Original Article

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Dentistry Section

Distribution of *Streptococcus mutans* and *Streptococcus sobrinus* in Dental Plaque of Indian Pre-School Children Using PCR and SB-20M Agar Medium

DIVYA SINGLA[1], ARUN SHARMA[2], VINOD SACHDEV[3], RADHIKA CHOPRA[4]

ABSTRACT

Introduction: Dental caries is one of the most common infectious diseases affecting the oral cavity. Among the oral bacteria, mutans streptococci have been implicated as major cariogenic bacteria as they can produce high levels of dental caries causing substances such as lactic acid and extracellular polysaccharides.

Aim: The aim of the study was to detect the presence of *Streptococcus mutans* and *Streptococcus sobrinus* in dental plaque by using Polymerase Chain Reaction (PCR) method, quantification of these micro-organisms using Modified Sucrose-Bacitracin (SB-20M) agar medium and to correlate their presence in Caries Active (CA) and Caries Free (CF) pre-school children.

Materials and Methods: Sixty-eight pre-school children, in the age group of 3-5 years were divided equally into 34 CA and 34 CF children. Dental plaque samples were obtained for detection of these microorganisms by PCR method and quantification was done using SB-20M culture medium.

The data was analyzed using statistical software SPSS version 16. For statistical analysis, the frequencies and means of Colony Forming Units (CFU) were used with CI = 95%. For bivariate analysis, Fisher exact test was used at 5% level of significance. The comparison of mean of number of CFU of *S. mutans* and *S. sobrinus* was made by Mann Whitney U test and Spearman's Rho test at 1% level of significance was used for correlation between dmft and CFU in CA group.

Results: The results showed that *S. sobrinus* was significantly higher in CA group as compared to CF group whereas *S. mutans* showed no significant difference. On quantification of these micro-organisms, *S. sobrinus* was present in significantly higher numbers in CA group as compared to CF group. On correlating the CFU/ml of the micro-organisms with the dmft index, both the micro-organisms showed a positive correlation.

Conclusion: We conclude that *S. mutans* and *S. sobrinus* were detected in higher numbers in CA children as compared to CF children. PCR is a sensitive, specific, rapid and an effective method for the detection of oral microorganisms.

Keywords: Age group, Analysis, Dental caries, Genetics, Oral microflora

INTRODUCTION

In spite of the advances in preventive dentistry, dental caries still remains a major dental health problem in several countries [1-4]. It is important to assess the microbial counts in saliva and plaque during primary and mixed dentition period to both predict and prevent dental caries.

Although a positive correlation has been shown between caries experience and mutans streptococci levels in an individual [5,6], some studies have also shown higher levels of these organisms in populations with a very low caries experience [7]. This can be explained by the presence of different species of mutans streptococci which exhibit variable virulence and adherence properties [8].

Out of the seven species of mutans streptococci group, *S. mutans* and *S. sobrinus* have been most commonly implicated in the pathogenesis of dental caries [9]. The association of these two species with dental caries has been assessed in many studies and a huge variation has been reported. While some researchers found *S. mutans* to be a better indicator for dental caries [10], other studies have shown *S. sobrinus* to be more closely associated [11,12].

To better understand the role of these organisms, we need efficient methods to detect and identify them. Various methods have been proposed to identify and differentiate oral streptococci including culture methods, biochemical tests, immunological and genetic methods with DNA probes, enzyme linked immunosorbent assay and Polymerase Chain Reaction (PCR) [11].

DNA based methods such as PCR method, have proved to be universally applicable for the detection of cariogenic bacteria in various epidemiological and clinical studies because of their high sensitivity, specificity and rapidity in obtaining results [13].

Several selective culture media have been developed for the enumeration of mutans streptococci in saliva and plaque. Modified Sucrose-Bacitracin (SB-20M) culture medium is a suitable media which shows morphological differences between colonies of *S. mutans* and *S. sobrinus* [14].

The aim of the present study was to detect the presence of *S. mutans* and *S. sobrinus* in dental plaque samples by PCR method, quantification of these micro-organisms using modified SB-20 culture medium and to correlate the presence of *S. mutans* and *S. sobrinus* with dental caries in Caries Active (CA) and Caries Free (CF) pre-school children.

MATERIALS AND METHODS

The present in-vivo study was conducted in the Department of Pedodontics and Preventive Dentistry at I.T.S., Centre for Dental Studies and Research, Ghaziabad, Uttar Pradesh, India, over a period of one year and was approved by Institutional Ethics Research Committee. Children in the age group of 3-5 years from various schools of Ghaziabad were screened using WHO diagnostic criteria to determine the dmft (decayed, missing, filled teeth) index. Children with good general health, CA (dmft≥4) and CF (dmft=0) were included in the study. Those who suffered from any systemic disease, had taken antibiotics in the past three months or medically compromised children were excluded from the study.

A pilot study was conducted and it was found that the Mean±S.D of number of CFU/ml for CA and CF groups were 88160.59±137207.5

and 9236.25±13634.45 respectively. With the help of software G*Power analysis version 3.1.9.2 for the power 0.95 and α err probability 0.05, sample size of 34 samples for each group was determined.

Sixty-eight children were selected for participation in the study based on their caries experience and were classified in two groups of 34 children each. Group A consisted of CA children (dmft≥4) and Group B consisted of CF children (dmft=0).

Supragingival plaque samples were collected by a single operator from each subject along the cervical margin of all teeth with the help of a sterile dental explorer. Each explorer was immediately placed in a sterile tube containing phosphate-buffered saline (pH=7) and transported in ice pack at 4°C to Codon Biotech Laboratory, Noida, Uttar Pradesh, India, where this sample was used for two independent procedures – PCR and plate count using SB-20M agar medium.

I) Detection of micro-organisms (*S. mutans* and *S. sobrinus*) by PCR method: DNA was extracted from the plaque sample using the standard procedure where bacterial cells from the suspension were first lysed with lysis solution containing lysozyme + Sodium Dodecyl Sulphate (SDS) + NaOH followed by addition of Potassium acetate (neutralizing solution) which precipitates the proteins. Then the chromosomal DNA was precipitated with isopropyl alcohol and stored in Tris buffer for further use in amplification.

Two sets of species specific primers synthesized by "Sigma Aldrich, USA" were used in the study. These were as follows -GTFB-F5'-ACTACACTTTCGGGTGGCTTGG,GTFB-R5' CAGTATAAGCGCCAGTTTCATC for amplification of 517 bp-DNA fragment of the gtf B gene of *S. mutans*, and GTFI-F5'-GATAACTACCTGACAGCTGACT,GTFI-R5'-AAGCTG CCTTAAGGTAATCACT for amplification of 712 bp-DNA fragment of the gtf I gene of *S. sobrinus*.

The PCR conditions used were denaturation at 95°C for 30 seconds, followed by annealing at 59°C for 30 seconds, and extension at 72°C for 1 minute. This amplification was repeated for 30 cycles. The final cycle was run at 72°C for 5 minutes. The PCR products obtained were subjected to electrophoresis on 1.5% agarose gel along with 100-bp DNA ladder marker. The gel was stained with ethidium bromide and illuminated under UV illuminator. The presence and absence of the type of bacteria was seen by the presence of amplified bands on the agarose gel.

II) Quantification of the two strains using SB-20M agar medium: Dental plaque samples were vortexed for 30 seconds, then serial diluted from 10^{-1} to 10^{-6} and spread plated on SB-20M culture medium.

The plates were incubated in an atmosphere of 10% CO_2 at room temperature for 48 hours. Since, SB-20M media is a differential media, both the bacterial cultures can be very easily differentiated on the agar due to their colonial and morphological difference. *S. mutans* colonies show a granular surface with polysaccharide drops while *S. sobrinus* colonies appear in the form of circular, opaque, milky drops.

The Colony Forming Units (CFU) were examined visually and counted using colony counter to verify the presence of CFU resembling *S. mutans* and *S. sobrinus* and results were described as CFU/ml.

STATISTICAL ANALYSIS

The data were analyzed using statistical software SPSS version 16. For statistical analysis, the frequencies and means of CFU were used with CI = 95%. For bivariate analysis, Fisher exact test was used at 5% level of significance. The comparison of mean of number of CFU of *S. mutans* and *S. sobrinus* was made by Mann Whitney U test. Spearman's Rho test at 1% level of significance was used for correlation between dmft and CFU in CA group.

RESULTS

The distribution of bacteria among CA and CF individuals found by PCR method is shown in [Table/Fig-1]. Sixty-eight children (37 boys, 31 girls) participated in the study with the mean age of 4.38 years.

Frequency of detection of *S. mutans* and *S. sobrinus* by PCR method (n=68) [Table/Fig-2]: The *S. mutans* was found to be present in 82.4% of CA children and 85.3% of CF children and this difference was found to be statistically non-significant (p>0.05). However, *S. sobrinus* was present in 67.6% of CA children and 11.8% of CF children and the difference was highly significant (p<0.001). In 58.8% of the CA children, both *S. mutans* and *S. sobrinus* were detected as against 11.7% children in CF group and this difference was also found to be statistically significant (p<0.05).

Quantification of *S. mutans* and *S. sobrinus* in both groups from SB-20M agar plates [Table/Fig-3]: The values of mean ranks for the CFU of *S. mutans* and *S. sobrinus* in both the groups. Mann Whitney statistics did not show any significant difference between two groups for the CFU of *S. mutans* (p>0.05).

On the other hand, colony counts for *S. sobrinus* were found to be significantly greater in the CA group (p<0.05).

Correlation of CFU of *S. mutans* and *S. sobrinus* with dmft in CA children [Table/Fig-4]: A negative correlation although statistically insignificant was found between the dmft and CFU of *S. mutans*. However, the correlation of dmft was positively correlated with CFU of *S. sobrinus* (p<0.001) and combined *S. mutans* and *S. sobrinus* (p<0.01).

Microorganisms Present		Caries Active	Caries Free
S. mutans	*S. sobrinus*	Frequency (%)	Frequency (%)
+	-	8 (23.5)	25 (73.5)
-	+	3 (8.8)	0
+	+	20 (58.8)	4 (11.7)
-	-	3 (8.8)	5 (14.7)
Total samples		34	34

[Table/Fig-1]: Distribution of bacteria among caries active and caries free individuals found by PCR method.

Variable			Caries Active Group	Caries Free Group	p-value
S. mutans	Present	Frequency	28	29	0.50*
		% within group	82.4	85.3	
	Absent	Frequency	6	5	
		% within group	17.6	14.7	
S. sobrinus	Present	Frequency	23	4	0.000***
		% within group	67.6	11.8	
	Absent	Frequency	11	30	
		% within group	32.4	88.2	
S. mutans & S. sobrinus	Present	Frequency	20	4	0.02**
		% within group	58.8	11.7	
	Absent	Frequency	3	5	
		% within group	8.8	14.7	

[Table/Fig-2]: Frequencies of identification of *S. mutans* and *S. sobrinus* from dental plaque by PCR in caries active and caries free children (n=68).
*NS , ** significant at 0.05, *** significant at 0.001 (Fisher exact test at 5% level of significance)

	Group	N	Mean Rank	Z value	p-value
CFU S. mutans	Caries Active	28	31.95	-1.582	0.11^{NS}
	Caries Free	29	25.05		
CFU S. sobrinus	Caries Active	23	15.24	-1.945	0.04*
	Caries Free	4	6.88		

[Table/Fig-3]: Distribution of mean ranks of CFU of *S. mutans* and *S. sobrinus* in caries active and caries free group.
*Significant p <0.05, NS Not Significant p > 0.05, (Mann Whitney U test used)

dmft Correlation	CFU *S. mutans*	CFU *S. sobrinus*	CFU *S. mutans* & *S. sobrinus*
Correlation Coefficient (r)	-0.296	0.895	0.602
p-value	0.126 NS	0.000*	0.005*

[Table/Fig-4]: Correlation of CFU of *S. mutans*, *S. sobrinus* and both with dmft index within caries active group.
*Significant p <0.05, NS Not Significant p > 0.05
(Spearman's Rho Test used at 1% level of significance)

DISCUSSION

Dental caries is a multifactorial infectious disease and is still considered as one of the most prevalent biofilm-mediated diseases affecting humans. The most important risk factor in any disease is the causative agent [15].

Among the mutans streptococci group, *S. mutans* and *S. sobrinus* are the most frequently isolated species from human dental plaque and are believed to be the major aetiological agents for tooth decay. These micro-organisms have the capability of adhering to the enamel surface and forming a bio-film facilitated by extracellular polysaccharides produced by using sugars in the diet as a substrate, which favours demineralization measured by the acid products from bacterial metabolism [16].

In the present study, dental plaque was used for the detection of cariogenic micro-organisms rather than saliva. Various previous studies have used saliva for detecting cariogenic bacteria; however, the tendency in recent studies has been towards the use of dental plaque. As the intention was to relate the presence of cariogenic bacteria and dental caries, it was found that using saliva as a source of cariogenic bacteria does not permit establishing an effective association. Although the presence of *S. mutans* is high in saliva, it is lower on the surface of enamel, where this bacterium actually manifests its capacity to produce acids and result in subsequent demineralization [16].

The present study showed that *S. mutans* alone was present in 23.5% of CA children and 73.5% of CF children. The distribution of *S. mutans* in previous studies was quite variable with the values ranging from 24-64% in CA children and was present up to 76% in CF children [11,17,18]. Comparison with other similar studies has been shown in [Table/Fig-5].

In the present study, *S. sobrinus* alone was present only in 8.8% of CA children and in none of the samples of CF children. However, in a previous study by Nurelhuda NM et al., [11], *S. sobrinus* was never present alone in any of the samples. Other comparative studies have been compiled in [Table/Fig-6].

A significant finding of the present study was presence of both *S. mutans* and *S. sobrinus* in 58.8% of CA group was almost five times more than CF group and this finding correlates with previous studies as well [11]. Thus, the presence of both the bacteria together can be considered an important pathogenic factor in the development of carious lesions.

At the same time, *S. sobrinus* was found six times more frequently in CA group when assessed by PCR method; whereas, the frequency of *S. mutans* was not statistically significant between the two groups. Thus, it is not only important to assess the total bacterial load but also the presence of individual species which can dictate the disease outcome. Similar results were obtained on comparing the CFU from SB-20M medium. The difference in CFU of *S. mutans* between the two groups was not statistically significant while it was significantly more for *S. sobrinus* in CA group. Thus, from this study it can be concluded that *S. sobrinus* has a higher cariogenic potential than *S. mutans*. Similar conclusions have been drawn from previous experiments done on animals [19] and also from studies carried out in pre-school children [12].

On comparing the results obtained from PCR method and SB-20M agar plates, similar distribution of *S. mutans* and *S. sobrinus* was found and the difference between the two methods was not statistically significant. There was only one sample from CF group where *S. mutans* was detected by PCR and missed in plate analysis. Thus, both the methods are equally effective in detecting the individual species.

In the CA group, a positive correlation was found between dmft index and CFU of *S. sobrinus* and *S. sobrinus*/*S. mutans* (combined). On the contrary, no correlation could be established between *S. mutans* and dmft; thus, again emphasizing on the fact that severity of the disease can also be affected by increase in amount of certain specific species of bacteria. This finding is supported by Choi EJ et al., who concluded that *S. sobrinus* was more related to dental caries than *S. mutans* [20].

Study	Caries Active Samples	Caries Free Samples	Result
Rodriguez J et al., (2007) [15]	75%	60%	NS
Choi EJ et al., (2009) [20]	100%	80%	NS
Carmona LE et al., (2011) [16]	76%	24%	S
Soni H and Vasavada M (2015) [21]	100%	100%	NS

[Table/Fig-5]: Other studies for the presence of *Streptococcus mutans*.

Study	Caries Active Samples	Caries Free Samples	Result
Rodriguez J et al., (2007) [15]	50%	22.5%	S
Choi EJ et al., (2009) [20]	43-60%	8.6%	S
Carmona LE et al., (2011) [16]	81.9%	18.1%	S
Soni H and Vasavada M (2015) [21]	60%	30%	S

[Table/Fig-6]: Other studies for the presence to *Streptococcus sobrinus*.

LIMITATION

The limitation in the present study was that the sample size was small and only two species of mutans streptococci were tested. More studies are needed to evaluate other species of mutans streptococci and correlate them with caries experience in children.

CONCLUSION

From the present study, it can be concluded that both SB-20M agar medium and PCR method are equally effective in detecting *S. mutans* and *S. sobrinus* from plaque samples. It is also concluded from this study that *S. sobrinus* might play a more active role in the pathogenesis of dental caries as compared to *S. mutans*; thus, giving importance to the detection of individual species while assessing caries susceptibility of an individual.

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PARTICULARS OF CONTRIBUTORS:
1. Senior Resident, Department of Dental Surgery, Dr. Ram Manohar Lohia Hospital, New Delhi, India.
2. Professor and Head, Department of Pedodontics and Preventive Dentistry, People's College of Dental Sciences and Research Centre, Bhopal, Madhya Pradesh, India.
3. Professor and Head, Department of Pedodontics and Preventive Dentistry, ITS – Centre for Dental Studies and Research, Muradnagar, Ghaziabad, Uttar Pradesh, India.
4. Reader, Department of Pedodontics and Preventive Dentistry, ITS – Centre for Dental Studies and Research, Muradnagar, Ghaziabad, Uttar Pradesh, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Divya Singla,
H.No. 426, Daulat Pura, Bhatia Road, Near Gurudwara, Ghaziabad-201001, Uttar Pradesh, India.
E-mail: divyasingla.2110@gmail.com

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