmol/L Tris-HCl, 1 mmol/L of EDTA, 0.5 % sodium dodecyl sulfate, 40 g of proteinase K, pH 8.0) with agitation at 37°C for 1hr. After addition of 50 μ l of 5M NaCl and 40 μ l of 4% cetyltrimmonium bromide, the suspension was incubated at 65°C for 10 min. The resultant lysate was extracted with phenol-chloroform and precipitated with isoprophyl alcohol. All DNA fractions were then stored in 30 μ l of Tris-EDTA buffer (10 mmol/L of Tris-HCl buffer, 1 mmol/L of EDTA, pH 7.4). Extraction of chromosomal DNA from reference bacteria was performed in a similar way.

PCR procedure

Each PCR mixture (50 μ l) consisted of 10 mM Tris-HCl buffer (pH 8.3), 1.5 mM MgCl₂, 50 mM KCl, 200 μ M each of dATP, dTTP, dGTP, and dCTP, 1 μ M oligonucleotide primers of both *S. mutans* and *S. sobrinus*, 2.5 units of Taq DNA polymerase (Takara Korea Co, Seoul, Korea), and 10 μ l of template solution. The mixture was denatured at 95°C for 3 min followed by a series of amplications: denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. The series was repeated for 32 cycles. The final cycle comprised of 95°C for 1 min, 55°C for 1 min, and 72°C for 5 min. The amplified products were electrophoresed on 1.0% agarose gels and stained with ethidium bromide.

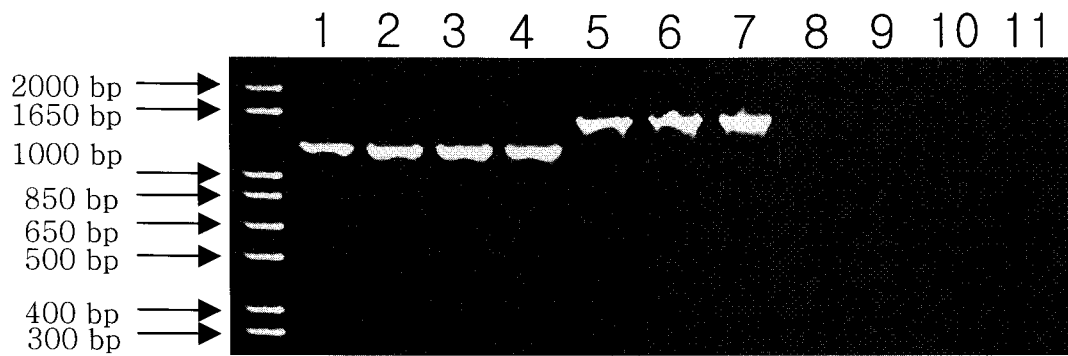


Fig 1. PCR amplification of the dextranase DNA sequence on chromosomal DNAs from known strains of streptococci. Lane 1, *S. mutans* Ingbritt; 2, *S. mutans* KPSK-2; 3, *S. mutans* LM-7; 4, *S. mutans* OMZ-175; 5, *S. sobrinus* B13; 6, *S. sobrinus* SL1; 7, *S. sobrinus* 6715; 8, *S. cricetus* E49; 9, *S. gordonii* DL1; 10, *S. rattii* BHT9; 11, *S. rattii* FA-1F. *S. mutans* produced a single DNA fragment of 1272 base pairs (bp) and *S. sobrinus* produced a single DNA fragment of 1610 bp.

RESULTS

Specificity and sensitivity of PCR

All the strains listed in Table 1 were examined using the specific primer pairs in order to evaluate the specificity of PCR. Of the 11 strains of mutans streptococci, only *S. mutans* strains and *S. sobrinus* strains produced a single DNA fragment of 1272 bp and 1610 bp, respectively (Fig 1). Amplified DNA was not detected in other mutans streptococci as well as in other gram-positive species (5 strains of 3 species) or gram-negative species (11 strains of 9 species). These results suggest that the primers used in this study are specific for *S. mutans* or *S. sobrinus*.

The sensitivity of PCR was examined using known numbers of cells of *S. mutans* Ingbritt and *S. sobrinus* 6715. The total cell count of each strain was adjusted to 10^6 cells per mL using a Petroff-Hauser counter (Hauser Scientific Partnership, Horsham, PA, USA). As a result, chromosomal DNA extracted from as few as 1×10^3 cells of each strain produced a detectable PCR product (Fig 2).

Results of the PCR analysis

The prevalence of cariogenic streptococci on the

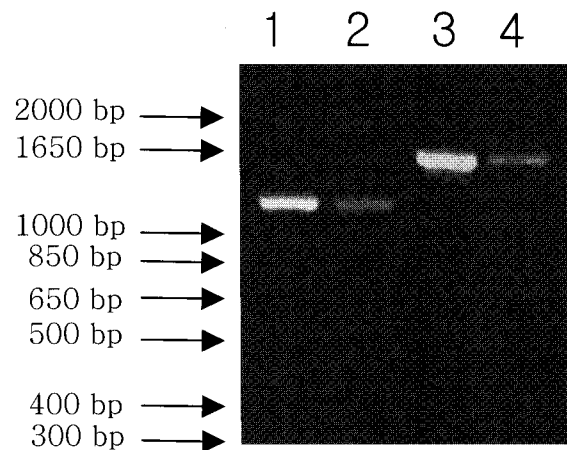


Fig 2. Sensitivity of the dextranase PCR with purified chromosomal DNA from known number of *S. sobrinus* 6715 and *S. mutans* Ingbritt. Number of lysed cells in lanes: 1, 10,000 cells of *S. mutans*; 2, 1,000 cells of *S. mutans*; 3, 10,000 cells of *S. sobrinus*; 4, 1,000 cells of *S. sobrinus*. Chromosomal DNA extracted from as few as 1×10^3 cells of each species produced a detectable PCR product.

maxillary incisor brackets differed from that found on the mandibular incisor brackets. In the maxilla, *S. mutans* was detected in 40 patients (50.0%) and *S. sobrinus* in 14 patients (17.5%). In the mandibular arch,

Table 2. Prevalence of *Streptococcus mutans* and *Streptococcus sobrinus* on incisor metal brackets (n = 80)

	Maxilla	Mandible	Both jaws
<i>S. mutans</i>			
Number of patients	40	27	16
Prevalence	50.0%	33.8%	20.0%
<i>S. sobrinus</i>			
Number of patients	14	12	5
Prevalence	17.5%	15.0%	6.3%
Both species			
Number of patients	7	5	4
Prevalence	8.8%	6.3%	5.0%

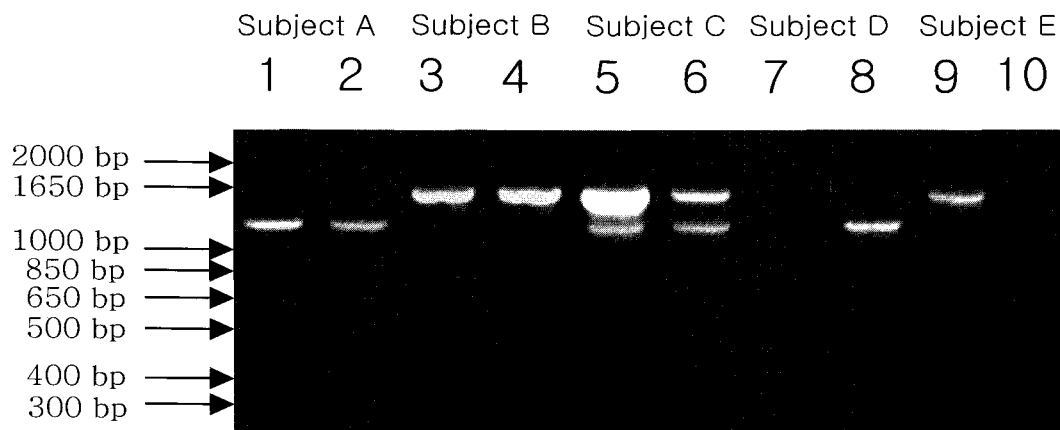


Fig 3. Direct detection of *S. mutans* and *S. sobrinus* from orthodontic metal brackets by PCR amplification. Lanes 1 and 2, upper and lower bracket samples from subject A, respectively; lanes 3 and 4, upper and lower bracket samples from subject B, respectively; lanes 5 and 6, upper and lower bracket samples from subject C, respectively; lanes 7 and 8, upper and lower bracket samples from subject D, respectively; lanes 9 and 10, upper and lower bracket samples from subject E, respectively.

S. mutans was detected in 27 patients (33.8%) and *S. sobrinus* in 12 patients (15.0%). Both species of streptococci were identified on the maxillary incisor brackets of 7 patients and on the mandibular incisor brackets of 5 patients. In only 4 patients, both species were detected on the maxillary and mandibular brackets (Table 2).

Representative results of PCR detection from the brackets are shown in Fig 3. Positive response suggests that the number of streptococci adhered to the incisor brackets was over 1×10^3 cells. In subject A,

S. mutans-specific product was observed on both upper and lower brackets (Fig 3; lanes 1 and 2), suggesting that the number of *S. mutans* were over 1×10^3 cells on both brackets. In subject B, only *S. sobrinus*-specific products were detected (Fig 3; lanes 3 and 4). Both *S. mutans*- and *S. sobrinus*-specific products were detected on upper and lower brackets of subject C (Fig 3; lanes 5 and 6). It can therefore be presumed that the number of *S. mutans* and *S. sobrinus* on both incisor brackets were over 1×10^3 cells respectively. In subject E, the *S. sobrinus*-specific

product was detected only on the upper brackets, while the *S. mutans*-specific product was only observed on the lower brackets in subject D.

DISCUSSION

Enamel white spot formation adjacent to the orthodontic brackets has been a great concern to orthodontists. Orthodontic brackets play a key role in enamel demineralization, because plaque accumulates around the brackets even in patients with good oral hygiene.¹⁴ Nevertheless, there are few reports on the biological aspects of the orthodontic brackets associated with the existence and prevalence of cariogenic MS on the orthodontic brackets *in vivo*. The present study was aimed to examine the prevalence of cariogenic MS on metal brackets of orthodontic patients.

The specific oligonucleotide primers, designed according to the nucleotide sequence of the dextranase gene, were used to detect *S. mutans* and *S. sobrinus* in this PCR method.^{18,19} The dextranase genes are suitable for the PCR primers, because these genes express important virulence factors of cariogenic streptococci. In addition, the primers used were highly sensitive for *S. mutans* and *S. sobrinus* and it was possible to diagnose as few as 1,000 cells of *S. mutans* or *S. sobrinus* (Fig 2). The capability of detecting fewer than 1,000 bacteria will be of particular importance in the identification of patients who may be at risk of developing enamel demineralization around orthodontic brackets. Furthermore, the presence of *S. sobrinus* and *S. mutans* can be determined within 1 day (6 ~ 7 hr), because the bacterial growth, isolation, and culture procedures are omitted.

This study revealed higher prevalence of *S. mutans* than that of *S. sobrinus* on the brackets of upper and lower anterior areas (Table 2). The prevalence of *S. mutans* is 50.0% on the maxillary incisor brackets and 33.8% on the mandibular incisor brackets. The prevalence of *S. sobrinus* is 17.5% and 15.0%, respectively. This could be mainly due to the difference in the prevalence between these species in the oral cavity. Many studies have shown that *S. mutans* is isolated more frequently than *S. sobrinus* in the oral

cavity.^{8,21} This could be also partly explained by fundamental differences between the mechanisms by which *S. mutans* and *S. sobrinus* attach to the bracket surfaces. Saliva can increase the binding of *S. mutans* more than *S. sobrinus* in the oral cavity, because *S. sobrinus* does not possess adhesins but, *S. mutans* possesses adhesins which bind to salivary components in the pellicles.²²

Difference in the prevalence between upper and lower incisor brackets was also found. *S. mutans* and *S. sobrinus* colonized on the maxillary incisor brackets to a somewhat higher degree than those of the mandible (Table 2). This result is consistent with another study, which showed the prevalence of MS is somewhat higher in the anterior region of the upper arch than in the lower arch.²³ The result may be due to the presence of major salivary glands, which clears the lower anterior portion more rapidly than the upper portion from easy access of saliva. The upper anterior region is located far from the major salivary glands and appears to receive very little saliva. Probably, these can give rise to the greater retention and slower elimination of food debris and sucrose, and provide better conditions for colonization of cariogenic MS. This can explain the result that upper incisors are more affected by enamel demineralization and white spot formation than lower incisors during orthodontic treatment.²⁴

The prevalence of both species on the anterior brackets was lower than currently reported in the oral cavity. The prevalence of *S. sobrinus* was reported to be about 80% in human oral cavities and that of *S. mutans* to be 100% by using the PCR method.^{18,19} This may be partly due to the low binding affinity of cariogenic MS to orthodontic brackets. A previous study reported that the binding affinity of cariogenic streptococci to orthodontic metal brackets was under 0.5% and the adhesion amount was significantly lower than other streptococci.²⁵

Recent studies showed that the presence of both *S. mutans* and *S. sobrinus* were strongly correlated with early caries lesions.⁵ In particular, the prevalence of *S. sobrinus* was closely associated with increase in smooth surface caries.^{10,11} Therefore, the prevalence of

S. sobrinus is more important than that of *S. mutans* in orthodontic patients, because orthodontic brackets are attached mainly to smooth buccal surfaces. This is proved by a previous study that incipient caries were developed on 3 upper incisors, where *S. sobrinus* harbored in the dental plaque around the maxillary incisor brackets.²⁶ The results obtained in this study did not find the clear relationship between the prevalence of cariogenic MS and enamel demineralization, because this study only provided some information on the prevalence of cariogenic streptococci. However, the level and presence of *S. mutans* and especially *S. sobrinus* on the anterior brackets indicate that orthodontic brackets may be a potential risk for enamel demineralization during orthodontic treatment.

The presence of *S. mutans* and *S. sobrinus* on orthodontic brackets may not necessarily be predictive of caries development in patients. However, the presence of cariogenic MS itself indicate that careful attention is required for prevention of white spot formation or enamel demineralization, particularly in the mouth of orthodontic patients who have poor oral hygiene or increased caries-activity. Cariogenic bacteria on orthodontic brackets would provide a distinct possibility that the remaining bacteria on the orthodontic brackets may continue to grow on the tooth surface surrounding the brackets, because microbial mass increases primarily as a result of cell division.²⁷

CONCLUSIONS

Cariogenic MS were detected and differentiated from the incisor brackets *in vivo* by PCR. The results showed that the prevalence of *S. mutans* was 50.0% (40 patients) on maxillary incisor brackets and 33.8% (27 patients) on mandibular incisor brackets, and those of *S. sobrinus* was 17.5% (14 patients) and 15.0% (12 patients), respectively. Both species were detected on maxillary incisor brackets of 7 patients and on mandibular incisor brackets of 5 patients. This study indicates that cariogenic MS may be a resident species on incisor brackets. The result stresses the need for careful hygiene control around orthodontic brackets.

- 국문초록 -

교정용 메탈 브라켓에서 자가중합효소연쇄반응을 통한 치아우식증 원인균의 검출

안석준 · 이신재 · 백승학 · 김태우 ·
장영일 · 남동석 · 임범순

*Streptococcus mutans*와 *Streptococcus sobrinus*는 교정장치 주위의 법랑질 탈회를 유발하는 중요한 원인균들이다. 본 연구는 중합효소연쇄반응법을 이용하여 생체 브라켓에 존재하는 이들 균주의 이환율을 평가하기 위해 고안되었다. 상악과 하악치열의 절치 브라켓 각각 4개를 교정치료 종료 시점에서 채취하였다. 브라켓에 부착된 세균들의 DNA를 추출하고 각각의 세균에 해당하는 프라이머를 이용하여 텍스트란 분해효소를 증폭시켰다. 본 연구의 결과는 *S. mutans*와 *S. sobrinus*가 하악절치 브라켓에 비해 상악절치 브라켓에 더 많이 존재함을 보여주었으며, 이들 브라켓에 존재하는 세균의 빈도는 이전의 연구에서 구강내에 존재하는 빈도에 비해 더 적음을 보여주었다. *S. mutans*의 상악절치 브라켓 이환율은 50.0%, 하악절치 브라켓 이환율은 33.8%였고, *S. sobrinus*는 각각 17.5, 15.0%였다. 상악절치 브라켓에 두 종류의 세균이 모두 이환된 환자는 7명(8.8%)였으며, 하악절치 브라켓에 두 종류의 세균이 모두 이환된 환자는 5명(6.3%)였다. 본 연구의 결과는 법랑질 탈회를 유발하는 치아우식증 원인균이 절치 브라켓에 부착하는 상주균일 가능성을 제시한다.

(주요 단어: 치아우식증 원인균, 검출, 메탈 브라켓, 자가중합효소연쇄소반응)

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