Differentiation of banding patterns between *Streptococcus mutans* and *Streptococcus sobrinus* isolates in rep-PCR using ERIC primer

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Background: *Streptococcus mutans* and *Streptococcus sobrinus* are considered to be important bacterial species in the initiation of human dental caries. Therefore, the establishment of a reliable genotyping method to distinguish *S. mutans* from *S. sobrinus* is of central importance.

Objective: We assessed the usefulness of repetitive extragenic palindromic polymerase chain reaction (rep-PCR) using ERIC primer banding patterns in differentiating *S. mutans* and *S. sobrinus*.

Design: Five *S. mutans* and two *S. sobrinus* prototype strains and 50 clinical isolates (38 *S. mutans* serotype *c*, 4 *S. sobrinus* serotype *d*, and 8 *S. sobrinus* serotype *g*) were examined. The banding patterns of amplicons generated were compared among the prototype strains and clinical isolates, to find common bands that distinguish *S. mutans* and *S. sobrinus*.

Results: Multiple banding patterns were seen with all strains tested. The representative strains of *S. mutans* tested revealed six unique, strong bands at 2,000 bp, 1,700 bp, 1,400 bp, 1,100 bp, 850 bp, and 250 bp, whereas *S. sobrinus* had seven strong bands at 2,000 bp, 1,800 bp, 1,100 bp, 900 bp, 800 bp, 600 bp, and 550 bp. The band at 1,100 bp was the only band that was observed in both *S. mutans* and *S. sobrinus*. Furthermore, most clinical *S. mutans* isolates revealed identical banding patterns. All *S. mutans* had amplicons at 1,700 bp, 850 bp, and 250 bp, whereas those of *S. sobrinus* were at 1,100 bp, 900 bp, and 800 bp.

Conclusions: These results indicate that using rep-PCR with the ERIC primers can distinguish between *S. mutans* and *S. sobrinus*.

Keywords: *rep-PCR*; *mutans streptococci*; *clinical isolates*; *dental caries*; *amplicons*

Dental caries is one of the most common worldwide infectious diseases (1), and the principal etiologic agents associated with developing initial dental caries in human are *Streptococcus mutans* and *Streptococcus sobrinus* (2). A variety of approaches for clonal characterizations of *S. mutans* isolates has revealed extensive genetic diversity (3, 4). Genotypic methods to discriminate strains of bacteria include arbitrary primed PCR, random amplified polymorphic DNA, pulsed field gel electrophoresis (PFGE), and repetitive extragenic palindromic PCR (rep-PCR). Among these, rep-PCR has been found to be a useful, effective, and relatively low cost method for discriminating and genotyping strains of *Escherichia coli* (5, 6), *Salmonella* sp. (7, 8), *Streptococcus pneumonia* (9, 10), and other bacterial species. Various primers have been identified and commonly used for rep-PCR such as the ERIC primers (5) that were developed for *E. coli*, but due to the ubiquitous nature of repetitive sequences, many are widely applicable to other species. This easy and rapid method is based on repetitive

sequences within bacteria and is widely applied to investigate taxonomy or epidemiology. However, this technology has not been vigorously applied to *S. mutans* genotyping. A recent report showed that rep-PCR with distinct α-hemolytic streptococci using (GTG)$_5$ primer was a fast and reliable method for identification of *S. mutans* (11). Other studies have reported that rep-PCR and PFGE result in similar conclusions (3). The purpose of this study is to determine the usefulness of rep-PCR with ERIC primers to differentiate between *S. mutans* and *S. sobrinus* using representative prototype strains and clinical isolates.

Materials and methods

Bacterial strains

The study protocol was approved by the Ethics Board of Nihon University School of Dentistry at Matsudo (EC03-011). *S. mutans* serotype *c* (JC2, PS14, and JCM5705), *e* (LM7), *f* (OMZ175), and *S. sobrinus* serotype *d* (OMZ176 and B13), and *g* (GTC278 and 6715) were used as representative prototype strains and were obtained from ATCC. Frozen (−80°C) stocks of clinical isolates were stored by the Department of Oral Microbiology at Nihon University School of Dentistry at Matsudo. All clinical isolates used in this study were randomly selected from *S. mutans* serotype *c*, *S. sobrinus* serotype *d*, and *g* (by serological tests) isolates obtained from plaque samples using standard methodology.

DNA isolation

Genomic DNA was isolated from cultures grown in 10 ml of Brain Heart Infusion broth (Becton Dickson and Company, Franklin Lakes, NJ, USA) at 37°C in candle jars for 24 hours, using a commercially available DNA extraction kit (QIAamp DNA Mini Kit, Qiagen, CA, USA) and some modifications from the manufacturer's instructions. Briefly, bacteria were harvested by centrifugation, and then 180 μl of lysozyme (20 mg/ml of lysozyme in 20 mM of Tris–HCl) and 50 units of mutanolysin (Sigma-Aldrich Co., St. Louis, MO, USA) were added, followed by overnight incubation at 37°C to lyse cells. The quality and quantity of DNA samples were confirmed with a Nano Drop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA) at 260/280 nm. The concentration of extracted DNA was adjusted to approximately 400 ng/μl and stored at −30°C until analyzed.

Repetitive extragenic palindromic PCR

DNA was used to perform rep-PCR with ERIC primers using the manufacturer's protocol. The primer pair ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC 2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) was used. The reaction mixture contained 10 μl of buffer components (FailSafe PCR PreMix, EPICENTER Technologies, Madison, WI, USA), 100 p mol of each primer, 2.5 U of enzyme (FailSafe PCR Enzyme Mix, EPICENTER Technologies, Madison, WI, USA), and 2 μl of DNA solution made up to 20 μl final volume. All amplification reactions were conducted in a Gene Amp 9700 thermal cycler (Applied Biosystems, Carsbad, CA, USA). The initial denaturing temperature was 95°C for 5 min, followed by 30 cycles of 95°C for 3 min, 48°C for 3 min, and 72°C for 2 min, with a final extension at 72°C for 7 min.

Electrophoresis

Approximately 20 μl of the postamplification reactions were separated by electrophoresis in a 2% agarose gel (Agarose, USB, Cleveland, OH, USA) in Tris–Borate–EDTA (TBE) buffer, stained with an ethidium bromide solution (10 μg/ml), and photo documented under UV light (Image Saver AE-6905C, ATTO, Tokyo, Japan). An optimized comparative study of the diversity of the obtained banding patterns showed the presence or absence of repetitive elements using photographic images.

Results

### The differentiation of banding patterns between S. mutans and S. sobrinus representative strains

The banding patterns obtained following rep-PCR of *S. mutans* JC2, PS14, JCM5705, LM7, OMZ175, and *S. sobrinus* OMZ176 and 6715 are shown in Fig. 1. *S. mutans* rep-PCR resulted in amplicons with sizes that ranged from 250 bp to 2,000 bp. *S. mutans* strains had six strong bands at 2,000 bp, 1,700 bp, 1,400 bp, 1,100 bp, 850 bp, and 250 bp, except OMZ175 that was missing the 1,400 bp band. Additionally, with JCM5705, the band at 1,100 bp was weaker than that was seen with other *S. mutans* strains. However, *S. sobrinus* had distinctly different banding patterns when compared with the *S. mutans* strains. *S. sobrinus* yielded seven bands at 2,000 bp, 1,800 bp, 1,100 bp, 900 bp, 800 bp, 600 bp, and 550 bp, except OMZ176 that was missing the band at 2,000 bp (Fig. 1).

Comparison of rep-PCR fingerprint banding patterns from clinical isolates

Common banding patterns were observed within the *S. mutans* clinical isolates (Fig. 2). Although the intensity of banding varied, most clinical isolates had the six strong bands that were seen with the representative strains (i.e. 2,000 bp, 1,700 bp, 1,400 bp, 1,100 bp, 850 bp, and 250 bp). The clinical isolates 1, 18, 23, 26, 27, and 31 were missing the band at 2,000 bp, isolates 1, 2, 25, 28, 29, and 30 were missing the 1,100 bp band, and isolates 3, 10, 16, 20, 25, 28, 29, 30, 32, 33, and 38 were missing the 1,400 bp band. All the isolates had bands at 1,700 bp, 850 bp,
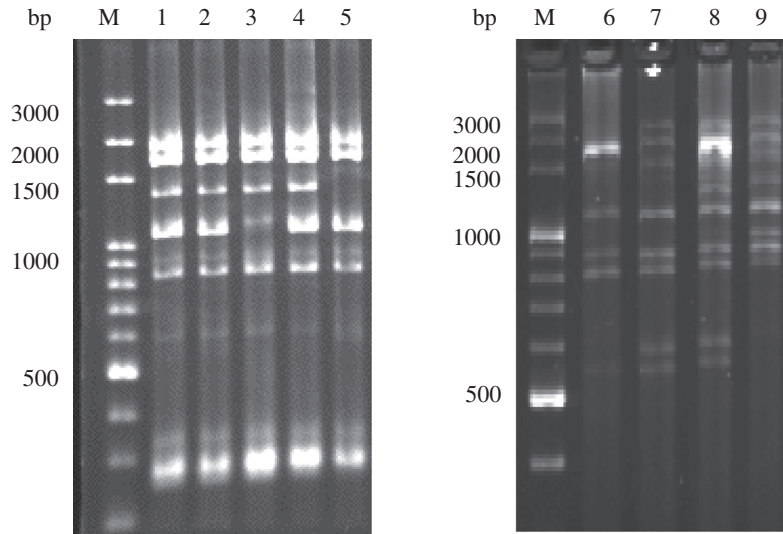
Fig. 1. Photographic images of gel banding patterns for *S. mutans* and *S. sobrinus* representative prototype strains. Lanes M, 100 bb ladder; 1, *S. mutans* JC2 (serotype *c*); 2, PS14 (*c*); 3, JCM5705(*c*); 4, LM7 (*e*); 5, OMZ175 (*f*); 6, *S. sobrinus* OMZ176 (*d*); 7, B13(*d*); 8, GTC278 (*g*); 9, 6715 (*g*).
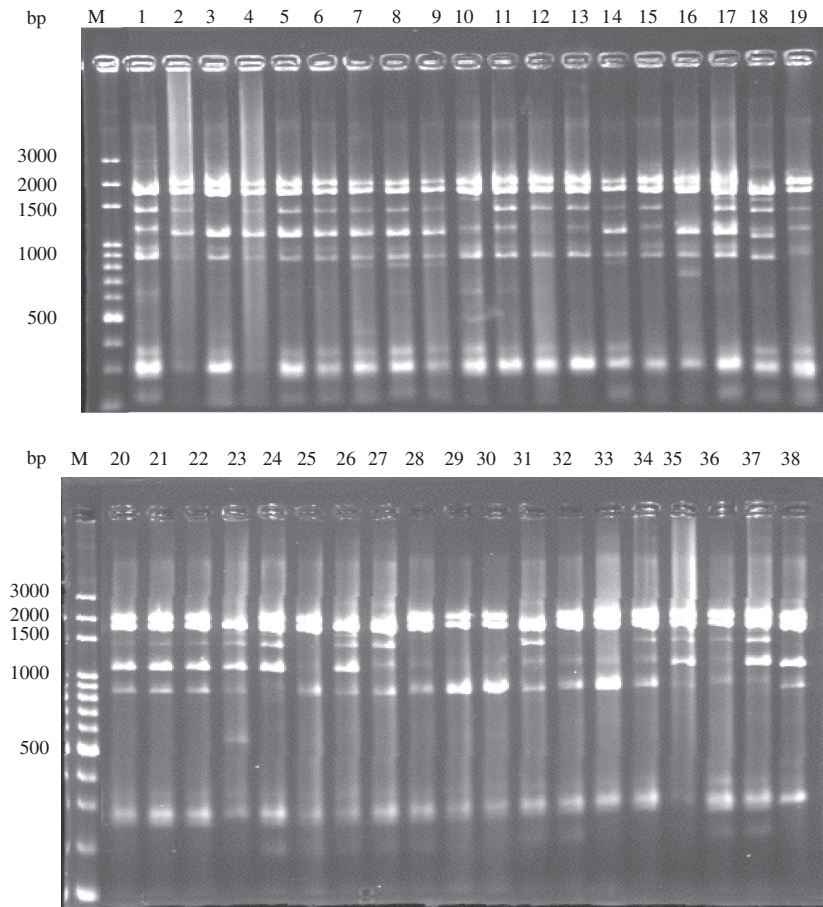
Fig. 2. The photographic image of gel banding patterns for *S. mutans* serotype *c* clinical isolates. Lanes 1–38, *S. mutans* (*c*) clinical isolates; M, 100 bp ladder.

and 250 bp. Figure 3 shows the patterns from *S. sobrinus* serotype *d* and *g* clinical isolates. Isolates 4 and 9 were missing the 550 bp band. Isolates 10, 12, 13, 14, and 15 were missing the bands at 600 bp and 550 bp. All the isolates had bands at 1,100 bp, 900 bp, and 800 bp.

Discussion

S. mutans and *S. sobrinus* are members of the mutans streptococci (MS). Although there are seven serotypes that compose the MS, *S. mutans* (serotypes *c*, *e*, and *f*) and *S. sobrinus* (serotypes *d* and *g*) are the only MS that have been found in humans (4). Therefore, a technique that can easily distinguish *S. sobrinus* from *S.mutans* would be useful for analysis of human oral samples.

In the process of examining the feasibility of an easy and reliable rep-PCR genotyping system for *S. mutans*, we used the common ERIC primers. Before we performed rep-PCR using ERIC primers, six different primers sets, i.e. BOX, ERIC, SERA, REP, M13, and $(GTG)_5$ primers, (Hokkaido system science, Sapporo, Hokkaido, Japan), enzyme, and buffer components (EPICENTER Technologies, TOYOBO, Promega, Takara and others) were screened for rep-PCR results using the manufacturer's protocol. The BOX primers were designed for not only analysis of *S. pneumoniae* strains but also to amplify a small number of bands from *S. mutans*, but not other oral streptococci (9). However, we did not observe any *S. mutans* PCR amplicons with BOX or REP primers (data not shown). Alam et al. compared ERIC, SERA, and REP primers with oral streptococci but did not evaluate *S. mutans*. They concluded that there

was no evidence of banding patterns or individual bands among α-hemolytic streptococci (4). Švec et al. investigated rep-PCR using $(GTG)_5$ primer for *S. mutans* and observed banding patterns. They concluded that using $(GTG)_5$ primer is one of the reliable methods for strain typing of *S. mutans* (11). However, we were not able to reproduce their findings using $(GTG)_5$, SERE, and M13 primers (data not shown). ERIC primers have been used for molecular-typing studies and clonal discriminations with both Gram-negative and Gram-positive bacteria (6). Indeed, there is no report that investigates *S. mutans* with rep-PCR using ERIC primers.

We examined *S. mutans* and *S. sobrinus* representative strains and serotype *c*, *d*, and *g* clinical isolates that are the most common serotypes in human decay (1) by rep-PCR using ERIC primers. Six strong bands at 2,000 bp, 1,700 bp, 1,400 bp, 1,100 bp, 850 bp, and 250 bp were observed in all *S. mutans* serotype *c* and *e* representative strains and clinical isolates, with some exception (Figs 1 and 2). The serotype *f* strain lacked the 1,400 bp band. Some *S. mutans* clinical isolates lacked a band at 2,000 bp, 1,400 bp, or 1,100 bp. All isolates and representative strains of *S. mutans* had bands at 1,700 bp, 850 bp, and 250 bp that might be specific bands for identification of *S. mutans*. Differentiation of *S. mutans* serotypes by rep-PCR using ERIC primers is at present not completely resolved, especially because serotype *f* representative strains were more variable than the other prototype strains evaluated. A clear differentiation of prototype strains and clinical isolates based on consistent specific banding patterns is necessary to validate this approach in
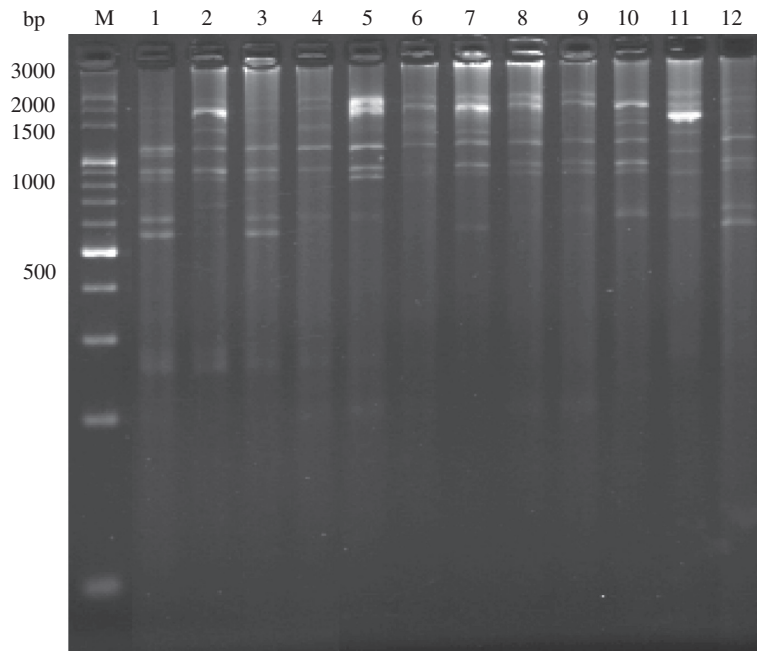
Fig. 3. The photographic image of gel banding patterns for *S. sobrinus* serotype *d* and *g*. Lanes 1–4, *S. sobrinus* (*d*) clinical isolates; 5–12, *S. sobrinus* (*g*) clinical isolates; M, 100 bp ladder.

specific differentiation. On the other hand, *S. sobrinus*, except OMZ176, had seven strong bands at 2,000 bp, 1,800 bp, 1,100 bp, 900 bp, 800 bp, 600 bp, and 550 bp. Almost all *S. sobrinus* clinical isolates had amplicons at 1,100 bp, 900 bp, and 800 bp. Therefore, the bands at 1,100 bp, 900 bp, and 800 bp could be the focus for differentiating between *S. mutans* and *S. sobrinus*. Among these bands, the band at 1,100 bp may be a common band for *S. mutans* and *S. sobrinus*. The results presented herein are from a method that was found to be most optimal for obtaining bands from amplicons generated with ERIC primers from *S. mutans* and *S. sobrinus*. Other primer sets concentrations of enzyme components and thermal cycles conditions were evaluated in effort to obtain the results presented (data not shown).

Compared with PFGE or multiple locus sequence typing, rep-PCR has less-defined results; however, we found that compared to other methods, rep-PCR with ERIC primers was able to differentiate representative as well as clinical *S. mutans* and *S. sobrinus* isolates. Due to a lack of serotype *e*, *f*, and non-streptococci clinical isolates, we were unable to analyze their banding patterns. Further studies to evaluate the banding patterns of these serotypes for comparison will follow. Using computer software to obtain dendrograms might be a more efficient and reproducible method to identify banding patterns of strains within these two species. Even though we did not use computer software to make dendrograms, we were able to distinguish between *S. mutans* and *S. sobrinus*. More detailed experimental research will be necessary to analyze the sequences of primers that obtain the strongest bands.

The slight difference in banding pattern sometimes seen with the same extracted DNA, ERIC primers, buffer, and enzyme amplifications may be due to the low specificity of primers, not only the ERIC but also other rep-PCR primers. Nevertheless, this technique is able to distinguish *S. mutans* from *S. sobrinus*. Thus, the demonstrated presence or absence of particular repetitive elements will be useful in genotyping *S. mutans* and *S. sobrinus*.

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There is no conflict of interest in the present study for any of the authors.

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