Distribution and Characterization of Serotype k *Streptococcus mutans*

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**Abstract**
*Streptococcus mutans*, consisting of serotypes *c*, *e* and *f* is an oral aciduric organism associated with the initiation and progression of dental caries. Recently a new serotype *k* *S. mutans* was proposed. The distribution of serotype *k* *S. mutans* was investigated in 244 subjects. The ratio of *S. mutans* and *Streptococcus sobrinus* against total streptococci was approximately 1–0.1%. Mutans streptococci were found in 212 subjects (86.9%), *S. mutans* in 74.2% and *S. sobrinus* in 12.7%. No mutans streptococci were found in 32 subjects (13.1%). The predominant serotype was *c* with 84.9% detection in *S. mutans*. Serotype *k* was found in only 3 subjects (1.4%): 1 of them had a coexisting serotype *c* strain and the other 2 were alone. Serotypes *d* and *g* of *S. sobrinus* were detected at equal percentages and those usually coexisted with *S. mutans* strains. Serotype *k* *S. mutans* isolates were confirmed by serological and genetical methods and characterized. The homologies of the *ngpF* gene sequence, which is related to the biosynthesis of serotype antigens, of isolates were 93–100% among serotype *k* type strains and 71% with serotype *c* to serotype *k* strains. Water-insoluble glucan synthesis from sucrose and artificial plaque formation of the serotype *k* strains were similar to those of the serotype *c* strain. However, acid production by the serotype *k* strains was comparatively lower than that of serotype *c*. A few years after the first investigation, serotype *k* *S. mutans* was confirmed to be maintained in the individual oral cavities of the same subjects by genetic analysis.

**Introduction**

*Streptococcus mutans* and *Streptococcus sobrinus* are the most cariogenic bacteria in humans (1). *S. mutans* is divided into 3 serotypes, *c*, *e* and *f* and *S. sobrinus* is divided into 2 serotypes *d* and *g*. Recently, serotype untypeable *S. mutans* strains were proposed and characterized as a new serotype *k* (2, 3). *S. mutans* serotype *k* strains were isolated from not only the oral cavity but also blood in infected endocarditis patients (4). The prevalence of the *c*, *e*, *f* and *k* strains in the oral cavity has been reported to be 78%, 17%, 3% and <2%, respectively (5). As for serotype *k* in cardiovascular specimens, the detection frequency was 12%, which was significantly higher than that for oral specimens from healthy subjects (2–5%). Thus, it was speculated that serotype *k* *S. mutans* may be associated with the development of cardiovascular diseases (6). Properties of serotype *k* *S. mutans* are lacking protein antigen (PA) and glucan-binding proteins (Gbps), and deduction of sucrose-dependent adhesion, dextran-binding and glucosyltransferase (GTF) activities (6–8).

The purpose of this study was to analyze the serotype distribution of strains isolated from Japanese subjects and whether serotype *k* strains were present.

**Materials and Methods**

**Subjects and clinical samples**

Saliva samples from 244 subjects between 2003 and 2005 were stocked at −80 °C and selected from the department of Oral Microbiology, School of Dentistry at Matsudo collection. This study was approved by the Ethics Committee of Nihon University
School of Dentistry at Matsudo (EC03–013, EC04–009 and EC05–003). Paraffin wax–stimulated saliva samples were collected in a sterile microcentrifuge tube from 244 volunteers. After sonication (50 W, 20 kHz, 30s, in ice), tenfold serial dilutions of each sample were prepared in 50 mM Tris–HCl buffer (pH 7.2) and appropriate dilutions were spread on Mitis Salivarius agar (MS, Becton–Dickinson), MS–MUTV (9) and MS–SOB (10) plates. The plates were cultured anaerobically at 37 °C for 3 days, and the colony–forming units (cfu) were calculated for total streptococci on MS, S. mutans on MS–MUTV and S. sobrinus on MS–SOB according to colony morphology with the aid of a microscope.

Identification of mutans streptococci from clinical samples.

Three colonies, which appeared to be mutans streptococci based on their colony morphology on each selective medium, per subject were subcultured to confirm the presence of mutans streptococci. For a different colony appearance in the same subject, three more colonies were subcultured. Pure cultures of each isolate were identified by: 1) colony morphology ; 2) an agar–gel immunodiffusion test using rabbit antisera raised against reference strains prepared in our laboratory as described previously (11, 12) ; 3) their fermentation pattern using the rapid ID 32 STREP (bio Merieux) for undecided strains ; and 4) PCR method using serotype–specific primers (2, 3).

Genetic analysis

Chromosomal DNA was extracted from bacterial cultures using a Promega Genome kit (Promega, Co.) according to the manufacturer’s instructions. The target regions of the genes in 16S rRNA and in biosynthesis of the serotype–specific polysaccharide were amplified according to previously described procedure (2, 3, 13). The amplicon size of the PCR products was confirmed by agarose gel electrophoresis after staining with ethidium bromide. The products were purified by Suprec–PCR™ (Takara, Co.), and sequencing with an ABI PRISM 3130 Genetic Analyzer using Big Dye Terminator v1.1 cycle sequencing kit (Life Technologies, Ltd.). Their 16S rRNA gene sequences and serotype–specific nucleotide were retrieved from DDBJ/EMBL/ GeneBank database searches.

Bacterial strains

S. mutans JC2, NUM–Smk 51, NUM–Smk 52 and NUM–Smk 89 were mainly used in this study. Bacteria were grown and maintained anaerobically at 37 °C on brain heart infusion (BHI, Becton–Dickinson) agar plates supplemented with 1% yeast extract (BHIY, Becton–Dickinson).

GTF preparation and assay

The bacteria were grown in M4 medium (14) at 37 °C for 20 h anaerobically. In the assay of GTF activity, the reaction mixture consisted of 1 ml of culture supernatant and 1 ml of 0.6 M acetate buffer (pH 5.5) containing 0.3 M sucrose. The mixture was incubated at 37 °C for 60 min.

Assay of plaque formation

The ability to form plaques in vitro was determined using a method described previously with some modifications (15). The bacteria were used to inoculate BHI broth containing 5% sucrose and were incubated anaerobically at 37 °C for 20 h at a 15° angle. The culture medium was then decanted. Four milliliters of phosphate–buffered saline (PBS) were added, and the tubes were then vortexed strongly for 5 s, decanted, and washed twice in 4 ml of PBS.

Acid production

Acid productions from glucose were examined using a method described previously, with some modifications (16). The bacteria were cultured at 37 °C for 20 h in BHI broth. The reaction mixture contained 0.4 ml of packed cells (approx. 1.5×10^10 cfu/ml) and 0.2 ml of 0.3 M glucose in Stephan’s buffer, and was incubated at 37 °C in a water bath with shaking. Aliquots of 25 µl were collected at timed intervals, and the pH was measured using a digital pH meter (Shindengen Co.).
Search into of serotype k S. mutans

Collection of clinical specimens from the same subjects with the detected serotype of k S. mutans a few years earlier was carried out with informed consent. Samples were treated by the same method as mentioned above.

Results
Detection of mutans streptococci

Fig. 1 shows the cfu numbers of total streptococci, S. mutans and S. sobrinus from subjects. Total streptococci were obtained at 4×10^7 cfu on average. S. mutans and S. sobrinus were detected at approximately 1% and 0.1% against total streptococci (Fig. 1). Table 1 shows the detection subject number of mutans streptococci. S. mutans was detected in 212 (86.9%) of 244 subjects and S. sobrinus in 31 (12.7%). Most S. mutans were detected alone, but S. sobrinus was usually coexistent with S. mutans. No mutans streptococci were found in the 32 subjects (13.1%).

Prevalence of mutans streptococci serotypes

Table 2 shows the prevalence of S. mutans and S. sobrinus serotypes from human oral samples. The most frequently occurring strains belonged to S. mutans serotype c. Eighty-two point five percent of subjects had serotype c as the predominant species. Serotype e and f were found in 14.6 and 4.7%, respectively. The new serotype k was also found in 3 subjects (1.4%). Serotypes d and g of S. sobrinus were found in 15 and 16 subjects, respectively.

Individual serotype distribution in 212 subjects with detected mutans streptococci

Subjects who showed a single serotype strain accounted for 78.3% and those who showed multiple serotypes strains 21.7%. Six individuals harbored S. mutans with different S. mutans serotypes. Two serotype k strains were pure, but, one was coexistent with the serotype c strain. One sample with only S. sobrinus detected showed serotype g. Serotypes d or g of S. sobrinus were each found in 15 subjects, coexisting with S. mutans. Fourteen subjects had 2 morphologically different types of S. mutans serotype c. One and 4 subjects had 2 types of morphological different S. sobrinus serotype d or g.
Table 3. Individual serotype distribution in 212 subjects

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<th>S. mutans</th>
<th>S. sobrinus</th>
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<tr>
<td>S. mutans alone</td>
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<tr>
<td>coexistent of c</td>
<td>136</td>
<td>23</td>
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<td>S. sobrinus alone</td>
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strains with the S. mutans serotype c, respectively. The coexistent of serotypes d and g of S. sobrinus was not found in any subjects.

Identification of the serotype k strain

Double immunodiffusion precipitation reaction patterns were obtained with anti serotype c, e and f serum tested against serotype-specific polysaccharides. The antigens of 3 isolates, designated as NUM-Smk 51, NUM-Smk 52 and NUM-Smk 89, did not react with these anti-sera. Each serotype antibody reacted with the corresponding antigen. These isolates by serological methods were identified as S. mutans by 16S rRNA gene sequencing. NUM-Smk 51, 52 and 89 were not amplified by serotype c, e and f-specific primers, but an amplicon band at 294 bp was obtained by PCR using a serotype k-specific primer (Fig. 2). The PCR products were further analyzed.

Fig. 3 shows the nucleotide alignment of rgpF gene sequences of S. mutans serotype k isolate strains compared with serotype c and k strains reported previously (4). The NUM-Smk 52 and NUM-Smk 89

![Figure 2](image-url)  
**Fig. 2.** PCR amplification of DNA of S. mutans using serotype-specific primers. A, serotype c (product size is 725 bp); B, serotype e (517 bp); C, serotype f (316 bp); D, serotype k (294 bp) specific primer. Lane 1, JCM5705 (serotype c); 2, NUM-Smk 51 (c); 3, NUM-Smk 52 (k); 4, NUM-Smk 89 (k); 5, LM7 (c); 6, OMZ717 (f); M, 100 bp ladder.

![Figure 3](image-url)  
**Fig. 3.** Multiple-sequence alignment of the serotype k-specific S' region in the rgpF gene. The nucleotide sequences and numbers from strain Xc (accession number AB010970) are shown. Dots indicate that the nucleotide is identical to that in strain Xc. Serotypes are indicated in parentheses. The data of TW295 and YT1 refer to 2.
strains were almost identical to the serotype k YT1 strain. The sequence of NUM-Smk 51 differed from that of strains NUM-Smk 52, and NUM-Smk 89 at 11 nucleotide positions in 240 nucleotide sequences.

Water-insoluble polysaccharide synthesis and artificial plaque formation

The water insoluble glucans from sucrose and plaque formations of the serotype k strains are shown in Fig. 4. The culture supernatant of a partially defined medium, M4, of serotype k strain produced a water-insoluble glucan after a 60 min incubation with sugar and was similar to that of the serotype c strain (Fig. 4A). The plaque formations that serotype k produced were similar to those of the serotype c strain (Fig. 4B).

Acid production

The time-course curves of pH reduction caused by S. mutans serotype c and strains are shown in Fig. 5. The pH of the reaction mixture decreased and reached critical pH (5.5) after incubation for 20 min in the serotype c strain. However, the pH reductions of the serotype k strains were very slow. The time taken to reach pH 5.5 in the serotype k strains was 40–45 min (data not shown).

Research into of serotype k S. mutans

Serotype k S. mutans were isolated from the same 3 subjects with detected serotype strains previously. All new isolates from each subject were identical to the serotype k S. mutans, as compared with the k strains isolated previously by rgpF gene sequence analysis.

Discussion

S. mutans is classified into serotypes c, e, f and k based on the chemical composition of serotype-specific polysaccharides. In general, the major serotype in the oral cavity is serotype c (approximately 70–80%), followed by serotype e (approximately 20%), while the prevalence of serotype f is low (<5%). The isolation frequencies of S. mutans serotypes c, e, and f in the present study were 79.7%, 14.3% and 4.6%, and were very similar to those reported previously. Serotype k has been found among strains isolated in Japan, Finland, Thailand and the UK (6, 17). Its distribution has been reported to range from 0 to 4% (2, 7). In this study, serotype k was isolated from 3 subjects (1.4%).

Serotype k strains showed low levels of sucrose-dependent adhesion, cellular hydrophobicity and dextran-binding ability (6, 17). Therefore, it is thought that serotype k strains have low cariogenicity. However, our results showed that the serotype k strains had the same levels of GTF activ-
ity and plaque formation, but a lower level acid production compared with the serotype c strain. Thus, these properties may depend on the serotype k strain.

The PCR method here will be useful for screening subjects with S. mutans serotype k strains. The 5' region of the rgpF gene (350 bp from the initial sequence) was shown to be specific for serotype k strains compared to the reference serotype c strains. PCR methods with primers constructed based on the rgp gene have been utilized for their rapid sensitive serotype k detection (4). However, this serotype k-specific primer set amplifies not only serotype k, but also serotype c, e and f of S. mutans DNA with minor bands. PCR using the serotype k primer may lead to false-positive results by the amplicons as serotype c, e, or f strains as the serotype k strain.

S. mutans is sometimes isolated from the blood of people with bacteremia or infective endocarditis (IE). IE is known to be initiated by an invasion of pathogenic bacteria into the bloodstream. The serotype k strains showed low levels of phagocytosis susceptibility due to a lack of PA or Gbps (6). Serotype k S. mutans was detected at a much higher frequency in the dental plaque specimens from the present subacute IE patients than in those from the non-IE patients, suggesting that those with IE may possess serotype k S. mutans in the oral cavity at a higher frequency (4). If they used a PCR method with a serotype k primer, the results could be false-positive. We think that it is important to identify subjects with serotype k strains in the oral cavity as they may carry a risk of IE caused by S. mutans.

In this study, the results confirmed the serotype k S. mutans was found at a very low level (1.2%) in the oral cavity.

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References


13. Takada K, Hirasawa M: Streptococcus orisuis sp. nov.