Method for rapid identification of oral streptococci by PCR using 16S-23S ribosomal RNA intergenic spacer gene

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Abstract Mutans group streptococci play an important role in the formation of dental caries, while mitis group streptococci are considered to be associated with subacute bacterial endocarditis. Both mitis and salivarius group streptococci, which are early colonizers in the human oral cavity, have an effect on the infection and colonization of mutans streptococci. In the present study, a rapid and exact PCR method to detect those oral streptococci was developed using the 16S-23S ribosomal RNA intergenic spacer gene, which is present between 16S rRNA and 23S rRNA within the RNA operon. The PCR analysis was able to correctly identify mutans and mitis group streptococcal species. Our results indicate that the combined PCR analysis method established in the present study is useful for detecting oral streptococcal species, as well as in clinical applications to predict and prevent dental caries and etiological studies of streptococcal infective endocarditis.

Key words

16S-23S rRNA intergenic spacer gene,
Oral streptococci,
PCR,
Species identification

Introduction

Bacteria of the genus *Streptococcus* are subdivided into major phylogenetic groups, which are the pyogenic, anginosus, bovis, salivarius, mitis, and mutans groups. Among them, some species of mutans group streptococci (*Streptococcus mutans* and *Streptococcus sobrinus*), mitis group streptococci (*Streptococcus mitis*, *Streptococcus sanguinis*, *Streptococcus oralis*, and *Streptococcus gordonii*), and salivarius group streptococci (*Streptococcus salivarius*, and others) are commonly found in the oral cavity. Further, mitis group streptococci are the most frequently isolated from the blood of patients with infective endocarditis¹⁾, while mutans group streptococci have been implicated as the prime causative organisms of human dental caries²⁾. Since both mitis and salivarius group streptococci are

earlier colonizers in the human oral cavity³⁾, they likely have an effect on the infection and colonization of mutans streptococci. Although the cariogenic potential of mitis group streptococci is low, those species may play an antagonistic role in the development of dental caries⁴⁾. The prevalence of mitis group streptococci has been shown to be different between caries-active and caries-inactive individuals⁵⁾, therefore, detection and identification of mutans and mitis group streptococci among the oral flora may be important for not only predicting and preventing dental caries but also the association with bacterial endocarditis.

The 16S-23S ribosomal RNA intergenic spacer (16S-23S spacer) gene is present between the 16S and 23S ribosomal RNA genes. This region is largely non-functional, thus it is likely to contain sequence variations from which taxonomically useful information might be obtained. Polymorphisms in the length and sequence of the 16S-23S spacer have been used for strain identification of *Streptococcus*

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Table 1 PCR Primers used in this study

Target	Name	Primer sequence	Annealing temp. (°C)
Universal	TI-F	5'GAAGTCGTAACAAGGTAGCCGT3'	60
16S-23S spacer	TI-R	5'TGCCAGGGCATCCACC3'	
<i>S. mutans</i>	MUT-F	5'CTCCTTTCTAAGGAAAAACGCA3'	60
16S-23S spacer	MUT-R	5'TGAACTCCAGACTGACTTATTAGAAAA3'	
<i>S. sobrinus</i>	SOB-F	5'CAATTACTTAGTAACGGAAGCACGTT3'	60
16S-23S spacer	SOB-R	5'TTTGAGAATAGTTGAACTCCGTATCCT3'	
<i>S. sanguinis</i>	SAN-F	5'CACACGGAATGCACTTGAGTC3'	65
16S-23S spacer	SAN-R	5'CTAGTCAAAGTAGATAATGGAGCCT3'	
<i>S. oralis</i> and <i>S. mitis</i>	O/M-F	5'AGGATAAGGAACTGCACATTGGTC3'	68
16S-23S spacer	O/M-R	5'TGCATTACTTGGTGATCTCTCACC3'	
<i>S. gordonii</i>	GOR-F	5'AAACGGAATGCACGATGGAGTCTTG3'	64
16S-23S spacer	GOR-R	5'CTCATTAATATTACAGAGTTTCGG3'	

anginosus and *Streptococcus constellatus* belonging to “milleri group” streptococci⁶⁾. However, few sequence data for the 16S-23S spacer from mutans and mitis group streptococci in samples from human oral cavities are available.

The purpose of this study was to analyze the 16S-23S spacer genes from oral streptococci, in order to design species specific polymerase chain reaction (PCR) primers based on the sequence data, and to develop useful methods to detect and identify oral streptococcal species in the human oral cavity.

Materials and Methods

Bacterial strains

The bacterial strains used in this study were *S. mutans* MT8148 (serotype *c*), GS5 (*c*), MT4245 (*e*), MT4467 (*e*), MT4251 (*f*), and OMZ175 (*f*), *S. sobrinus* OMZ176 (*d*), B13 (*d*), 6715 (*g*), and MT10186 (*g*), *S. salivarius* NCTC 8618, and HHT, *S. sanguinis* ATCC 10556, and ST3, *S. oralis* ATCC 10557, and SK23, *S. gordonii* ATCC 10558, and SK51, and *S. mitis* SK24, and ATCC 903, which came from our culture collection. Organisms were routinely cultured in brain heart infusion broth (Difco Laboratories, Detroit, Mich., USA).

Sequence analysis of the 16S-23S rRNA intergenic spacer genes from oral streptococci

For sequencing of the 16S-23S spacer genes, primers TI-F and TI-R (Table 1) were designed from the conserved region of 16S rRNA⁷⁾ and 23S rRNA⁶⁾.

Preparation of chromosomal DNA from oral streptococci was performed as described previously⁸⁾. PCR amplification was performed using chromosomal DNA from each oral streptococcal strain as a template. The PCR product was prepared using a Centricon 100 spin column (Millipore, Bedford, Mass., USA) for a DNA-sequencing template. A dideoxy dye termination reaction was performed using an ABI PRISM cycle sequencing kit (Applied Biosystems, Foster City, Calif., USA) in a GeneAmp 2400 thermal cycler (Applied Biosystems), following manufacturer's protocols. The product was then analyzed using an automated DNA sequencer model 373 (Applied Biosystems).

Sequence analysis and design of primers

Analyses of sequence data and the multiple-sequence alignment were performed with nucleic acid sequence and protein sequence analysis software, GeneWorks (IntelliGenetics Inc., Mountain View, Calif., USA). The primers were designed with the help of PrimerExpress software (Applied Biosystems).

PCR condition and agarose gel electrophoresis

PCR assays of the 16S-23S spacer genes were carried out in 20 μ l of reaction mixture containing 0.5 U AmpliTaq Gold DNA polymerase (Applied Biosystems), 0.5 μ M of oligonucleotide primers, 10 ng of oral streptococcal chromosomal DNA, and 1.5 mM MgCl₂, following the manufacturers' protocols. Amplification was performed with the GeneAmp 2400 thermal cycler using the following

		16S Ribosomal RNA										>	
<i>S. mutans</i>	<u>GAAGTCGTAA</u>	<u>CAAGGTAGCC</u>	<u>GTATCGGAAG</u>	GTGCGGCTGG	ATCACCTCCT	<u>TTCTAAGGAA</u>	<u>AAAC---GCA</u>	-----	-AAG-----C	ACAT-----T	76		
<i>S. sobrinus</i>	<u>GAAGTCGTAA</u>	<u>CAAGGTAGCC</u>	<u>GTATCGGAAG</u>	GTGCGGCTGG	ATCACCTCCT	<u>TTCTAAGGAT</u>	<u>AAACTAAGTA</u>	ACGAAGAGCT	TAAGAAATCC	GTATGAAAT	100		
<i>S. salivarius</i>	<u>GAAGTCGTAA</u>	<u>CAAGGTAGCC</u>	<u>GTATCGGAAG</u>	GTGCGGCTGG	ATCACCTCCT	<u>TTCTAAGGAA</u>	<u>AAAC-----</u>	-----	-----	-----	64		
<i>S. sanguinis</i>	<u>GAAGTCGTAA</u>	<u>CAAGGTAGCC</u>	<u>GTATCGGAAG</u>	GTGCGGCTGG	ATCACCTCCT	<u>TTCTAAGGA-</u>	-----	-----	---G---TCC	GTA-----	66		
<i>S. gordonii</i>	<u>GAAGTCGTAA</u>	<u>CAAGGTAGCC</u>	<u>GTATCGGAAG</u>	GTGCGGCTGG	ATCACCTCCT	<u>TTCTAAGGAA</u>	<u>AAAC-----</u>	-----	-----	-----	64		
<i>S. oralis</i>	<u>GAAGTCGTAA</u>	<u>CAAGGTAGCC</u>	<u>GTATCGGAAG</u>	GTGCGGCTGG	ATCACCTCCT	<u>TTCTAAGGAT</u>	<u>AA-----</u>	-----	-----	-----	62		
<i>S. mitis</i>	<u>GAAGTCGTAA</u>	<u>CAAGGTAGCC</u>	<u>GTATCGGAAG</u>	GTGCGGCTGG	ATCACCTCCT	<u>TTCTAAGGAA</u>	<u>AA-----</u>	-----	-----	-----	62		
<i>S. mutans</i>	AGGGGA----	-----	-----	-----	-----	-----	-----	-----	--ACTT----	-----	89		
<i>S. sobrinus</i>	AGGGGACGCG	CAGAAAATCC	TGATTTTCTA	GGACGGCCAT	CTTTTTCCTA	GGATTTTAGT	CTGAGTACAA	TTACTTAGTA	ACGGAA-GCA	CGTTTCGGGG	199		
<i>S. salivarius</i>	-----	-----	-----	-----	-----	-----	-----	-----	---GAAATGTA	C---TTGAGT	79		
<i>S. sanguinis</i>	-----	-----	-----A	GGAC-----	-----	-----	-----	-----	--AC-----	ACGGAATGCA	C---TTGAG-	89	
<i>S. gordonii</i>	-----	-----	-----	-----	-----	-----	-----	-----	---GGAATGCA	CG-ATGGAG-	80		
<i>S. oralis</i>	-----	-----	-----	-----	-----	GGA---A-	CT-GCACA-	---TT---	--GG-----	-----	77		
<i>S. mitis</i>	-----	-----	-----	-----	-----	GGA---A-	CT-GCGCA-	---TT---	--GG-----	-----	78		
<i>S. mutans</i>	--CTTGTGTTA	GTTTGTAGAG	GGT-AAGTGG	GGCCTTAGCT	CAGCTGGGAG	AGCGCCTGCT	TTGCACGCAG	GAGGTCACGC	GTTTCGATCCC	GCTAGGCTCC	183		
<i>S. sobrinus</i>	ACGCTATTTA	GTTTTCAGAG	GTT-TTGTTG	GGCCGTAGTT	CAGGTGGGAG	AGCGCCTGCT	TTGCACGCAG	GAGGTCACGC	GTTTCGATCCC	GCTAGGCTCC	298		
<i>S. salivarius</i>	TTCTTATTTA	GTTTGTAGAG	GTC-TTGTTG	GGCCTTAGCT	CAGCTGGGAG	AGCGCCTGCT	TTGCACGCAG	GAGGTCACGC	GTTTCGATCCC	GCTAGGCTCC	178		
<i>S. sanguinis</i>	-TCTTGTGTTA	GTTTGTAGAG	GGCTATGTGG	GGCCTTAGCT	CAGCTGGGAG	AGCGCCTGCT	TTGCACGCAG	GAGGTCACGC	GTTTCGATCCC	GCTAGGCTCC	188		
<i>S. gordonii</i>	-TCTTGTGTTA	GTTTGTAGAG	GTC-TTGTTG	GGCCTTAGCT	CAGCTGGGAG	AGCGCCTGCT	TTGCACGCAG	GAGGTCACGC	GTTTCGATCCC	GCTAGGCTCC	178		
<i>S. oralis</i>	-TCTTGTGTTA	GTCTTGAGAG	GTC-TTGTTG	GGCCTTAGCT	CAGCTGGGAG	AGCGCCTGCT	TTGCACGCAG	GAGGTCACGC	GTTTCGATCCC	GCTAGGCTCC	175		
<i>S. mitis</i>	-TCTTGTGTTA	GTCTTGAGAG	GTC-TTGTTG	GGCCTTAGCT	CAGCTGGGAG	AGCGCCTGCT	TTGCACGCAG	GAGGTCACGC	GTTTCGATCCC	GCTAGGCTCC	175		
<i>S. mutans</i>	ATTAATAACAG	GATACTAACA	GACGGTTCGC	TTGCGAATAC	TTCTGTAGAA	GATTATCTAT	<u>TTTCTAAGA</u>	<u>AGTCAGTCTG</u>	<u>GAGTTCAACT</u>	TCATATCTTG	283		
<i>S. sobrinus</i>	ATTA-----G	GATAC-----	-----	-----	-----	-----	-----	-----G	GAGTTCAACT	--AT-TCT--	324		
<i>S. salivarius</i>	ATT-----	-----	-----	---GAA---	-TC-----	GA-----	-----	---AAGA	-----	---TTCAA--	197		
<i>S. sanguinis</i>	ATTA-----	-----	-----	-----	-----	-----	-----	-----	-----	-----TCTAG	197		
<i>S. gordonii</i>	ATT-----G	GA-----	-ACG-----	-----	---GAA	G-----	---TTC-----	---CA-----	---AACT-----	-----	200		
<i>S. oralis</i>	ATT-----	-----	---GG-----	-----	---TG-AG-A	GATCACC--	-----	-----	-----	-----	192		
<i>S. mitis</i>	ATT-----	-----	---GG-----	-----	---TG-AG-A	GATCACC--	-----	-----	-----	-----	192		
<i>S. mutans</i>	TTTAAGACAA	GGAAGTCTAT	AAAATCCACT	TAGGATATGT	TAAGTATCCT	A-----	GA---G---	ATGGTTCATT	GACAATTGAA	TAGCTA-GTA	366		
<i>S. sobrinus</i>	-----CAA	AG-AG-C---	-AGTGCACC	-----T	T---TATCCT	AAGCGCAAC	GACGTTGCCA	ATTGTTTCATT	GAAAATTGAA	TATCTA--TC	396		
<i>S. salivarius</i>	-----	-----	-----	-----	-----	-----	---GT---	ATTGTCCATT	GAAAATTGAA	TATCTATATC	229		
<i>S. sanguinis</i>	TTT--GAC-	-----	-----	TA-GATA---	-----	AAG---AAC	-----	---TTGTCATT	GAAAATTGAA	TACCGATATC	244		
<i>S. gordonii</i>	-----	-----	-----	-----	-----	-----	-----	A--GTCCATT	GAAAATTGAA	TACTATATATC	228		
<i>S. oralis</i>	-----	--AAGT---	--AATGCA-	-----	-----	-----	-----	---CATT	GAAAATTGAA	TATCTATATC	226		
<i>S. mitis</i>	-----	--AAGT---	--AATGCA-	-----	-----	-----	-----	---CATT	GAAAATTGAA	TATCTATATC	226		
		< 23S ribosomal RNA											
<i>S. mutans</i>	AATAAGGTT-	AAGTT-AATA	AGGGCGCACG	<u>GTGGATGCCC</u>	<u>TGGCA</u>	478							
<i>S. sobrinus</i>	AATAAGGTT-	AAGTTCAATC	AAGGCGCACG	<u>GTGGATGCCC</u>	<u>TGGCA</u>	498							
<i>S. salivarius</i>	AATAAGGTT-	AAGTT-AATA	AGGGCGCACG	<u>GTGGATGCCC</u>	<u>TGGCA</u>	363							
<i>S. sanguinis</i>	AATAAGGTT-	AAGTT-AGTA	AGGGCGCACG	<u>GTGGATGCCC</u>	<u>TGGCA</u>	354							
<i>S. gordonii</i>	AATAAGGTT-	AAGTT-AGTA	AGGGCGCACG	<u>GTGGATGCCC</u>	<u>TGGCA</u>	336							
<i>S. oralis</i>	AATAAGGTTG	AAGTT-AATA	AGGGCGCACG	<u>GTGGATGCCC</u>	<u>TGGCA</u>	337							
<i>S. mitis</i>	AATAAGGTT-	AAGTT-AAT-	AAGGCGCACG	<u>GTGGATGCCC</u>	<u>TGGCA</u>	338							

Fig. 1 Multiple alignment of 16S-23S ribosomal RNA intergenic spacer sequence of oral streptococci

As no inter-strain differences were observed among the tested strains, only 1 sequence is shown. The nucleotide sequences have been deposited in the DDBJ as follows: *S. mutans* strains MT8148, AB050989; MT4245, AB050988; MT4251, AB050990; *S. sobrinus* strains 6715, AB051014; OMZ176, AB051015; *S. salivarius* NCTC 8618, AB051016; *S. sanguinis* ATCC 10556, AB051017; *S. oralis* ATCC 10557, AB051018; *S. gordonii* ATCC 10558, AB051019; and *S. mitis* SK24, AB051020. Gaps are indicated by hyphens and the sequences of the designed PCR primers are underlined. End point of 16S ribosomal RNA and start point of 23S ribosomal RNA sequence was indicated by ">" and "<", respectively.

parameters; a preincubation step at 95°C for 9 minutes, followed by 30 cycles of a denaturing step at 94°C for 30 seconds, and a primer-annealing and extension step at 60°C to 68°C for 1 minute. The primer-annealing and extension temperature was changed with every primer set (Table 1). The PCR products were visualized by 2.0% agarose gel electrophoresis using ethidium bromide staining. For molecular weight standards, a 100bp DNA

ladder (Promega, Madison, Wis., USA) was used.

Nucleotide sequence accession numbers

The nucleotide sequences of the 16S-23S rRNA intergenic spacer gene have been deposited in the DDBJ database as follows: *S. mutans* strains MT8148, AB050989; MT4245, AB050988; MT4251, AB050990; *S. sobrinus* strains 6715, AB051014; OMZ176, AB051015; *S. salivarius* NCTC 8618,

Fig. 2 PCR amplification of 16S-23S ribosomal RNA intergenic spacer genes

PCR amplification was performed with the primer sets from whole 16S-23S ribosomal RNA genes (A), as well as species-specific primer sets against *S. mutans* (B), *S. sobrinus* (C), *S. sanguinis* (D), *S. oralis* and *S. mitis* (E), and *S. gordonii* (F). Lanes: M, 100bp molecular weight marker; 1, *S. mutans*; 2, *S. sobrinus*; 3, *S. salivarius*; 4, *S. sanguinis*; 5, *S. oralis*; 6, *S. gordonii*; and 7, *S. mitis*.

AB051016; *S. sanguinis* ATCC 10556, AB051017; *S. oralis* ATCC 10557, AB051018; *S. gordonii* ATCC 10558, AB051019; and *S. mitis* SK24, AB051020.

Results

DNA sequence analyses of the 16S-23S spacer genes

The DNA sequences of the 16S-23S spacer genes from *S. mutans*, *S. sobrinus*, *S. salivarius*, *S. sanguinis*, *S. oralis*, *S. gordonii*, and *S. mitis* found to be 388 bp, 407 bp, 273 bp, 264 bp, 246 bp, 246 bp, and 249 bp, respectively. There was no significant difference among serotype *c/e/f* of *S. mutans* and *d/g* of *S. sobrinus* strains, and no inter-strain difference was observed in the tested oral streptococci (data not shown). The multiple alignment revealed that the 16S-23S spacer genes were different among the species except between *S. oralis* and *S. mitis*, which were shown to be nearly identical (Fig. 1).

Design of specific primers using the 16S-23S spacer genes

The aligned sequences of the 16S-23S spacer genes were manually compared, and species-specific primers based on the alignment of the 16S-23S spacer genes

were designed and synthesized, as shown in the Table 1 and Fig. 1. However, the species-specific region of the 16S-23S spacer gene from *S. salivarius* was too short to design a species-specific PCR primer set.

PCR amplification of the 16S-23S spacer genes

Figure 2 shows representative results of PCR amplification of the 16S-23S spacer genes. Using primers based on the 16S-23S spacer gene, species-specific amplification was observed for all of the species, except for *S. salivarius*. PCR products were amplified from both *S. oralis* and *S. mitis* chromosomal DNA with the primer set of O/M-F and O/M-R, which was against the identical 16S-23S spacer genes of *S. oralis* and *S. mitis*. Each species-specific amplification was confirmed by direct sequencing of the amplicon. The concentration of streptococcal DNA available for the PCR analysis was approximately 50 pg/ml.

Discussion

In the present study, we designed primers corresponding to the 16S-23S spacer genes to enable identification of oral streptococci using a simple

and reliable PCR method. Species identification of streptococci is usually determined from phenotypic characteristics such as colony morphology, hemolysis, carbohydrate fermentation, and serological properties⁹). Since the biochemical, physiological, serological characteristics of mitis group streptococci are very similar⁹), classification of those strains has been difficult. In addition, many nucleic acid-based typing methods have been applied to studies of the epidemiology^{10,11}), including restriction endonuclease analysis¹²), probe-based fingerprinting^{13,14}), and PCR-based fingerprinting^{15,16}). PCR methods offer several advantages over other nucleic acid-based typing methods, such as: speed, the need for fewer cells, and protocols that involve fewer steps.

Although DNA sequencing is the best method of quantitatively determining bacterial taxonomy, it requires an expensive automated DNA sequencer apparatus and complex experimental procedures. A comparison of 16S rRNA sequences is well established as a standard method for the identification of bacterial species, genera, and families, though it may not be useful for subtyping. Since the homology of 16S rRNA sequences among oral streptococcal species is more than 96%⁷), it is impossible to design a species-specific PCR primer for that region.

The 16S-23S spacer gene is a stretch of DNA that lies between the 16S and 23S rRNA region, and is under minimal selective pressure because of its non-coding function, and should have accumulated a higher percentage of mutations than rRNA genes¹⁷). Therefore, it may be useful for accurate identification of species, due to the enhanced variations between species within a genus.

The multiple sequence alignment of the 16S-23S spacer genes revealed that this region was a species-specific region in the present oral streptococci, except for *S. oralis* and *S. mitis*. Using a PCR analysis method with the primers for the 16S-23S spacer genes, we were able to identify all of the present oral streptococcal species, except for *S. oralis* and *S. mitis*. In addition, no species-specific primer against *S. salivarius* was found.

Recently, we developed a PCR method that utilizes the hypervariable region of the gene encoding glucosyltransferase (*gtf*) as the target gene¹⁸). Nearly all oral streptococci, with the exception of *S. mitis*, produce extracellular polysaccharide⁹) and possess the *gtf* gene¹⁹). Therefore, the differentiation of *S. mitis* and *S. oralis* as well as the detection of *S. salivarius* can be performed by a combination

of these *gtf* based primers. In the epidemiologic study of sepsis and endocarditis caused by oral streptococci, the identification of mitis group streptococci is very important, since it is reported that viridans streptococci, including mitis group streptococci, is most frequently associated with infective endocarditis²⁰). Thus, it is thought that the method presented here is very available for the clinical research of endocarditis, since it is possible to distinguish those streptococci in species level.

Several investigators have developed PCR methods to detect the species of mutans group streptococci using the dextranase gene²¹), and the insoluble glucan synthesizing *gtf* gene²²). However, the information presented in those studies is only useful for identifying species of mutans group streptococci.

We reported that glucosyltransferase from *S. oralis*, one of early colonizing streptococci, plays an important role in the inhabitation of *S. mutans* on tooth surface⁸). This result indicated that commensal streptococci were necessary to the formation of cariogenic dental plaque. Thus, it is important to perform research of symbiosis among mutans group streptococci and the other group streptococci. Especially, the understanding of the change of oral microflora from predentate infant to dentate child might be useful for the control of the inhabitation of mutans streptococci and the prevention of caries in early childhood. It seems to be very significant that the combined method, described above, is applied in clinical investigations of pediatric dentistry.

The development of real-time PCR methods has made it possible to directly determine the amount of bacteria in a sample without culturing, such as *Porphyromonas gingivalis*²³). In addition, using a real-time reverse-transcription/polymerase reaction, we previously determined the expression of 3 different *gtf* genes²⁴). Currently, we are attempting quantitative analyses of streptococci in saliva and plaque, in order to determine which streptococcal species affect to the infection and colonization of mutans group streptococci. We were able to identify oral streptococci with the PCR analysis developed in the present report, though further studies are required to establish a quantitative method of streptococcal species identification using a real-time PCR method.

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