Dental Research Journal

Original Article

Correlation between dental caries experience and mutans streptococci counts by microbial and molecular (polymerase chain reaction) assay using saliva as microbial risk indicator

S. G. Damle[1], Ashish Loomba[1], Abhishek Dhindsa[1], Ashu Loomba[2], Vikas Beniwal[3]

[1]Department of Pediatric and Preventive Dentistry, M.M. College of Dental Sciences and Research, M.M. University, [3]Department of Biotechnology, M.M. Engineering College, M.M. University, Mullana, Haryana, [2]Department of Public Health Dentistry, Dr. Loomba's Family Dental Clinic, Mohali, Punjab, India

ABSTRACT

Background: The aim of this study was to assess the relationships of quantitative salivary levels of mutans streptococci (MS) in children, aged 3–6 years and 12–15 years, exhibiting variable patterns of caries activity, and to compare the association of MS in saliva using microbial and molecular (polymerase chain reaction [PCR]) assay.

Materials and Methods: In this cross-sectional observational study, eighty children were included, forty children each in Group I (3–6 years) and Group II (12–15 years). Children were further divided into two subgroups (Group IC, INC and Group IIC, IINC) based on their dental caries status. Saliva samples were collected and plated onto Mitis Salivarius-Bacitracin agar plates. After detection of MS, DNA was isolated and purified, and MS were evaluated using the PCR and AP-PCR.

Results: Of the selected 80 children, 42 were male and 38 were female. In Group IC, the mean colony count was $2.27 \pm 0.54 \times 10^5$ CFU/ml, and in Group INC, the mean colony was found to be $1.61 \pm 0.54 \times 10^5$ CFU/ml. In Group II, where mean colony count of $3.31 \pm 0.85 \times 10^5$ CFU/ml and $2.44 \pm 0.54 \times 10^5$ CFU/ml was observed in Group IIC and Group IINC, respectively.

Conclusion: The mean colony count increased with increasing age and was also more in children with dental caries. Based on the matrices generated by the PCR analysis using coefficient slipped-strand mispairing, wide range of genetic diversity was seen in cases of children with and without clinically detectable caries.

Key Words: Dental caries, polymerase chain reaction, saliva

Received: May 2016
Accepted: October 2016

Address for correspondence:
Dr. Ashish Loomba,
Department of Pediatric and Preventive Dentistry,
M.M. College of Dental Sciences and Research,
M.M. University, Ambala,
Mullana - 133 207, Haryana,
India.
E-mail: ashish.loomba23@gmail.com

INTRODUCTION

Human oral cavity is a diverse environment with hard and soft tissues comprising a total area of 215 cm^2 bathed in saliva.[1] The oral environment is thus optimal for microorganisms to grow, similar in their architecture and characteristics to biofilms in nature.[2] Oral microbes colonize an infant's oral cavity soon after the birth.[3] Their number increases gradually, owing to the exposure from the external microbial sources.[4] With the eruption of primary teeth, the number and complexity of the microflora in the oral environment

Access this article online

Website: www.drj.ir
www.drjjournal.net
www.ncbi.nlm.nih.gov/pmc/journals/1480

This is an open access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 3.0 License, which allows others to remix, tweak, and build upon the work non-commercially, as long as the author is credited and the new creations are licensed under the identical terms.

For reprints contact: reprints@medknow.com

How to cite this article: Damle SG, Loomba A, Dhindsa A, Loomba A, Beniwal V. Correlation between dental caries experience and mutans streptococci counts by microbial and molecular (polymerase chain reaction) assay using saliva as microbial risk indicator. Dent Res J 2016;13:552-9.

increase progressively.[5] Mutans streptococci (MS) are of special interest in cariogenesis.[6] They are a group of bacterial species characterized by their ability to produce extracellular glucans from sucrose and by their acid production in animal and human studies.[7]

Based on a strong correlation between microorganism counts in saliva and plaque, MS determination in saliva has been recommended as a suitable method for identifying patients at high risk of dental caries.[7-9] As saliva is continuously in contact with all the teeth, it provides a better reflection of the colonization of MS.

Over the last two decades, several molecular typing methods have become available. Molecular methods allow determining intraspecies differences with higher reproducibility than the phenotypic ones. In particular, polymerase chain reaction (PCR)-based methods and denaturing gradient gel electrophoresis have been widely used.[9] PCR proficiently detects the presence of MS stains in saliva, using specific primers for the genes that encode glucosyltransferase (gtfB in *Streptococcus mutans* and gtfI in *Streptococcus sobrinus*), which can be used to assess the prevalence of these organisms in the sample.

Therefore, the present study was designed and carried out to assess the distribution of MS in children with and without clinically detectable dental caries between the age group of 3–6 years and 12–15 years by microbial and molecular (PCR) assay and to check the association between socioeconomic class, frequency of tooth brushing, rinse after meals, diet, and mean MS count.

MATERIALS AND METHODS

This institutionally approved, cross-sectional observational study using stratified random sampling consisted of 80 children (40 children of 3–6 years of age and 40 of 12–15 years). A total of 600 schoolchildren, from 2 schools (300 children from a school in Barara City and 300 children from a school in Mullana City, Ambala District) situated in the vicinity of MM College of Dental Sciences and Research, Mullana, were screened, and of them, 80 children (42 males and 38 females) met the inclusion criteria and who were ready to comply with the study protocol were finally included. The sample size was calculated for α error fixed at <5% ($P < 0.05$) and β fixed at 20%, expected mean difference = 2.691 and standard deviation = 2.319. The study objective and the protocol were explained in detail to parents in their local language. The participation in the study was completely voluntary, and the parents who gave written consent for participation of their children were included. Saliva samples of the enrolled children were collected, and the ground water fluoride assessment was done. It found to contain 0.03 ppm of fluoride. The selected children had same cultural practices, living standards, and dietary habits.

Children belonging to two sets of age groups were analyzed for the study, i.e., 3–6 years (having complete deciduous dentition) and 12–15 years (having complete permanent dentition except third molars). Dental caries was recorded as per criteria described by the WHO 2004. The included children were divided into four groups.

- Group IC – Twenty children in the age group of 3–6 years, having 3 or more clinically detectable carious lesions
- Group INC – Twenty children with no clinically detectable caries, in the age group of 3–6 years
- Group IIC – Twenty children, in the age group of 12-15 years, having 3 or more clinically detectable carious lesions
- Group IINC – Twenty children with no clinically detectable caries, in the age group of 12–15 years.

Inclusion and exclusion criteria

The children exhibiting good general health, with a consistent attendance in school and no history of intake of antibiotics and fluoride for the last 6 months, were selected for the study. Medically compromised children and those with a physical limitation or motor in-coordination were excluded from the study. Furthermore, the ones undergoing orthodontic therapy, which might preclude normal tooth brushing, were not included. Children having more than 3 clinically detectable caries were included in the caries-active group and children without any clinically detectable caries were included in caries-free group.

Collection of saliva and bacterial culturing

The saliva samples of the children were collected using a sterile cotton swab, kept below the ventral surface of the tongue until saturated, and were transferred immediately to the duly labeled screw capped sterile polypropylene tubes. The samples were then dispersed from swabs and diluted in Todd Hewitt Broth.

Mitis Salivarius-Bacitracin (MSB**)** agar was prepared by adding 0.2 units of bacitracin/ml and sucrose to a

concentration of 20%. Aliquots of each dilution were inoculated in MSB agar plates, and the plates were then incubated at 37°C for 48 h in the candle jar containing 5% CO_2. For examination, smears on plain slides were prepared, Gram staining was performed, and stained sections were examined using a Nikon research microscope (ECLIPSE 80i), with CCD video camera (NIKON DS.U2, 5.03) at ×100 resolution in oil immersion.

The colonies were identified by their morphology, i.e., whether they were round/spherical/raised/blue in color, ranged from pinpoint to pinhead size with a rough surface and detachable from the agar surface [Figure 1]. A few colonies from the cultures, selected randomly, were tested biochemically using mannitol fermentation, oxidase and catalase tests. The colony counts of the children with and without active carious lesions in CFU/ml were counted using colony counter.

DNA isolation

The procedure of DNA isolation, digestion, and electrophoresis and gel analysis was done as per the method described by Bert *et al.*[10] The bacterial identification of MS was performed by PCR.

DNA from strains was extracted using a simple DNA preparation. The cells were washed twice in TE buffer (1 M Tris-Cl, pH 8.0, 0.5 M ethylenediaminetetraacetic acid [EDTA], pH 8.0), centrifuged, and resuspended in a TE buffer. DNA was released from bacterial cells by incubation with 30 µl of 10% sodium dodecyl sulfate and 10 mg/ml lysozyme for 30 min at 60°C, followed by incubation with 10 mg/ml proteinase K for 1 h at 65°C. Then, 100 µl of 5 M NaCl and 80 µl of cetyltrimethylammonium bromide/NaCl were added and mixed thoroughly and incubated at 65°C for 10 min. The pellets were treated several times with phenol:chloroform:isoamyl alcohol (25:24:1), and finally, DNA was precipitated with cold ethanol. DNA was dried, suspended in TE buffer, and stored in −20°C. This procedure was followed for all the cultures [Figure 2].

Polymerase chain reaction identification and arbitrarily primed-polymerase chain reaction typing

The DNA samples from MS isolates were identified by PCR with primers: *S. mutans* primer pair GTFB-F, 5’-ACT ACA CTT TCG GGT GGC TTG G-3’ and GTFB-R, 5’-CAG TAT AAG CGC CAG TTT CAT C-3’ (517 base pair [bp]). PCR amplification

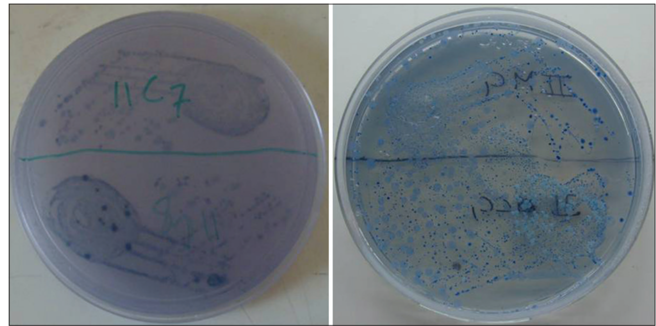

Figure 1: Photograph showing Mitis Salivarius-Bacitracin agar plates with the growth of mutans streptococci.

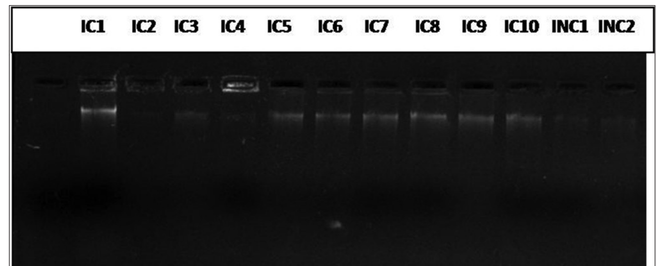

Figure 2: Photograph showing images obtained after DNA extraction.

was performed with an Applied Biosystems, 5720 Thermocycler (PCR system), under thermal conditions. The PCR amplification products were separated by electrophoresis in 0.8% agarose gel with Tris-acetate-EDTA running buffer (pH 8.0).

Strains identified as MS by PCR method were used for genotyping. The arbitrarily primed-PCR (AP-PCR) fingerprinting was performed with primer OPA-13 (5’-CAGCACCCAC-3’). The temperature profile in an Applied Biosystems, 5720 thermocycler, was 45 cycles of denaturation at 94°C for 30 s, annealing at 36°C for 30 s and 72°C for 1 min, with an initial denaturation at 94°C for 5 min and a final extension at 72°C for 5 min. Amplification products were analyzed electrophoretically with a 0.8% agarose gel with Tris-acetate-EDTA running buffer (pH 8.0) [Figure 3].

Analysis of the gels

1000 bp DNA ladder (BIORON) was used as a molecular marker. Each gel had 13 wells. The molecular marker was run in the first well, and the DNA isolates of each of the children with and without active carious lesion were run in remaining 12 wells.

The band patterns obtained on the gels were viewed on an ultraviolet viewer, and the gels were photographed using a digital camera [Figure 4]. The

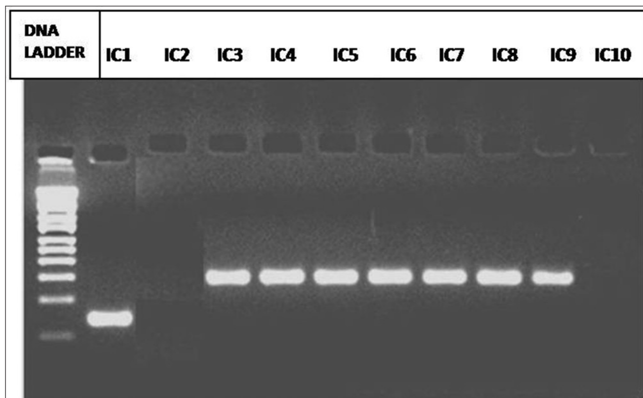

Figure 3: Photograph showing images obtained after polymerase chain reaction.

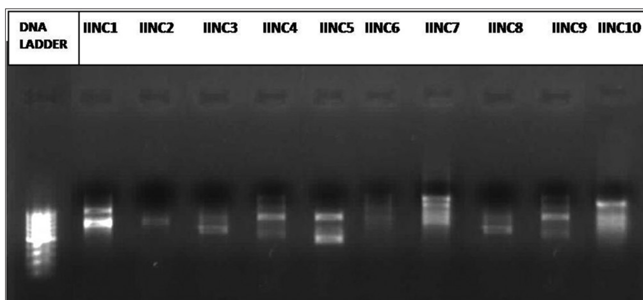

Figure 4: Photograph showing images obtained after arbitrarily primed-polymerase chain reaction.

band patterns for each of the children were compared with the standard (molecular marker) for the similarity in appearance.

RESULTS

The age of children in Group I ranged from 3 to 6 years with a mean age of 4.8 ± 0.6 years while that of Group II ranged from 12 to 15 years with a mean age of 13.6 ± 0.5 years. In Group I, the number of males was 18 (10 in Group IC and 8 in Group INC) and females was 22 (10 in Group IC and 12 in Group INC), whereas in Group II, the number of males was more than females, i.e., 24 males (12 in Group IIC and 12 in Group IINC) and 16 females (8 in Group IIC and 8 in Group IINC); the difference in gender-wise distribution was statistically insignificant ($P = 0.761$).

The mean MS count in female subjects (1.97 ± 0.62 × 10^5 CFU/ml) of Group I was slightly more than that of male subjects (1.91 ± 0.65 × 10^5 CFU/ml). In Group II, males had higher colony count (2.90 ± 0.91 × 10^5 CFU/ml) than females (2.83 ± 0.72 × 10^5 CFU/ml). On statistical analysis, the difference in gender was not found to be significant ($P = 0.328$). With the increase in age, children demonstrated a significant increase in the mean colony count. It was observed that the mean colony count in Group I was 1.941 ± 0.63 × 10^5 CFU/ml, whereas in Group II, the mean colony count was 2.876 ± 0.85 × 10^5 CFU/ml ($P = 0.031$).

The difference of mean MS count in children of Group I with clinically detectable dental caries was significantly more than the children without clinically detectable dental caries ($P < 0.001$). In Group IC, the mean colony count was 2.27 ± 0.54 × 10^5 CFU/ml, and in Group INC, the mean colony was found to be 1.61 ± 0.54 × 10^5 CFU/ml. A similar trend was seen in Group II, where mean colony count of 3.31 ± 0.85 × 10^5 CFU/ml and 2.44 ± 0.54 × 10^5 CFU/ml was observed in Group II C and Group II NC, respectively [Table 1].

The mean dmft (decayed, missing and filled teeth in deciduous dentition) of 40 children of Group I was found to be 3.90 ± 0.509, which was comparatively greater than the DMFT (decayed, missing and filled teeth in permanent dentition) of 40 children in Group II which was 2.95 ± 2.891. This difference in the mean dmft/DMFT in both groups was not found to be statistically significant ($P = 0.063$).

The mean colony count among children with clinically detectable dental caries showed an increase with the increasing age, whereas dmft/DMFT was found to be more in children between the age group of 3 and 6 years. The mean colony count in Groups IC and IIC was 2.27 ± 0.54 × 10^5 CFU/ml and 3.31 ± 0.85 × 10^5 CFU/ml, respectively. The mean dmft/DMFT in Groups IC and IIC was 6.55 ± 2.30 and 5.55 ± 1.57, respectively. Comparison between mean dmft/DMFT and mean colony count was statistically significant for both groups. A similar trend was seen among children with no clinically detectable dental caries. The mean colony count of children in Group INC was found to be 1.61 ± 0.52 × 10^5 CFU/ml compared to 2.44 ± 0.54 × 10^5 CFU/ml in Group IINC. The mean dmft of children in Group INC was 1.25 ± 1.07, whereas in Group IINC, the mean DMFT was 0.35 ± 0.67. Comparison between mean dmft/DMFT and mean colony count was statistically insignificant for both groups.

No significant association was found between socioeconomic class, frequency of tooth brushing,

Table 1: Comparison of mean colony count of mutans streptococci and dmft/DMFT in Group I and Group II

Study groups	Mean colony count (CFU/ml×10^5)	*P*	Subgroups	Number of children	dmft/DMFT and CFU/ml×10^5	Mean	SD	Range
Group I	1.941	0.014	IC	20	dmft	6.55	2.30	4-12
					CFU/ml×10^5	2.27	0.54	1.27-3.54
			INC	20	dmft	1.25	1.07	0-3
					CFU/ml×10^5	1.61	0.54	0.54-2.71
Group II	2.876		IIC	20	DMFT	5.55	1.57	3-9
					CFU/ml×10^5	3.31	0.85	1.83-5.34
			IINC	20	DMFT	0.35	0.67	0-2
					CFU/ml×10^5	2.44	0.54	1.56-3.80

DMFT: Decayed, missing and filled teeth in permanent dentition; dmft: Decayed, missing and filled teeth in deciduous dentition; SD: Standard deviation

rinse after meals, diet, and mean MS count. However, there was a bleakly noticeable association between the socioeconomic status and the mean colony count (*P* = 0.132). It was observed that the mean colony count was maximum in child belonging to a lower socioeconomic class, i.e., 4.0 × 10^5 CFU/ml. Association of the frequency of tooth brushing was statistically insignificantly correlated to the mean MS count. Maximum colony count was observed in children who do not brush everyday (2.58 ± 0.52 × 10^5 CFU/ml) followed by children who brush once daily (2.49 ± 0.65 × 10^5 CFU/ml) and twice a day (2.41 ± 0.57 × 10^5 CFU/ml). Association of rinse after meals and diet on mean colony count was also found to be statistically insignificant [Table 2].

When frequencies of identification of MS from MSB and PCR were evaluated, it was observed that 8 children in Group IC, 9 children in Group INC, 9 children in Group IIC, and all 10 children in Group IINC showed positive results [Table 3].

Dendrogram analysis of arbitrarily primed-polymerase chain reaction fingerprinting profile analysis

The AP-PCR fingerprinting profile analysis with primer OPA-13 showed distinct genotypes patterns of MS obtained from saliva samples of children with and without clinically detectable dental caries.

Based on the matrices generated by the PCA analysis using coefficient slipped-strand mispairing (SSM), the genetic similarity levels were obtained among the MS strains [Figure 5]. In this study, the same genotypic pattern was considered as identical or related samples with genetic similarity (SSM) ≥0.650 (threshold).

A wide range of genetic diversity was seen in cases of children with and without clinically detectable caries with SSM value ranging from 0.04 to 0.371. Only four out of twenty children with clinically

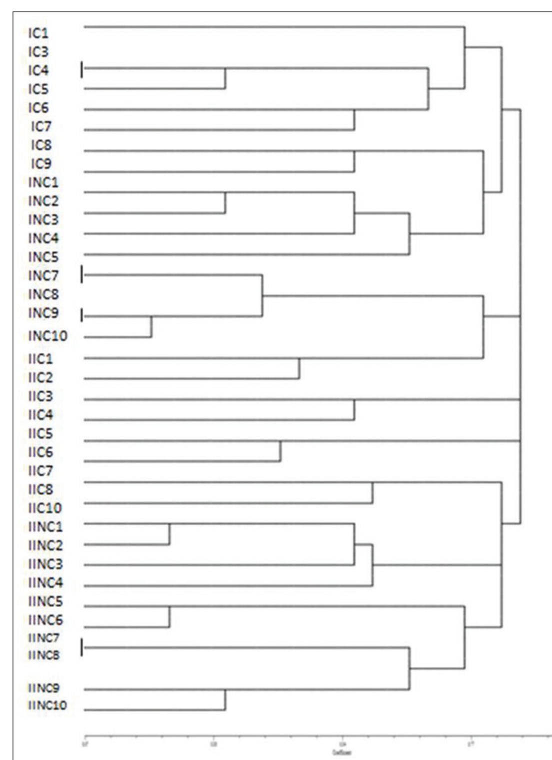

Figure 5: Dendrogram analysis of arbitrarily primed-polymerase chain reaction findings.

detectable dental caries showed two genotypes, whereas in children without clinically detectable dental caries, only two children out of twenty showed two genotypes in AP-PCR.

DISCUSSION

A group of phenotypically similar organisms collectively called as MS has been implicated as the principal microbial component liable for dental caries. MS adhere to the tooth surfaces enmeshed in dental plaque. The presence of MS continues to be one of the best indicators of dental caries development. Therefore, MS levels in children deliver valuable

information in relation to the development of caries patterns.[11-14]

Various methods have been utilized to identify the oral MS. A selective medium, Mitis-Salivarius Sucrose Bacitracin agar (MSB), has been the outmoded method for assessing microbial or epidemiological studies related to isolation, identification, and quantification of MS bacteria.[9,15] With the advent of modern techniques in molecular biology, the use of phenotypic methods has been largely replaced by genotypic techniques, which are more rapid and reliable. The characterization of oral bacteria by DNA probes,[16] 16S rDNA sequencing comparison methods,[17] PCR-restriction fragment length polymorphism,[18] PCR, AP-PCR, or DNA fingerprinting has become well accepted.[19,20]

The age group of 3–6 years was preferred as at this age, all the primary teeth are present in the oral cavity and also exposed to the oral environment continuously for 1–3 years. The other age group of 12–15 years was preferred as during this age, all the permanent teeth except third molars are present and exposed to the oral environment for a considerable duration.

Table 2: Association of socioeconomic class, frequency of tooth brushing, diet, and rinse after meals with mean colony count in all children

Variable	Number of cases	Mean CFU/ml × 10^5	SD	Significance of association (*P*)
SEC				
1 - upper	1	3.91	-	Mann-Whitney U-test 0.132
2 - upper middle	53	2.34	0.64	
3 - lower middle	12	2.52	0.73	
4 - upper lower	13	2.62	0.56	
5 - lower	1	4.0	-	
Frequency of brushing				
1 - once daily	61	2.49	0.65	0.989
2 - twice a day	12	2.41	0.57	
3 - not everyday	7	2.58	0.52	
Rinse after meals				
0 - no	22	2.34	0.88	0.831
1 - yes	58	2.55	0.56	
Diet				
1 - vegetarian	59	2.28	0.61	0.791
2 - mixed	21	2.47	0.49	

SEC: Socioeconomic classification; SD: Standard deviation

MS were identified in all the children (100%) in both groups by the microbial assay. No significant difference in the mean MS colony count was observed in male and female children. The observations were in accordance with the studies reported by Zickert *et al*.[21] and Hegde *et al*.[22] in the age group of 13–15 years. This may be due to the fact that newly emerged teeth represent a "virgin" habitat which enables MS to colonize the teeth, avoiding competition with other indigenous bacteria already established or better suited in adhering to enamel surface. The observations are in accordance with the findings reported by Wan *et al*.[23]

In the present study, a highly significant difference in mean MS colony count was observed between all the children in both the age groups (with and without clinically detectable dental caries). The observations are in complete agreement with the study reported by Twetman *et al*.[24] who reported that children with no detectable or low levels of MS had less caries experience irrespective of fluoride level in drinking water.

In the present study, a significant difference between the mean dmft/DMFT was recorded among the children with and without clinically detectable dental caries in both the age groups. Similar findings were reported by Bader *et al*. and Motohashi *et al*.[25]

A highly statistically significant linear correlation was observed in the mean dmft/DMFT and MS colony count, i.e., an increase in mean colony count with increasing dmft/DMFT was observed. These findings are in accordance with Loyola-Rodriguez *et al*.,[26] who investigated the distribution of MS in caries-free and caries-active preschool (3–6-year-old) Mexican children by microbial and molecular assay and found that dmft was positively correlated with mutans streptococci. In 12–15-year-old children with clinically detectable dental caries, a mean DMFT of 5.55 with MS colony count of 3.31 × 10^5 CFU/ml compared

Table 3: Frequencies of identification of mutans streptococci from mitis salivarius-bacitracin confirmed by polymerase chain reaction

Mutans streptococci confirmed by PCR	Group I		Group II		Total	
	Group IC	Group INC	Group IIC	Group IINC	Carious	Noncarious
Positive	8	9	9	10	17	19
Negative	2	1	1	0	3	1

PCR: Polymerase chain reaction

to mean DMFT of 0.35 with MS colony count of 2.44×10^5 CFU/ml in children with no clinically detectable dental caries was observed. Similar findings were observed by Hegde *et al*.[22] in 13–15-year-old schoolchildren. They also reported a highly significant relation between mean DMFT and MS load in saliva.

Statically no significant association was found between socioeconomic class, frequency of tooth brushing, rinse after meals, diet, and mean MS count. There was a slight association of socioeconomic class with mean colony count ($P = 0.132$, $P > 0.05$). It was observed that the mean colony count was maximum in children belonging to the lower socioeconomic class.

In the present study, of the total eighty saliva samples, forty were subjected to PCR assay (10 from each group). It was observed that MS were detected in 90% of the saliva samples. PCR showed no results in four children, i.e., in IC2, IC10 (children with clinically detectable dental caries in 3–6 years of age), INC6 (children with no clinically detectable dental caries in 3–6 years of age), and in IIC9 (children with clinically detectable dental caries in 12–15 years of age). When MSB method was compared with PCR technique, there was no statistical difference, and the results are similar to the findings of Franco e Franco *et al*.,[27] who reported a prevalence of 85.7% MS in plaque samples by molecular assay.

The results gained with the present AP-PCR method suggested an insignificant relationship between dental caries activity and genetic diversity. The observations are in accordance with the previous studies carried out by Lembo *et al*.[28] and Kreulen *et al*.,[29] who reported significant genetic variability of MS. The genotypic similarity was less than previously reported study done by Pieralisi *et al*.,[30] in which 17 out of 28 samples confirmed genotypic similarity with more than 1 genotype.

Despite some paucity, such as the difficulty to visualize low-intensity bands and the need for more than one primer to increase the technique accuracy, studies have shown the efficacy of AP-PCR in the detection of genetic polymorphism of various bacterial species, obtaining similar results to more sophisticated techniques.[21]

CONCLUSIONS

Based on the observations, the following conclusions can be made:

- The number of MS increases with increasing age
- Number of MS is more in children with clinically detectable dental caries than children without any clinically detectable dental caries
- Molecular methodology (PCR) is a useful tool in molecular epidemiology for dental caries studies and is effective in detecting and identifying MS from saliva in children
- Based on the matrices generated by the PCR analysis, a wide range of genetic diversity was seen using coefficient SSM in cases of children with and without clinically detectable caries.

Financial support and sponsorship

Nil.

Conflicts of interest

The authors of this manuscript declare that they have no conflicts of interest, real or perceived, financial or non-financial in this article.

REFERENCES

1. Nanda J, Sachdev V, Sandhu M, Deep-Singh-Nanda K. Correlation between dental caries experience and mutans streptococci counts using saliva and plaque as microbial risk indicators in 3-8 year old children. A cross sectional study. J Clin Exp Dent 2015;7:e114-8.
2. Auschill TM, Arweiler NB, Netuschil L, Brecx M, Reich E, Sculean A. Spatial distribution of vital and dead microorganisms in dental biofilms. Arch Oral Biol 2001;46:471-6.
3. Saraithong P, Pattanaporn K, Chen Z, Khongkhunthian S, Laohapensang P, Chhun N, *et al. Streptococcus mutans* and *Streptococcus sobrinus* colonization and caries experience in 3- and 5-year-old Thai children. Clin Oral Investig 2015;19:1955-64.
4. Pearce C, Bowden GH, Evans M, Fitzsimmons SP, Johnson J, Sheridan MJ, *et al.* Identification of pioneer viridans streptococci in the oral cavity of human neonates. J Med Microbiol 1995;42:67-72.
5. Rotimi VO, Duerden BI. The development of the bacterial flora in normal neonates. J Med Microbiol 1981;14:51-62.
6. Seki M, Yamashita Y, Shibata Y, Torigoe H, Tsuda H, Maeno M. Effect of mixed mutans streptococci colonization on caries development. Oral Microbiol Immunol 2006;21:47-52.
7. Marsh PD. Are dental diseases examples of ecological catastrophes? Microbiology 2003;149(Pt 2):279-94.
8. Fejerskov O. Changing paradigms in concepts on dental caries: Consequences for oral health care. Caries Res 2004;38:182-91.
9. Aas JA, Griffen AL, Dardis SR, Lee AM, Olsen I, Dewhirst FE, *et al.* Bacteria of dental caries in primary and permanent teeth in children and young adults. J Clin Microbiol 2008;46:1407-17.
10. Bert F, Picard B, Branger C, Lambert-Zechovsky N. Analysis of genetic relationships among strains of groups A, C and G streptococci by random amplified polymorphic DNA analysis. J Med Microbiol 1996;45:278-84.

11. Bobu LI, Bârlean L, Bârlean M. Levels of mutans streptococci among schoolchildren in Iasi, Romania. Rev Med Chir Soc Med Nat Iasi 2015;119:996-1000.
12. Mitrakul K, Vongsawan K, Sriutai A, Thosathan W. Association between *S. mutans* and *S. sanguinis* in severe early childhood caries and caries-free children a quantitative real-time PCR analysis. J Clin Pediatr Dent 2016;40:281-9.
13. Lif Holgerson P, Öhman C, Rönnlund A, Johansson I. Maturation of oral microbiota in children with or without dental caries. PLoS One 2015;10:e0128534.
14. Lynch DJ, Villhauer AL, Warren JJ, Marshall TA, Dawson DV, Blanchette DR, *et al.* Genotypic characterization of initial acquisition of *Streptococcus mutans* in American Indian children. J Oral Microbiol 2015;7:27182.
15. Svensäter G, Larsson UB, Greif EC, Cvitkovitch DG, Hamilton IR. Acid tolerance response and survival by oral bacteria. Oral Microbiol Immunol 1997;12:266-73.
16. Cangelosi GA, Iversen JM, Zuo Y, Oswald TK, Lamont RJ. Oligonucleotide probes for mutans streptococci. Mol Cell Probes 1994;8:73-80.
17. Kawamura Y, Hou XG, Sultana F, Miura H, Ezaki T. Determination of 16S rRNA sequences of *Streptococcus mitis* and *Streptococcus gordonii* and phylogenetic relationships among members of the genus *Streptococcus*. Int J Syst Bacteriol 1995;45:406-8.
18. Igarashi T, Ichikawa K, Yamamoto A, Goto N. Identification of mutans streptococcal species by the PCR products of the dex genes. J Microbiol Methods 2001;46:99-105.
19. Liu Y, Zou J, Shang R, Zhou XD. Genotypic diversity of *Streptococcus mutans* in 3- to 4-year-old Chinese nursery children suggests horizontal transmission. Arch Oral Biol 2007;52:876-81.
20. Napimoga MH, Kamiya RU, Rosa RT, Rosa EA, Höfling JF, Mattos-Graner R, *et al.* Genotypic diversity and virulence traits of *Streptococcus mutans* in caries-free and caries-active individuals. J Med Microbiol 2004;53(Pt 7):697-703.
21. Zickert I, Emilson CG, Krasse B. *Streptococcus mutans*, lactobacilli and dental health in 13-14-year-old Swedish children. Community Dent Oral Epidemiol 1982;10:77-81.
22. Hegde PP, Ashok Kumar BR, Ankola VA. Dental caries experience and salivary levels of *Streptococcus mutans* and lactobacilli in 13-15 years old children of Belgaum city, Karnataka. J Indian Soc Pedod Prev Dent 2005;23:23-6.
23. Wan AK, Seow WK, Purdie DM, Bird PS, Walsh LJ, Tudehope DI. A longitudinal study of *Streptococcus mutans* colonization in infants after tooth eruption. J Dent Res 2003;82:504-8.
24. Twetman S, Mattiasson A, Varela, Bratthall D. Mutans streptococci in saliva and dental caries in children living in a high and a low fluoride area. Oral Microbiol Immunol 1990;5:169-71.
25. Motohashi M, Yamada H, Genkai F, Kato H, Imai T, Sato S, *et al.* Employing dmft score as a risk predictor for caries development in the permanent teeth in Japanese primary school girls. J Oral Sci 2006;48:233-7.
26. Loyola-Rodriguez JP, Martinez-Martinez RE, Flores-Ferreyra BI, Patiño-Marin N, Alpuche-Solis AG, Reyes-Macias JF. Distribution of *Streptococcus mutans* and *Streptococcus sobrinus* in saliva of Mexican preschool caries-free and caries-active children by microbial and molecular (PCR) assays. J Clin Pediatr Dent 2008;32:121-6.
27. Franco e Franco TC, Amoroso P, Marin JM, de Avila FA. Detection of *Streptococcus mutans* and *Streptococcus sobrinus* in dental plaque samples from Brazilian preschool children by polymerase chain reaction. Braz Dent J 2007;18:329-33.
28. Lembo FL, Longo PL, Ota-Tsuzuki C, Rodrigues CR, Mayer MP. Genotypic and phenotypic analysis of *Streptococcus mutans* from different oral cavity sites of caries-free and caries-active children. Oral Microbiol Immunol 2007;22:313-9.
29. Kreulen CM, de Soet HJ, Hogeveen R, Veerkamp JS. *Streptococcus mutans* in children using nursing bottles. ASDC J Dent Child 1997;64:107-11.
30. Pieralisi FJ, Rodrigues MR, Segura VG, Maciel SM, Ferreira FB, Garcia JE, *et al.* Genotypic diversity of *Streptococcus mutans* in caries-free and caries-active preschool children. Int J Dent 2010;2010:824976.