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

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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 ± 0.54 × 10⁵ CFU/ml, and in Group INC, the mean colony was found to be 1.61 ± 0.54 × 10⁵ CFU/ml. In Group II, where mean colony count of 3.31 ± 0.85 × 10⁵ CFU/ml and 2.44 ± 0.54 × 10⁵ 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

INTRODUCTION

Human oral cavity is a diverse environment with hard and soft tissues comprising a total area of 215 cm² 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...
increase progressively.\textsuperscript{[5]} Mutans streptococci (MS) are of special interest in cariogenesis.\textsuperscript{[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.\textsuperscript{[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.\textsuperscript{[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.\textsuperscript{[9]} PCR proficiently detects the presence of MS stains in saliva, using specific primers for the genes that encode glucosyltransferase (gtfB in \textit{Streptococcus mutans} and gtfI in \textit{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 \( \alpha \) error fixed at \(<5\% \ (P < 0.05) \) and \( \beta \) 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.

Mutis 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% CO₂. 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. 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 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].
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 ($1.97 \pm 0.62 \times 10^5$ CFU/ml) of Group I was slightly more than that of male subjects ($1.91 \pm 0.65 \times 10^5$ CFU/ml). In Group II, males had higher colony count ($2.90 \pm 0.91 \times 10^5$ CFU/ml) than females ($2.83 \pm 0.72 \times 10^5$ 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 $1.941 \pm 0.63 \times 10^5$ CFU/ml, whereas in Group II, the mean colony count was $2.876 \pm 0.85 \times 10^5$ 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 $2.27 \pm 0.54 \times 10^5$ CFU/ml, and in Group INC, the mean colony was found to be $1.61 \pm 0.54 \times 10^5$ CFU/ml. A similar trend was seen 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 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 $3.90 \pm 0.509$, which was comparatively greater than the DMFT (decayed, missing and filled teeth in permanent dentition) of 40 children in Group II which was $2.95 \pm 2.891$. 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 $2.27 \pm 0.54 \times 10^5$ CFU/ml and $3.31 \pm 0.85 \times 10^5$ CFU/ml, respectively. The mean dmft/DMFT in Groups IC and IIC was $6.55 \pm 2.30$ and $5.55 \pm 1.57$, 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 $1.61 \pm 0.52 \times 10^5$ CFU/ml compared to $2.44 \pm 0.54 \times 10^5$ CFU/ml in Group IINC. The mean dmft of children in Group INC was $1.25 \pm 1.07$, whereas in Group IINC, the mean DMFT was $0.35 \pm 0.67$. 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,
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., $4.0 \times 10^5$ 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 ($2.58 \pm 0.52 \times 10^6$ CFU/ml) followed by children who brush once daily ($2.49 \pm 0.65 \times 10^6$ CFU/ml) and twice a day ($2.41 \pm 0.57 \times 10^6$ 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) $\geq 0.650$ (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 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.\textsuperscript{[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.\textsuperscript{[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,\textsuperscript{[16]} 16S rDNA sequencing comparison methods,\textsuperscript{[17]} PCR-restriction fragment length polymorphism,\textsuperscript{[18]} PCR, AP-PCR, or DNA fingerprinting has become well accepted.\textsuperscript{[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.

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 \textit{et al.}\textsuperscript{[21]} and Hegde \textit{et al.}\textsuperscript{[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 \textit{et al.}\textsuperscript{[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 \textit{et al.}\textsuperscript{[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 \textit{et al.} and Motohashi \textit{et al.}\textsuperscript{[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 \textit{et al.},\textsuperscript{[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 \(3.31 \times 10^5\) CFU/ml compared

\begin{table}[h]
\centering
\caption{Association of socioeconomic class, frequency of tooth brushing, diet, and rinse after meals with mean colony count in all children}
\begin{tabular}{|l|c|c|c|c|}
\hline
\textbf{Variable} & \textbf{Number of cases} & \textbf{Mean CFU/ml} & \textbf{SD} & \textbf{Significance of association (P)} \\
\hline
\textbf{SEC} & & & & \\
1 - upper & 1 & 3.91 & - & Mann-Whitney \\
2 - upper middle & 53 & 2.34 & 0.64 & U-test 0.132 \\
3 - lower middle & 12 & 2.52 & 0.73 & \\
4 - upper lower & 13 & 2.62 & 0.56 & \\
5 - lower & 1 & 4.0 & - & \\
\hline
\textbf{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 & \\
\hline
\textbf{Rinse after meals} & & & & \\
0 - no & 22 & 2.34 & 0.88 & 0.831 \\
1 - yes & 58 & 2.55 & 0.56 & \\
\hline
\textbf{Diet} & & & & \\
1 - vegetarian & 59 & 2.28 & 0.61 & 0.791 \\
2 - mixed & 21 & 2.47 & 0.49 & \\
\hline
\end{tabular}
\end{table}

SEC: Socioeconomic classification; SD: Standard deviation

\begin{table}[h]
\centering
\caption{Frequencies of identification of mutans streptococci from mitis salivarius-bacitracin confirmed by polymerase chain reaction}
\begin{tabular}{|c|c|c|c|c|}
\hline
\textbf{Mutans streptococci confirmed by PCR} & \textbf{Group I} & \textbf{Group II} & \textbf{Total} \\
& \textbf{Group IC} & \textbf{Group INC} & \textbf{Group IIC} & \textbf{Group IINC} & \textbf{Curious} & \textbf{Noncurious} \\
\hline
Positive & 8 & 9 & 9 & 10 & 17 & 19 \\
Negative & 2 & 1 & 1 & 0 & 3 & 1 \\
\hline
\end{tabular}
\textbf{PCR: Polymerase chain reaction}
\end{table}
to mean DMFT of 0.35 with MS colony count of $2.44 \times 10^5$ CFU/ml in children with no clinically detectable dental caries was observed. Similar findings were observed by Hegde et al.[23] 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 Piteralisi 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.

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**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.

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