Original Article

Isolation and Typing of *Streptococcus mutans* and *Streptococcus sobrinus* from Caries-active Subjects

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Abstract

Background: *Streptococcus mutans* and *Streptococcus sobrinus* are main etiological agents of dental caries. **Aim:** The aim of the study was to isolate, identify, characterize, and determine the minimum inhibitory concentration (MIC) of *S. mutans* and *S. sobrinus* from caries-active subjects. **Materials and Methods:** Sixty-five plaque samples were collected from caries-active subjects aged between 35 and 44 years, processed and cultured on mitis salivarius bacitracin agar. All the bacterial isolates were subjected to morphotyping and the suspected colonies were identified by 16S rDNA sequencing. The *S. mutans* and *S. sobrinus* strains were characterized by biotyping and phylogenetic analysis. The MIC of ampicillin and erythromycin was determined by microtiter plate method. **Results:** Of the study population, 41 isolates displayed typical colony morphologies of *S. mutans* and *S. sobrinus*. The 16S rDNA sequencing results revealed that 36 isolates were *S. mutans* and 5 isolates were *S. sobrinus*. The biotyping of these isolates demonstrated three biotypes, namely, biotype I (n = 35), biotype III (n = 1), and biotype IV (n = 2). However, 3 isolates exhibited variant biotypes. The phylogenetic analysis revealed that the clinical strains of *S. mutans* and *S. sobrinus* clustered independently along with respective reference strains. The average MIC of ampicillin and erythromycin against *S. mutans* and *S. sobrinus* was 0.047 μg/ml and 0.39 μg/ml, respectively. **Conclusion:** The 16S rDNA sequencing was an impeccable method for *S. mutans* and *S. sobrinus* identification when compared with morphotyping and biotyping methods. The study also suggested that nonspecific bacteria might be involved in caries formation.

Keywords: *Biotyping, decay, missing, and filled teeth, minimum inhibitory concentration, morphotyping, mutans streptococci, phylogenetic*

Introduction

Dental caries is a pandemic disease affecting all the age groups of humans. Several studies indicated that >90% of the residents in developed and undeveloped countries are affected by dental caries.[1,2] Accumulative reports showed that among mutans streptococci (MS), *Streptococcus mutans* and *Streptococcus sobrinus* were the most isolated microorganisms from the majority of human dental caries.[3] *S. mutans* is also known to cause systematic diseases such as cardiovascular and infective endocarditis.[4,5] Recently, *S. mutans* have been considered as novel Gram-positive model bacteria due to its various virulence factors and direct association with the human host.[6]

It is well established that dental caries is a multifactorial disease and the heterogeneity among the members of MS is one of the prime factors in the initiation of the disease. The detection of *S. sobrinus* species in caries-active subjects was often correlated with high caries activity, though higher prevalence rates of *S. mutans* was reported.

The accurate identification of pathogens has been a key parameter as it directly involved in the treatment strategies. Over years, MS have been identified by various methods, including culturing, direct microscopy,[7] biochemical tests,[8] enzyme-linked immunosorbent assays,[9] polymerase chain reaction (PCR)-restriction fragment length polymorphism-PCR-RFLP,[10] PCR-based species-specific primer[11] and PCR-based 16S rRNA gene.[12] It is generally accepted that the 16S rDNA sequencing give more reliable results for identification of MS species.[13]

As a precautionary measure, antibiotics are generally prescribed by the dentists before commencing with the treatment, to prevent any systemic infections arising following

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cavity filling or tooth extraction. At present, adverse reactions of the antibiotics such as bacterial resistance and the rise of multidrug-resistant bacteria are the worldwide concern of public health.[14-16] In this background, the evaluation of antibiotic susceptibility of MS species is of fundamental importance.

A real-time understanding of the heterogeneity of *S. mutans* and *S. sobrinus* prevailing in caries-active subjects is essential to develop better treatment strategies. Additionally, this would aid in encountering other systemic infections arising from caries. In this background, the core objective of the present study was to isolate, identify, characterize, and also to determine the minimum inhibitory concentration (MIC) of *S. mutans* and *S. sobrinus* from caries-active subjects.

Materials and Methods

Study population

The ethical approval (Ref. no. 2576) of the present study was issued by the Institutional Ethics Committee of the PMNM Dental College, Bagalkot, India. It was ensured that all the qualified subjects of the study population had neither a chronic disease nor had received antibiotic therapy for at least 6 weeks before sampling.[17] The dental plaque samples were collected from 65 caries-active subjects included 37 males and 28 females. The subjects, aged 35–44 years as per the World Health Organization (WHO) guidelines.[18] The study was fully explained to every subject and formal written informed consent was obtained. The status of clinical oral health was measured using the decay, missing, and filled teeth (DMFT) index for dentitions. Reference strains employed in the study were *S. mutans* ATCC 25175, *S. mutans* MTCC 497, *S. mutans* MTCC 890, and *S. sobrinus* ATCC 33478.

Isolation and screening of *Streptococcus mutans* and *Streptococcus sobrinus*

The plaque samples were collected using the tips of sterile wooden toothpicks from carious lesions. The toothpicks were cut off and instantly dipped into 1 ml sterile phosphate-buffered saline (HiMedia, India) and stored at 4°C. Plaque samples were vortexed for a minute to disperse the plaque and obtain a homogeneous suspension. The samples were diluted by 100-fold in 1x sterile phosphate-buffered saline and plated on Mitis Salivarius Bacitracin (MSB) agar. The MSB agar composed of mitis salivarius agar (HiMedia, India) and supplemented with 15% of sucrose, 1% of agar, 0.0001% potassium tellurite solution and 0.2 units/ml of bacitracin (HiMedia, India). The plates were incubated anaerobically at 37°C for 48 h.[19]

After the incubation period, the colonies were identified on the basis of colony morphology.[20] The typical colonies from each sample plate were transferred to brain–heart infusion (BHI) broth (HiMedia, India) and incubated at 37°C for 18 h. After the incubation period, the broth cultures were streaked on MSB agar and anaerobically incubated at 37°C for 48 h. The overnight bacterial cultures were stored in 80% glycerol stock at −20°C.

Identification by 16S rDNA sequencing

DNA extraction and purification of the isolates were performed by cetyl trimethyl ammonium bromide method as fully explained by Salman *et al*.[21] PCR amplification of 16S rDNA region was done in 20 µl of reaction mixture containing 10.75 µl of nuclease free water, 2 µl of 10x reaction buffer with 1.5 mM $MgCl_2$, 2 µl of 2.5 mM dNTP mix, 2 µl of 10 picomoles 16S forward primer (5'-AGAGTTTGATCCTGGCTCAG-3'), 2 µl of 10 picomoles 16S reverse primer (5'-AAGGAGGTGATCCAGCCGCA-3'), 0.25 µl of 5 U *Taq* DNA polymerase and 1 µl of 50 ng/µl DNA template. The PCR temperature conditions for 30 cycles were as follows: Initial denaturation 94°C for 2 min, denaturation 94°C for 50 s, annealing 48°C for 30 s, extension 72°C for 90 s, and final extension 72°C for 6 min. The identification of the isolates was carried out by comparing the nucleotide sequences with the NCBI BLAST website database (http//www.ncbi.nlm.nih.gov/blast). The GenBank accession numbers of the clinical strains were also obtained.

Characterization of *Streptococcus mutans* and *Streptococcus sobrinus*

Biochemical characterization

Biochemical tests were performed to determine the biotypes of the clinical isolates as described by Shklair and Keene[22] and Yoo *et al*.[19] A phenol red broth base (HiMedia, India) was used as the basal medium for fermentation of mannitol, melibiose, sorbitol and raffinose (HiMedia, India). Arginine dihydrolase broth (HiMedia, India) was also employed in biochemical characterization. The test organisms were inoculated into the sterile broth and anaerobically incubated at 37°C for 48 h. The positive result was indicated by a color change as described by the manufacturer. The biochemical tests were confirmed with reference strains and repeated thrice to confirm the reproducibility and reliability.

Phylogenetic analysis

The clinical strains sequences of *S. mutans* and *S. sobrinus* were subjected to phylogenetic analysis along with the reference strains (*S. mutans* ATCC 25175, *S. sobrinus* ATCC 33478, and *S. downei* ATCC 33748). The reference strain sequences were recovered from GenBank nucleotide sequence database. The phylogenetic analysis was carried out employing a program named Phylogeny.fr.[23] MUSCLE program was used for sequence alignments, the further Gblocks program was applied to eliminate the poorly aligned position and also the regions of divergence in aligned DNA. The branch support value and a number of bootstrap <50% and 85% were collapsed, respectively.

The maximum likelihood method using PhyML 3.0 software was utilized to construct the phylogenetic tree.[24] TreeDyn was used to draw and render the tree.

Determination of minimum inhibitory concentration

The MIC profile of ampicillin and erythromycin (HiMedia, India) was determined by microdilution method. Twenty-four-well microtiter plate was used, 1 ml of BHI broth was dispensed to each well.[25] 1 ml of BHI broth containing 50 µl of antibiotic stock was added to the first well and serially 2-fold diluted, up to 14 wells. The 100 µl of adjusted inoculum (0.5 McFarland standards) was added into the wells. Two wells of control were also included, one was growth control (without antibiotic) and the other one was negative control (only broth). The plate was covered with a lid and anaerobically incubated at 37°C for 24 h. MIC is the lowest concentration of antibiotic that inhibits the growth of the microorganism; hence, wells were compared with the controls to determine the MIC. The experiment was performed in triplicate, along with the reference strains and repeated to confirm the reliability and reproducibility of the results.

Statistical analysis

The means and standard deviation of the age of the subjects and DMFT were determined. Shapiro–Wilk test was applied to check the normality of the variables. Since all the variables were not normally distributed, Chi-square test was applied to test the DMFT between male and female. The DMFT was correlated with the age of the subjects using non parametric rank correlation. All the statistical analyses were performed using the Statistical Package for the Social Sciences software version 21 (IBM Corporation, USA). The statistical analysis result with $P \leq 0.05$ was considered statistically significant.

Results

The mean average of the age of the subjects in the present study was 39.36 ± 2.09 years while the mean average of the DMFT was 3.98 ± 1.17. The rank correlation between DMFT and age was $\rho = 0.284$ and moderate relation existed between DMFT and age ($P < 0.05$). There were no significant differences between gender and DMFT ($\chi^2 = 4.8$ and $P > 0.05$); however, males were found to be more affected by DMFT.

Among the study population, 36 (55.38%) and 5 (7.69%) were identified as *S. mutans* and *S. sobrinus*, respectively, based on 16S rDNA sequencing. However, 24 (36.92%) isolates were other species of MS or non-MS. The NCBI GenBank accession numbers of *S. mutans* and *S. sobrinus* sequences are presented in Table 1.

The morphotyping and 16S rDNA sequencing results were compared with each other to validate the morphotyping [Table 2]. The results clearly demonstrated that morphotyping is a nonreliable method for differentiating between *S. mutans* and *S. sobrinus*.

Table 1: 16S rDNA sequencing identification and GenBank accession numbers of *Streptococcus mutans* and *Streptococcus sobrinus*

Serial number	Sample number	16s rDNA identification	GenBank accession number
1	H1	*S. mutans*	KP975166
2	H2	*S. mutans*	KP975167
3	H3	*S. mutans*	KP975168
4	H5	*S. mutans*	KP975169
5	H6	*S. mutans*	KP975170
6	H7	*S. mutans*	KP975171
7	H8	*S. mutans*	KP975172
8	H9	*S. mutans*	KP975173
9	H10	*S. mutans*	KP975174
10	H11	*S. mutans*	KP975175
11	H13	*S. mutans*	KP975176
12	H16	*S. sobrinus*	KP975179
13	H17	*S. mutans*	KP975180
14	H18	*S. mutans*	KP975181
15	H19	*S. mutans*	KP975182
16	H20	*S. mutans*	KP975183
17	H21	*S. sobrinus*	KP975184
18	H23	*S. mutans*	KP975185
19	H24	*S. mutans*	KP975186
20	H25	*S. mutans*	KP975187
21	H26	*S. mutans*	KP975188
22	H27	*S. mutans*	KP975189
23	H28	*S. mutans*	KP975190
24	H29	*S. sobrinus*	KP975191
25	H32	*S. mutans*	KP975192
26	H33	*S. mutans*	KP975193
27	H34	*S. mutans*	KP975194
28	H35	*S. mutans*	KP975195
29	H36	*S. mutans*	KP975196
30	H37	*S. mutans*	KP975197
31	H38	*S. mutans*	KP975198
32	H39	*S. mutans*	KP975199
33	H40	*S. mutans*	KP975200
34	H41	*S. mutans*	KP975201
35	H42	*S. mutans*	KP975202
36	H43	*S. sobrinus*	KP975203
37	H52	*S. mutans*	KP975207
38	H56	*S. mutans*	KP975208
39	H58	*S. mutans*	KP975209
40	H60	*S. mutans*	KP975210
41	H65	*S. sobrinus*	KP975213

S. sobrinus: *Streptococcus sobrinus*; *S. mutans*: *Streptococcus mutans*

Among the 41 clinical strains of *S. mutans* and *S. sobrinus*, 35 (85.36%), 1 (2.43%), and 2 (4.87%) strains were biotypes I, III, and IV, respectively, while 3 (7.31%) strains were variant biotypes. The biotypes of the clinical strains of *S. mutans* and *S. sobrinus* are outlined in Table 3.

The phylogenetic analysis of the 16S rDNA sequences constructed by maximum likelihood method is illustrated in

Table 2: Colony morphotyping of *Streptococcus mutans* and *Streptococcus sobrinus*

16s rDNA sequencing identification		Colony-morphotyping
***S. mutans* (*n*=36)**	***S. sobrinus* (*n*=5)**	
22	4	Glistening bubble colony
2	0	Extracellular Polysaccharide surrounded colony
12	1	Mulberry shaped colony

S. sobrinus: *Streptococcus sobrinus*; *S. mutans*: *Streptococcus mutans*

Figure 1. The clinical strains of *S. mutans* and *S. sobrinus* clustered independently along with respective reference strains in the phylogenetic tree. However, *S. sobrinus* strains were found to be more closely related to *S. downei* than *S. mutans*.

The average MIC of ampicillin and erythromycin was 0.047 μg/ml and 0.39 μg/ml, respectively, for both *S. mutans* and *S. sobrinus*.

Discussion

Dental caries is a major public health concern affecting all age groups of human beings.[26] The present study focused on middle age subjects (35–44 years) as a standard monitoring group among the five different age groups of WHO guidelines.[18] However, despite the importance of epidemiological studies in this age group, limited research has been carried out. In addition, 80% of Indian population aged 35–44 years reported to be affected by dental caries.[27]

As *S. mutans* and *S. sobrinus* are most commonly isolated species from caries subjects, the study focused further on these organisms. Among the sampling methodologies, plaque sample was preferred over saliva as detection levels of MS species were stated to be higher in plaque.[28,29]

Based on 16S rDNA sequencing, the present study revealed that the prevalence of *S. mutans* was higher than *S. sobrinus*. Although the differences in the geographical areas, detection methods and the age groups of the subjects employed in the previous studies, the prevalence and the distribution of MS showed a similar tendency to those of the current study.[19,20,30] However, this result was not in accordance with a study conducted in Japanese subjects aged 6–30 years old, where the prevalence of *S. sobrinus* was higher than *S. mutans*.[31] The reason might be due to the differences in the age group, the health condition of the subjects, sample processing, and identification methodologies.

Both *S. mutans* and *S. sobrinus* were found to be negative in 24 (36.92%) subjects. The reason for not detecting these bacteria might be an absence of bacteria from the sample collection sites, the number of *S. mutans* and *S. sobrinus* were below the detection limits in plaque samples, or involvement of nonspecific bacteria in caries formation supporting nonspecific plaque hypothesis.[32]

As the colony morphology was the basis of primary screening of *S. mutans* and *S. sobrinus*. The dominant colonies have recovered in the present study were surrounded by extracellular polysaccharides. Both *S. mutans* and *S. sobrinus* exhibited similar colony morphologies [Table 2]; hence, morphotyping was noted to be an unreliable for species identification which was in agreement with the earlier report.[20]

Table 3: Biotyping of the *Streptococcus mutans* and *Streptococcus sobrinus*

Sample number	Mn	Mb	Sb	Rf	Ar	Biotype
H1	+	+	+	+	-	I
H2	+	+	+	+	-	I
H3	+	+	+	+	-	I
H5	+	+	+	+	-	I
H6	+	+	+	+	-	I
H7	+	+	+	+	-	I
H8	+	+	+	+	-	I
H9	+	+	+	+	-	I
H10	+	+	+	+	-	I
H11	+	+	+	+	-	I
H13	+	+	+	+	-	I
H16	-	-	+	-	+	Variant
H17	+	+	+	+	-	I
H18	+	+	+	+	-	I
H19	+	+	+	+	-	I
H20	+	+	+	+	-	I
H21	+	-	-	-	-	IV
H23	+	+	+	+	-	I
H24	+	+	+	+	-	I
H25	+	+	+	+	-	I
H26	+	+	+	+	-	I
H27	+	+	+	+	-	I
H28	+	+	+	+	-	I
H29	-	-	+	-	-	Variant
H32	+	+	+	+	-	I
H33	+	+	+	+	-	I
H34	+	+	+	+	-	I
H35	+	+	+	+	-	I
H36	+	+	+	+	-	I
H37	-	+	+	+	-	III
H38	+	+	+	+	-	I
H39	+	+	+	+	-	I
H40	+	+	+	+	-	I
H41	+	+	+	+	-	I
H42	+	+	+	+	-	I
H43	-	-	-	-	-	Variant
H52	+	+	+	+	-	I
H56	+	+	+	+	-	I
H58	+	+	+	+	-	I
H60	+	+	+	+	-	I
H65	+	-	-	-	-	IV

Mn: Mannitol; Mb: Melibiose; Sb: Sorbitol; Rf: Raffinose; Ar: Arginine; +: Fermention positive; -: Fermentation negative

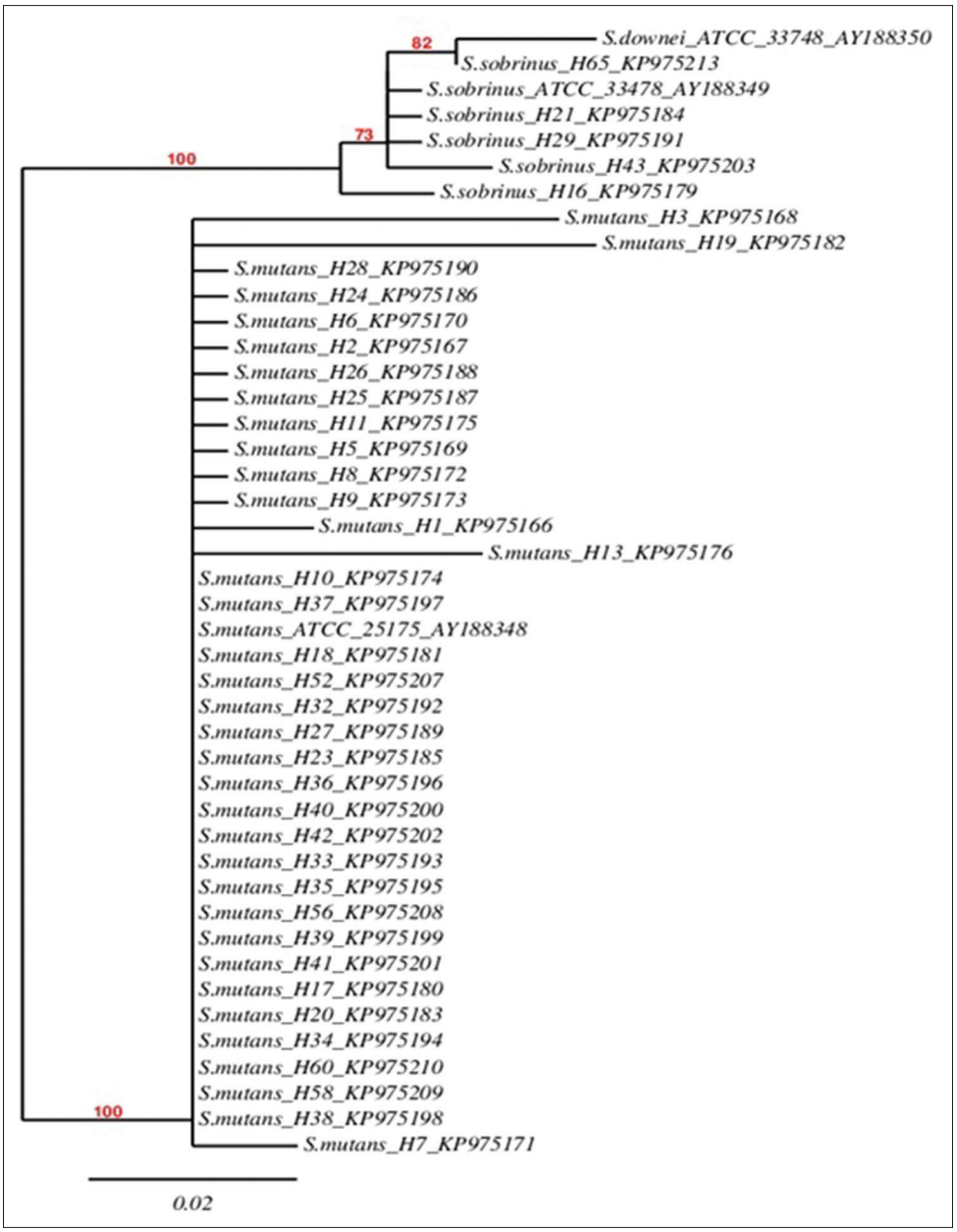

Figure 1: Phylogenetic analysis of the *Streptococcus mutans* and *Streptococcus sobrinus* strains based on 16S rDNA sequences. The generated tree was constructed by maximum likelihood method. The strain and accession numbers are shown next to the species

The biotyping data of the present study demonstrated biotype I (85.36%) as the most common biotype among the isolates of the study. This finding is in accordance with the reports of Imran and Senthilkumar[33] in the Indian population and Yoo *et al*.[19] in the Korean population. The current investigation suggested that biotype I was the most cariogenic biotype. In addition, biotypes III (2.43%), IV (4.87%), and variants biotype (7.31%) were also detected in the present study [Table 3]. The heterogeneity of biotypes among species was observed in the present investigation [Table 3]. The reason could be due to the differences in the host's oral environments and dietary habits of the studied subjects.

Phylogenetically, *S. sobrinus* and *S. downei* were noted to be more closely related to each other than to *S. mutans* [Figure 1], this finding was in accordance with the earlier reported study.[34]

The present investigation revealed that *S. mutans* and *S. sobrinus* were susceptible to ampicillin and erythromycin, MIC 0.047 μg/ml and 0.39 μg/ml, respectively. The determined susceptibility of the isolates to the antibiotics was higher than the previous report.[4] Although the finding of the study was in agreement with the results of recent report,[35] nevertheless, *S. mutans* was earlier reported with resistance to multiple antibiotics.[36]

Conclusion

16S rDNA sequencing was highly sensitive for differentiation between *S. mutans* and *S. sobrinus* compared to the conventional methods. Biotype I was found to be the predominant among the study population. All the strains of *S. mutans* and *S. sobrinus* were susceptible to the ampicillin and erythromycin.

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Conflicts of interest

There are no conflicts of interest.

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