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BIOTYPING AND MOLECULAR DETECTION OF *STREPTOCOCCUS MUTANS* AND *STREPTOCOCCUS SOBRINUS* IN CARIES ACTIVE SUBJECTS

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ABSTRACT

Dental caries is one of the most common prevailing disease in humans and a significant public health problem worldwide. Mutans streptococci are a group of oral bacteria, among this group *Streptococcus mutans* and *Streptococcus sobrinus* are frequently associated with dental caries. The aim of the present study was (i) To investigate the reliability of morphological, biochemical and multiplex PCR based methods to differentiate *S. mutans* and *S. sobrinus* (ii) To determine the proportion of *S. mutans*, *S. sobrinus* and their biotypes present in caries subjects. Dental plaque samples were collected from caries subjects and cultured on Mitis Salivarius Bacitracin agar. The bacteria were identified based on colony morphology, biotyping and multiplex PCR. The results showed that the multiplex PCR was found to be a simple and reliable method for detection and differentiation of *S. mutans* and *S. sobrinus* when compared to morphological and biotyping methods. The proportion of *S. mutans* (65.78%) were higher than *S. sobrinus* (21.05%) *and* biotype I (60.52%) was most frequently isolated from the study population.

KEY WORDS: ***Streptococcus mutans***, ***Streptococcus sobrinus,*** **Biotyping, Dental Caries and Multiplex PCR**

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INTRODUCTION

Dental caries is a polymicrobial infectious disease that is prevailing among all the age groups[1]. The prevalence and distribution of dental caries vary greatly in developed and developing countries and can reach over 90%[2, 3]. Dental caries prevalence in India is reported to be 50 - 60%[4]. Most of the studies in India on dental caries have focused mainly on children and limited studies have been carried out among adults. The understanding of the genotypes of mutans streptococci and their diversity may aid in developing new treatment methods of caries so as to promote health and to prevent the disease. There are about 19 distinct species of oral *Streptococci*, of these mutans *streptococci* are implicated with dental caries[5, 6]. Mutans streptococci are a group of cariogenic species consisting of *Streptococcus mutans* (serotype *c, e, f* and *k*), *Streptococcus sobrinus* (*d* and *g*), *Streptococcus downei* (*h*), *Streptococcus cricetus* (*a*), *Streptococcus rattus* (*b*), *Streptococcus ferus* (*c*), and *Streptococcus macacae* (*c*) [7, 8, 9]. Among mutans streptococci, the prime causative organisms which are involved in human dental caries are *S. mutans* and *S. sobrinus* and they are commonly isolated from dental plaques. Both *S. mutans* and *S. sobrinus* produce large amounts of acids and extra polysaccharides that cause demineralization of hydroxyapatite component of enamel and dentine thereby leading to dental caries[10, 11, 12]. Previous studies have reported variations in detection frequencies of mutans streptococci in caries active subjects[13, 14, 15]. Few investigators have reported that *S. mutans* is more prevalent than *S. sobrinus* in dental plaque samples [16], but the prevalence of *S. sobrinus* is strongly associated with high caries activity[17, 18, 19]. The identification and differentiation of mutans streptococci by traditional methods involves morphological, biochemical and immunological tests[13, 14, 20]. However, these conventional techniques are laborious, time consuming and require expertise, with results sometime being unsatisfactory[21]. Over the years, several investigators have preferred to use the PCR as it is simple, rapid and accurate identification method [22]. The diversity of mutans streptococci associated with caries subjects give rise to different virulence factors and these factors can be considered as vaccine candidates. Identification of pathogenic genotypes that colonize at specific sites in dental caries will help to predict the vulnerable sites for caries. Diversity identification is important to understand quorum sensing behavior among the genotypes. For the above reasons, determining the proportion of mutans streptococci and reliable methods of identification are in pressing need. In this background an investigation was carried out with the following objectives: (i) To investigate the reliability of morphological, biochemical and multiplex PCR based methods to differentiate *S. mutans* and *S. sobrinus* (ii) To determine the proportion of *S. mutans*, *S. sobrinus* and their biotypes present in caries active age group of 35-44years.

MATERIALS AND METHODS

STUDY POPULATION

Ethical approval was obtained by standard procedures from M.S. Ramaiah Dental College, affiliated to Rajiv Gandhi University of Health Sciences, Bangalore. The total study comprised of 38 subjects, which includes 19 males and 19 females respectively ranging from age group of 35 to 44 years as per the WHO guidelines. The study was carried out with a formal written informed consent of each subject after fully explaining the nature of the work to be followed in the present study. The subjects were screened using a pathfinder survey and who volunteered in the study was interviewed using a questionnaire. The qualified subjects for clinical examination were ensured of not having chronic disease or had not received antibiotic therapy for at least 6 weeks[23]. The clinical examinations were performed in duplicate by two calibrated dentists to evaluate intra-examiner reliability. Detection of cavity, undermined enamel or soft floor wall was considered as active caries and DMFT index (Decay, Missing, and Filled Teeth) was recorded[13, 24].

DENTAL PLAQUE COLLECTION

A sterile tongue depressor was used to aid the vision and to avoid contamination from other mouth parts. The plaque samples were collected from sites nearer to the carious lesions with the tips of sterile wooden toothpicks. The toothpicks were cutoff and immediately transferred aseptically into 1ml of sterile phosphate buffer saline and stored at 4ºC[13].

BACTERIAL ISOLATION FROM DENTAL PLAQUES

The samples were vortexed for a minute to disperse the plaque sample and to obtain a homogeneous suspension. 100µl of the sample was used to culture on Mitis Salivarius Bacitracin Agar (MSB) [25] [Himedia] containing 20% sucrose (Himedia) and 0.2 units/ml bacitracin (Himedia). All plates were incubated anaerobically at 37ºC for 48h.

MORPHOLOGICAL IDENTIFICATION

After the incubation period, the colonies of mutans streptococci were identified on the basis of colony morphology[13, 26, 27, 28, 29, 30]. From each sample plate, typical colonies of mutans streptococci were picked up and transferred to 2 ml of Brain Heart Infusion (BHI) broth (Himedia) and grown at 37ºC for 18h. The Gram's nature and morphology of the bacteria were determined by Gram's staining.

BIOTYPING

Colonies were further confirmed by biochemical scheme proposed by Shklair and Keene[14, 31] for fermentation of mannitol, sorbitol, melibiose, raffinose and hydrolysis of arginine (Himedia). Positive reaction of fermentation was indicated by a color change from red to yellow while arginine hydrolysis was detected by the color change from purple to yellow and then back to purple. The biochemical results were validated with reference strains (MTCC 497, ATCC 25175, MTCC 890, ATCC 33478 and KCOM 1221) and repeated in order to confirm reproducibility and reliability. After identification, the cultures were maintained at -20°C in 10% glycerol BHI broth (Himedia).

DNA EXTRACTION AND PURIFICATION

DNA was extracted and purified according to Spolidorio *et al.*[32] and Bert *et al.*[33] with some modifications. Briefly, single colony forming units of each strain was grown in BHI broth and incubated overnight at 37ºC. The cells were pelleted down and washed twice with TE buffer (50 mM Tris, 1 mM EDTA, pH 8)[Sigma Aldrich], centrifuged to pellet down the cells and resuspended in buffer containing 10 mM Tris-HCl, 50 mM EDTA (Sigma Aldrich), and 25% sucrose (Himedia). DNA was released from bacterial cells by incubation with 10 mg/ml lysozyme (Himedia) and 100 µg/ml RNAase (Sigma Aldrich) for 30 minutes at 60ºC followed by incubation with 50µl of 10 mg/ml proteinase K (Himedia) and 50µl of 10% sarkosyl (Sigma Aldrich) for 2h at 37ºC and then for 30 min at 68ºC. The pellet was treated twice with phenol: chloroform: isoamyl alcohol (25: 24:1) [Qualigens]. The tubes were centrifuged at 10,000rpm for 10 minutes. DNA was precipitated with double volume of ice cold ethanol (Himedia) and stored at -20ºC for 30 minutes. The vials were centrifuged at 14,000rpm for 10 minutes. The pellet was washed with 70% alcohol at 5000rpm for 5 min. The pellet was air dried and suspended in TE buffer and stored at -20º C. The DNA was further purified using column purification kit (Qiagen). Concentrations of eluted DNA samples were determined by measuring A260nm using UV spectrophotometer (UV-1800 Shimadzu). The purity of DNA was evaluated from A260/A280 ratio[26]. The isolated DNA was electrophoretically checked by loading 10µl of DNA sample with 5µl of DNA loading dye on 0.8% agarose gel.

PCR EXPERIMENTS

SPECIES IDENTIFICATION BY MULTIPLEX PCR

S. mutans and *S. sorbinus* were identified according to the modified method of Wu *et al.*[13]. Specific oligonucleotide primers (Eurofins) were used for species identification of *S. mutans* and *S. sorbinus* (Table 1). The primers based on the *gtf*B sequence (GTFB-F and GTFB-R) and *gtf*I sequence (GTFI-F and GTFI-R) were used for identification of *S. mutans* to amplify 517-bp DNA and *S. sobrinus* 712-bp DNA fragments.

Table 1
PCR primers for identification of S. mutans and S. sobrinus

Species	Target gene	Primer	Sequence	Location	Product Size(bp)
S. mutans	*gtf*B	GTFB-F	5'–ACTACACTTTCGGGTGGCTTGG	793-814	517
		GTFB-R	5'–CAGTATAAGCGCCAGTTTCATC	1288-1309	
S. sobrinus	*gtf*I	GTFI-F	5'-GATAACTACCTGACAGCTGACT	871-892	712
		GTFI-R	5'-AAGCTGCCTTAAGGTAATCACT	1561-1582	

Each PCR reaction mixture (50 µl) consisted of 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 50 mM KCl, 200µM each of dATP, dTTP, dGTP and dCTP (Aristrogene), 10µM oligoneucleotide primers (GTFB-F, GTFB-R, GTFI-F and GTFI-R) [Eurofins], 3 units of Taq polymerase (Aristrogene) and 10ng of DNA. The PCR (PerkinElmer 2009) conditions were initial denaturation at 94ºC for 2 minutes followed by denaturation at 94 ºC for 30 seconds. Annealing at 60 ºC for 30 seconds and extension at 72ºC for 30 seconds. This amplification was repeated for 29 cycles. The final extension cycle was run at 72 ºC for 5 minutes. For *S. mutans* MTCC 497, ATCC 25175, MTCC 890 and for *S. sobrinus* ATCC 33478 and KCOM 1221 were used as positive controls for PCR. Sterile distilled water was used as negative control. A 100bp DNA ladder (Aristrogene) was used as the marker. The PCR products were electrophoresed at 100V on 1.5% of agarose gel and stained with ethidium bromide (Sigma). The gel images were captured with digital imaging system (G: BOX - Syngene 2009).

STATISTICAL ANALYSIS

Dental data were recorded on data collection sheets and subjected to distribution analysis. Since the data did not follow normal distribution, the differences in DMFT were evaluated using a non parametric Mann-Whitney test using SPSS software V18.0. Morphological and biochemical methods for differentiation of *S. mutans* and *S. sobrinus* were then compared with the PCR approach, using the kappa statistics as a method of agreement between them.

RESULTS

The average age and DMFT of the study population was 39.09 ± 0.35 and 3.26 ± 0.22 respectively. Mann-Whitney test clearly showed there is a significant difference between females and males on DMFT and the females are more affected than males in terms of cavities (Mann-Whitney value is 89, Z test= -2.79, P value is .005). Glistening bubble and extracellular polysaccharide surrounded colonies were more prevalent among the variants of *S. mutans* and *S. sobrinus* respectively. The *S. mutans* and *S. sobrinus* colonies identified in the study population are presented in table 2. The biotyping data of 38 strains showed that 23(60.52%), 9(23.68%) and 2(5.26%) strains were of biotypes I, IV, V and 4 strains showing mulberry colonies remained unidentified based on the biochemical scheme. Fig. 1 presents the distribution of mutans streptococci biotypes in the study population. The proportion of unidentified biotypes is presented in table 3.

Table 2
Identification of clinical isolates based on colony morphology, biochemical tests and multiplex PCR method.

Colony Morphology	No. of colonies isolated n=38		Biochemical Test		Unidentified by Biochemical test	Multiplex PCR		Unidentified by Multiplex PCR
	S. mutans	*S. sobrinus*	*S. mutans*	*S. sobrinus*		*S. mutans*	*S. sobrinus*	
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
Glistening bubble colony	17(44.73)	0	17(100)	0	0	17(100)	0	0
Extracellular polysaccharide surrounded colony	0	8(21.05)	1(12.5)	7(87.5)	0	1(12.5)	6(75)	1(12.5)
Mulberry-shaped colony	13(34.21)	0	7(53.84)	2(15.38)	4(30.76)	7(53.84)	2(15.38)	4(30.76)

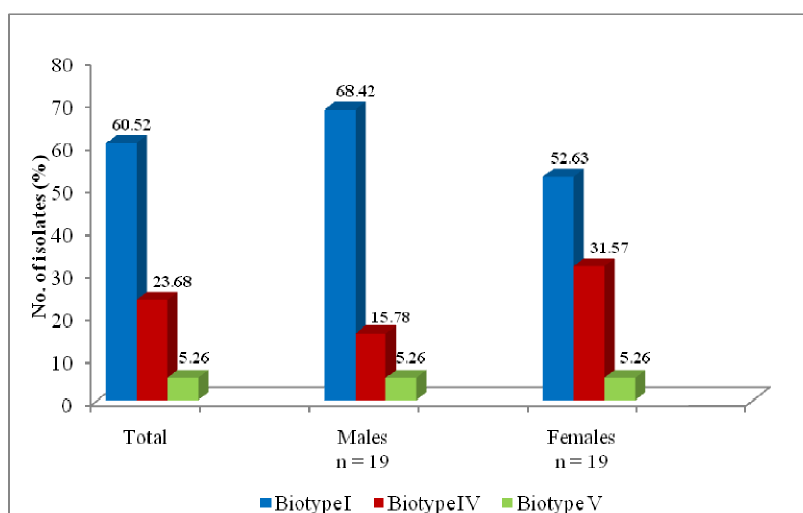

Figure 1
Distribution of mutans streptococci biotypes in dental caries subjects

Table 3
Proportion of unidentified biotypes present in the study population

Biotypes	Frequency of occurrence n = 38	Males n = 19	Females n = 19
	n (%)	n (%)	n (%)
Unidentified	4(10.52)	2(10.52)	2(10.52)

The GTFB and GTFI primers used in present study detected *S. mutans* and *S. sobrinus* by multiplex PCR. The detection of *S. mutans* and *S. sobrinus* resulted in a single DNA fragment at 517 and 712 bp. Fig. 2 shows the detection the *S. mutans* and *S. sobrinus* using positive and negative controls while Fig. 3, 4, & 5 are the representative results of clinical isolates.

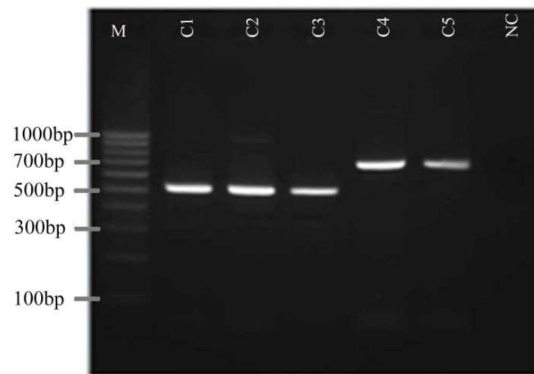

Figure 2
Detection of S. mutans and S. sobrinus by PCR amplification in control DNA samples. Lanes: 1, M-DNA marker; 2, C1- S. mutans MTCC 497; 3, C2- S. mutans ATCC 25175; 4, C3- S. mutans MTCC 890; 5, C4- S. sobrinus ATCC 33478; 6, C5- S. sobrinus KCOM 1221 and 7, NC- Negative control.

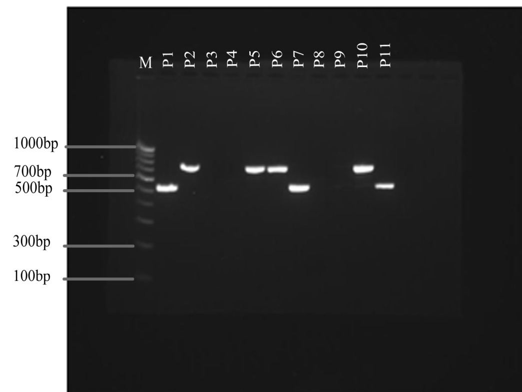

Figure 3
Detection of S. mutans and S. sobrinus in dental plaque samples by PCR amplification. Lanes: 1, (M) DNA marker; 2 to 12, (P1-P11) PCR products of the clinical isolates.

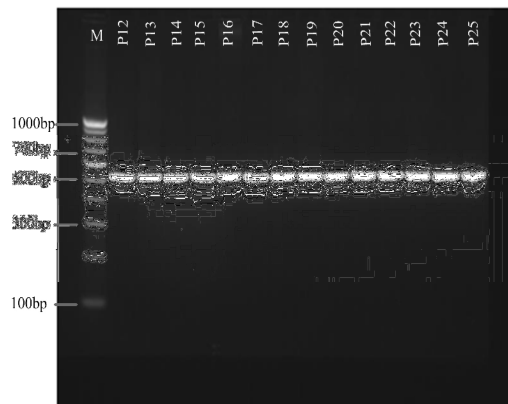

Figure 4
Detection of S. mutans and S. sobrinus in dental plaque samples by PCR amplification. Lanes: 1, (M) DNA marker; 2 to 15, (P12-P25) PCR products of the clinical isolates.

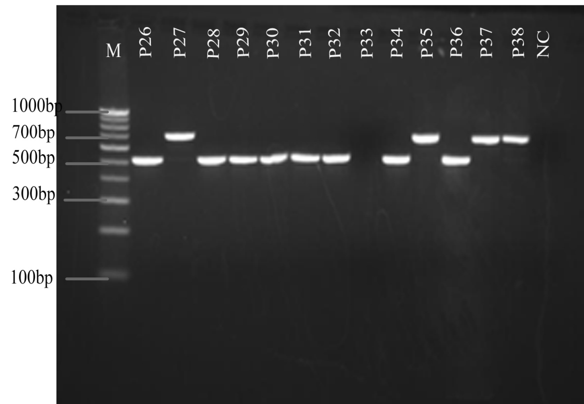

Figure 5
Detection of S. mutans and S. sobrinus in dental plaque samples by PCR amplification. Lanes: 1, (M) DNA marker; 2 to 14, (P26-P38) PCR products of the clinical isolates and 15, NC- Negative control.

Based on PCR, the proportion of *S. mutans* and *S. sobrinus* were detected at 65.78% and 21.05% of the subjects respectively. 13.15% of subjects were negative for both the species (Fig. 6). The proportion of *S. mutans* and *S. sobrinus* by gender is presented in Fig. 7. The number of males positive for *S. mutans* was 14(73.68%) higher than females 11(57.89%) while more females 5(26.31%) were found to be positive for *S. sobrinus* than males 3(15.78%).

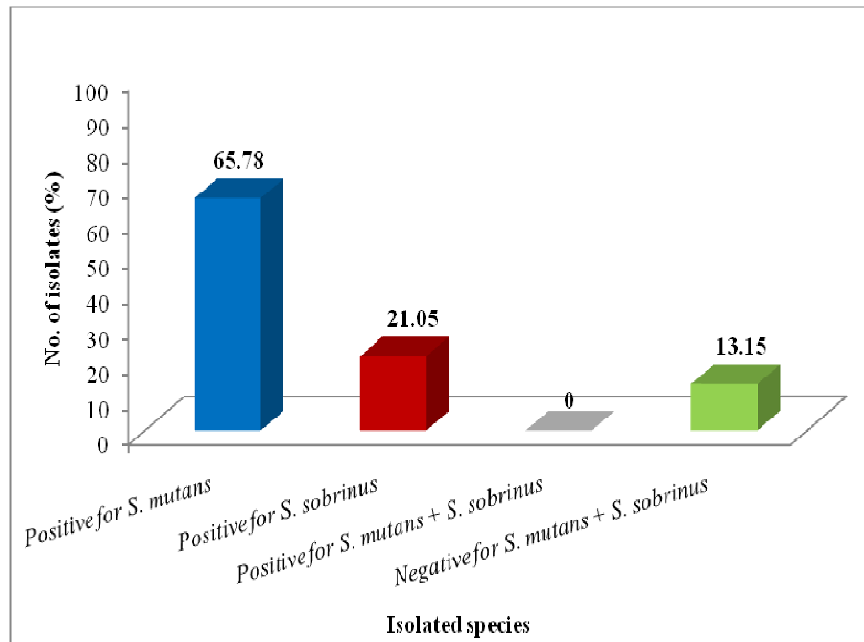

Figure 6
Proportion of S. mutans and S. sobrinus in study population

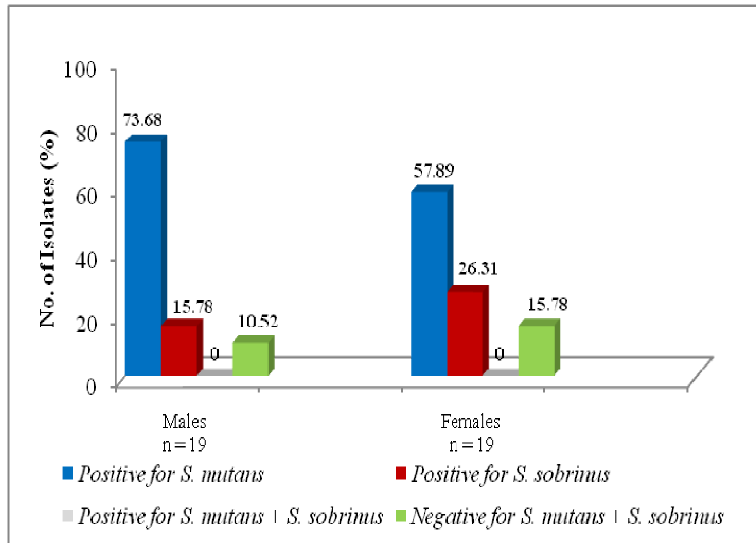

Figure 7
Proportion of S. mutans and S. sobrinus by gender

Kappa test 1 performed to find agreement between two methods morphology vs PCR is tabulated in table: 4. 96% *S. mutans* properly matched between morphology and PCR and for *S. sobrinus,* 75% is rightly matched.

Table 4
Kappa test 1- Agreement between two methods morphology vs. PCR cross tabulation

			PCR		
			S. mutans	***S. sobrinus***	**Total**
Morphology	***S. mutans***	Count	24	2	26
		%	96.0%	25.0%	78.8%
	S. sobrinus	Count	1	6	7
		%	4.0%	75.0%	21.2%
Total		Count	25	8	33
		%	100.0%	100.0%	100.0%

Symmetric measures between morphology vs PCR are presented in table 5 (Kappa value 0 .742, standard error =0.140 and P value is .000). There is a significant agreement between two methods for differentiation between *S. mutans* and *S. sobrinus.*

Table 5
Symmetric measures between morphology vs PCR

		Value	**Asymp. Std. Error[a]**	**Approx. T**	**P value**
Measure of agreement between morphology vs PCR	Kappa	0.742	0.140	4.276	0.000
N of Valid Cases		33			

Kappa test 2 performed to find agreement between two methods biochemical test vs PCR is tabulated in table 6. There was 100% match on both *S. mutans* and *S. sobrinus* between biochemical test and PCR method.

Table 6
Kappa test 2 - Agreement between two methods Biochemical test vs. PCR cross tabulation

			PCR			
			S. mutans	***S. sobrinus***	**Unidentified**	**Total**
Biochemical	***S. mutans***	Count	25	0	0	25
		% within PCR	100.0%	.0%	.0%	65.8%
	S. sobrinus	Count	0	8	1	9
		% within PCR	.0%	100.0%	20.0%	23.7%
	Unidentified	Count	0	0	4	4
		% within PCR	.0%	.0%	80.0%	10.5%
	Total	Count	25	8	5	38
		% within PCR	100.0%	100.0%	100.0%	100.0%

Symmetric measures between biochemical vs PCR are presented in table 7. (Kappa value is 0.948, standard error is 0.05, P value is 0.000). There is significant agreement between two methods for differentiation between *S. mutans* and *S. sobrinus.*

Table 7
Symmetric measures between Biochemical test vs PCR

		Value	Asymp. Std. Error[a]	Approx. T	P value
Measure of Agreement	Kappa	0.948	0.050	7.646	0.000
N of Valid Cases		38			

DISCUSSION

In the present study, 35-44 years of age group was selected as a standard monitoring group among the five age groups as per WHO guidelines[24]. The study was focused only on the *S. mutans* and *S. sobrinus* as these species are strongly associated with human dental caries[10]. It was observed that males had lower DMFT value than females which is in accordance with other studies[34, 35]. Although it is unclear why the caries incidence is more in females, but it is believed that they may be due to difference in dietary habits[35]. The finding of this study revealed the occurrence of *S. mutans* was 65.78% and *S. sobrinus* was 21.05%. In earlier studies investigators have reported varying proportion of *S. mutans* and *S. sobrinus*. There are several factors involved in the variations in the detection rate of *S. mutans* and *S. sobrinus* which include sample collection site, sample processing methodologies, media used in the study, ethnic diversity, dietary habits, dental anatomy and oral hygiene of the subjects[13]. From the kappa test it is understood both morphology vs PCR method and biochemical test vs PCR method is significant, that is both morphology and biochemical test goes well with PCR method. However, biochemical test does better agreement with PCR than morphology. In our study, among 38 strains, five strains remained unidentified. Four strains (P3, P4, P8 and P33) could not neither be identified by biotyping[14, 31] nor by multiplex PCR targeting *gtf*B and *gtf*I genes while one strain (P9) identified as *S. sobrinus* by biotyping remained unidentified by multiplex PCR. The possible explanation for not identifying four strains by biotyping and PCR methods is that they may belong to non-mutans streptococci in accordance to the results of Yoo *et al.,*[36] and Russell[37]. One strain (P9) which was identified as *S. sobrinus* by biotyping method did not respond for PCR method. Since it is expected that all *S. sobrinus* strains should respond for a GTFI primers[13], the above strain couldn't be confirmed as *S. sobrinus.* Further molecular techniques like 16Sr DNA cloning and sequencing techniques have to be carried out, to identify the unidentified strains.

CONCLUSION

In conclusion, the multiplex PCR was found to be a simple and reliable method for detection and differentiation of *S. mutans* and *S. sobrinus* when compared to morphological identification and biotyping methods. The proportion of *S. mutans* was higher than *S. sobrinus* and biotype I was most frequently isolated from the study population.

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