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Genotypic Diversity and Horizontal Transmission of *Streptococcus mutans* in Lower Socio-economic Caries in Active Children

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Abstract: This reports provides glimpses on genomic diversity of *Streptococcus mutants* among school going children which may help in the development of new treatment strategies for dental caries in order to prevent disease and promote health in addition to standard prevention treatments. Hence, this study was aimed to assess the genotypic diversity and to investigate the transmission of *Streptococcus mutans* genotypes among school going children. It was observed that detection of *S. mutans* isolates with high genotypic diversity in different individual indicated occurrence of transmissibility. A total of 179 isolates of *S. mutans* from caries active subjects were obtained from saliva, dental plaque, and carious material, and identify by PCR. The isolates were submitted to AP-PCR to establish the genotypic diversity and transmission. RFLP technique was used only on those isolates which were showing identical amplitypes in AP-PCR to confirm the transmission. RAPD analysis showed the high diversity of *S. mutans* genotypes within lower socioeconomic school going children which might have experienced multiple infections. The occurrence of 4 isolates with 100 % genetic similarity is indicative of horizontal transmission. This study may be important for leading the development caries prevention program worldwide.

Key words: *Streptococcus mutans*, genotypic diversity, RAPD analysis, RFLP technique.

Introduction

Presence of microorganisms is a natural part of the proper oral health. However, an imbalance in the microbial flora can lead to damage the teeth and gums. Dental caries is a transmissible infectious disease and *S. mutans* is generally considered to be the principal etiological agent for dental caries. The widely accepted theory for causing dental caries by *S. mutans* is production of water-insoluble glucan from sucrose by glucosyltransferase (GTF) in biofilm matrix [10], and the bacteria in the dental biofilm produce acids that decrease the pH and increase the biofilm potential in promoting dental demineralization [1]. It has been proved by several phenotyping and genotyping studies that mother is the chief and primary source of infection for children [11].

Some evidences have been reported of horizontal transmission among children of nursery school [5]. Zhan *et al.* [20] also observed nonmaterial as well maternal mutans Streptococci transmission. Apart from, other studies for other vertical

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sources like father and care taker are also been studied and found that, detection of genotypes of children was not found in their mothers or relatives indicated the existence of other sources of transmission [8],

Genomic diversity of *S. mutans* may be present because of above mentioned transmission from different sources or modification in base pair by deletion or insertion of new genetic sequencing [15]. Several techniques have been used to prove this genotypic diversity such as AP-PCR [3,14] multilocus enzyme electrophoresis (MLEE) [17], restriction fragment length polymorphism (RFLP) [5] and their relation with caries activity. All the above studies concluded that there is high degree of genomic diversity in children of 5-8 years. Therefore, present research was aimed to evaluate horizontal transmission of dental caries among school going children and genomic diversity of *S. mutans*.

Materials and methods

Subjects

The study groups consisted of 200 children, divided in two groups (A and B). Each group consisted 50 boys and 50 girls, aged 4 to 6 years, belonging to a low economic level. They stayed at the nursery school five days a week, 7-8 h per day. Preliminary examination was done by a trained examiner by using only a mouth mirror and light. The teeth were cleaned, dried with a cotton-wool roll and all surfaces were examined visually for dental caries. Written informed consent was obtained from parents of all individuals. All consent and experimental procedures were approved by the research committee of Barkatullah University of Bhopal, Bhopal, India.

Sampling

All children's underwent dental examination for the presence of teeth caries. Dental examinations were performed under natural light, using a plane dental mirror and explorer. Samples were collected from 62 caries active children by swab for 10 second or by scraping of plaque by spoon excavator after one hour of taking mid day meal. Sample collection was done in transporting medium (thioglycolate broth).

Microbiological processing

In order to detect *S. mutans*, samples were diluted in a saline solution (0.9 % NaCl). Aliquots of each dilution were inoculated in thioglycolate agar supplemented with 20 % sucrose, 2 discs of bacitracin (each contains 10 U) and 2 ml of blood. Plates were incubated at 37°C for 48 hrs in an atmosphere of 10 % CO_2. After incubation period, colonies were examined. Number of colonies with mutans-like morphology were obtained from the culture media and sub-cultured on mitis salivarius and tryptic soy agar plates, and pure cultures were then frozen at -70°C in 10 % skim milk. These strains were identified to species level biochemically.

Isolation of DNA and PCR analysis

DNA from a total of 180 *S. mutans* isolates was purified using Master Pure DNA purification kit (Hi-Media) according to the manufacturer's instructions. These DNA samples were identified as *S. mutans* by PCR using primers designed by Oho et al [13] to amplify a 517 bp sequence of the glucosyltransferase B gene (gtfB). The sequences of these primers were 5'-ACTACACTTTC-GGGTGGCTTGG-39 and 5'-CAGTATAAGCG-CCAGTTTCATC-3'. The PCR was processed in 25 µl of a reaction mixture containing 1 µl reaction buffer *Taq* polymerase, 1.5 mM $MgCl_2$, 0.1 mM deoxynucleoside triphosphate, 0.2 µM each primer, 1.5 U of *Taq* DNA polymerase, and 2.5 µl of DNA sample.

All PCR reagents were obtained from Hi-Media Biochem Life Sciences Genexy. Besides the samples, positive and negative controls were used in each experiment: purified genomic DNA from *S. mutans* (ATCC 25175) was used as positive controls, and distilled water was used as a negative control. Amplicons were separated by electrophoresis in 1.5 % agarose gels in Tris-borate-EDTA running buffer. Ethidium bromide-stained gel images were captured with a digital imaging system. A 100 base pair DNA ladder was used as molecular size marker.

RAPD analysis

Out of 180 isolates, 179 Identified as *S. mutans* were genotyped by AP-PCR. The sequences of

the primer and OPA 13 (5'CAGCACCCAC3') was used. The PCR reactions were performed as follows: 1x PCR buffer (10 mM Tris-HCl pH 8.4;) with 3.5 mM of $MgCl_2$, 0.2 mM of each dNTPs, 0.4 mM of primers, 1U of Taq DNA polymerase and 1.0 µl of DNA sample. The PCR conditions included 35 cycles of denaturation at 94ºC for 1 min, annealing at 36ºC for 2 min, extension at 72ºC for 2 min, with initial denaturation at 94ºC for 5 min and a final extension at 72ºC for 5 min. The electrophoresis was carried out as described above; however, the amplification products were analyzed in 2.5 % agarose gel.

16S rRNA restriction fragment length polymorphism

16S rRNA RFLP was done to confirm genotypic diversity for those subjects who were representing same RAPD profile.

RFLP patterns

The *Hae* III enzyme was used to digest the 16S rRNA gene of *S. mutans* yielding RFLP patterns in accordance with GeneBank. The gene contains 13 potential *Hae*III restriction sites. The primer sequences were as follows: 27 F 5'-AGAGTTTGATCCTGGCTCAG-3' 1492R 5'-TACGGGTACCTTGTTACGACTT-3'. A 10 µl of PCR products were transferred to the tubes. A 2 µl loading dye were added to all tubes and then loaded into agarose wells. Amplicons were resolved by electrophoresis and profiles visualize. Digestion master mix was prepared as follows: 195.6 µl sterile DH_2O was transferred to a fresh 0.5 ml tube; 24 µl 10x buffer was added, followed by 2.4 µl acetylated bovine serum albumin (10 mg/ml), making a total volume of 222 µl, and about 18.5 µl master mix was transferred to a series of fresh 0.5 ml tubes. One microliter of PCR product and about 0.5 µl *Hae* III was added to all preparations apart from one of the controls (i.e. containing neither DNA nor restriction enzyme), Samples were incubated at 37°C for 2.5 h. Restriction fragments were resolved by electrophoresis using 2 % agarose gel.

Data analysis

RAPD and RFLP bands were used to generate a binary (presence/absence) matrix, from which a genetic similarity matrix was computed using the Jaccard coefficient, as implemented in NTSYS 2.11 (Numerical Taxonomy System of Multivariate Programs). The UPGMA (Unweighted Pair-Group Method with Arithmetical Average) hierarchical method was used to group the units and to build the dendrogram. The software Boot 3.04 (6) was used for bootstrap analysis, which assesed the reliability of each grouping after 10.000 pseudo replicates.

Results

Prevalence and status of dental caries in low socioeconomic children

According to severity in caries active children, only 19.5 % girls were having severe dental caries with pain involve pulp, 50 % were having moderate, and 30.7 % were having low, involve only enamel. In boys, only 22.2 % were having severe dental caries, 55.5 % were having moderate, and 63.8% were low dental caries (Fig. 1).

Genomic diversity of *S. mutans* in school children

Strains of *S. mutans* produced characteristic colonies of about 1 mm in diameter, with beads, droplets or puddles containing soluble extracellular polysaccharide [6] with á or ã hemolysis. All identified 100 strains of section A and 79 strains from section B were genotyped by RAPD. Amplification of DNA from the *S. mutans* with the OPA 13 primer resulted in 6-9 fragments (amplicons), ranging from 0.5 to 2 Kb in size (Fig. 2a).

RAPD fingerprinting

In group A, out of 35 caries active children, one boy and three girls were carried only one genotype, remaining children were having more than one distinct amplitype. Among 100 *S. mutans* strains isolated from the children's oral cavity, 79 different genotypes were found. In group B, 54 different amplitypes were identified out of 76 strains. We observed high genetic diversity in caries active children. 2 girls and 3 boys carried more than two distinct amplitypes. One amplitype was found in one girl and 3 boys. Two amplitypes were identified in each of other children.

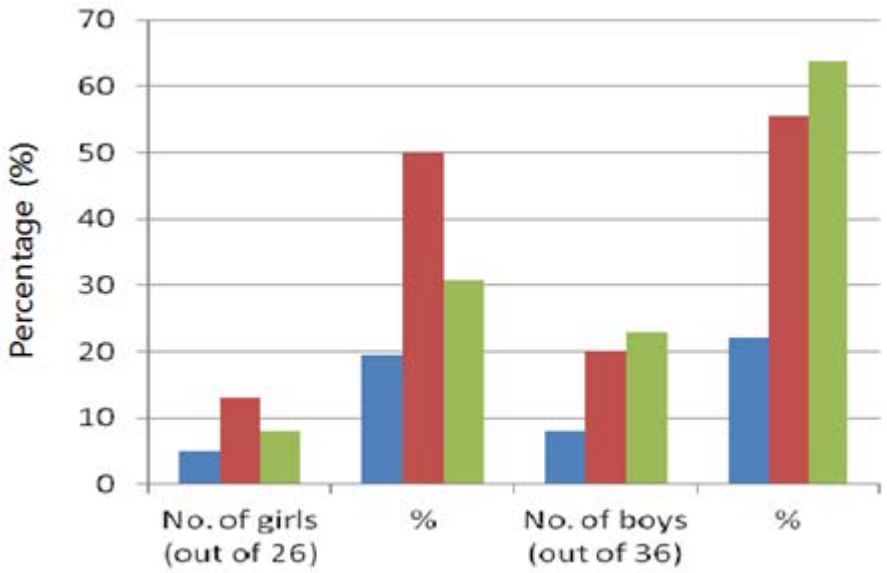

Fig. 1. Caries status in children. [ ] Severe with pain involve enamel, dentine and pulp; [ ] Moderate with no pain involve dentine; and [ ] low with no pain involve only enamel.

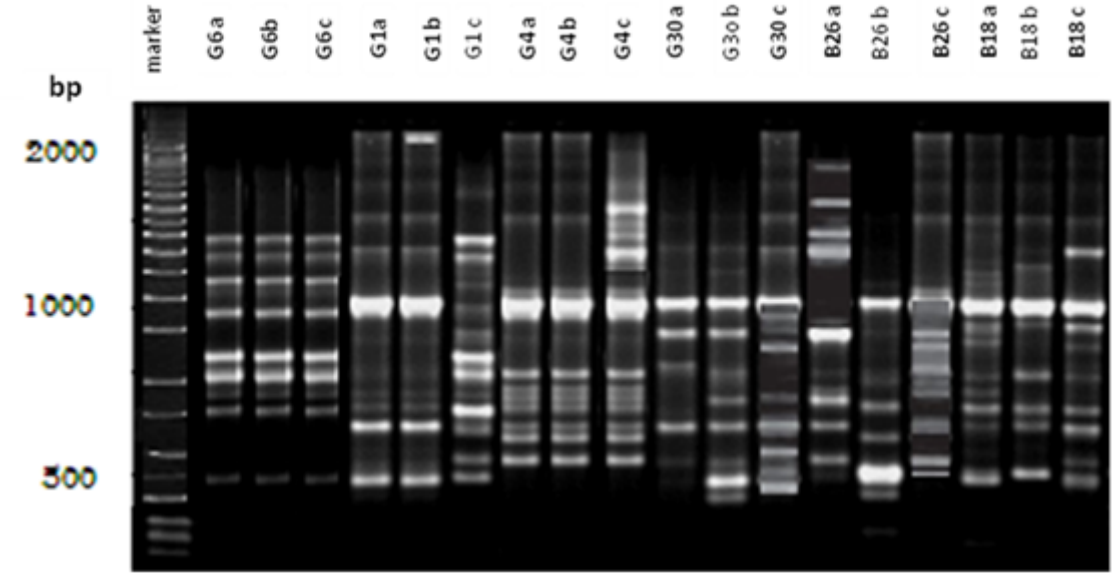

Fig. 2a. RAPD amplification profile obtained from *Streptococcus mutans* using the OPA 13 primer. Strains isolated from children; Marker: 100 bp DNA ladder

On RAPD analysis, we found in group A, B19 boys carried 3 genotypes in which one genotype was similar to B21 boy (B23 carried two genotype), and another pair of boys B26 and B27 girl shared same genotype in group B, B10 shared same genotype with C strains of B11 (Fig. 2b). Digestion of 16s rDNA of children pairs with *Hae III* enzyme showed similar result as in RAPD (Fig. 3).

Discussion

In the present study, children were selected from a low socioeconomic level received similar mid-day meal. Most of the children had chapatti or bread in their breakfast or nothing. In spite of belonging low socioeconomic status, the prevalence of dental caries was found to be very low in children. The overall prevalence of dental caries in age group of 4-5 year old children was

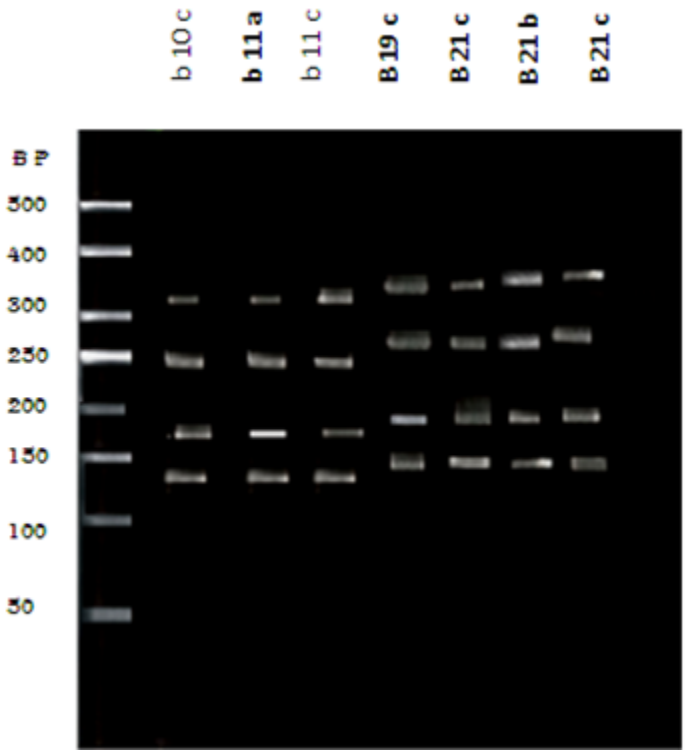

Fig. 2b. Restriction enzyme Hae III treated 16S rDNA segments of isolated strains of *Streptococcus mutans*

31 %, 26 % girls and 36 % boys were caries active. As compared to other studies which showed high prevalence of caries in school going children [4,7], present study observed no association between economic status and prevalence of dental caries. In dental caries, *S. mutans* plays very important role and its prevalence increases as child develops. The result of our study showed that *S. mutans* isolates displayed genomic diversity. Many children colonized with more than one genotype of *S. mutans*. In addition to this previously Napimoga *et al.* [12] proved that individual could be colonized with 8 genotypes of *S. mutans*. Similarly our present study showed higher genomic diversity from 176 isolates 133 distinct genotypes, which were properly identified. There are many reviews on positive relationship between genomic diversity and caries activity [21]. Lembo *et al.* [9] demonstrated that there was no significant relationship between genomic diversity and caries activity and also the genomic diversity increases with the age of the children.

Multiple genotypes found in oral cavity of one individual showed acquiesce of *S. mutans* from different sources such as through mother, father, sibling, care taker, spouse, and class mates etc. Studies using phenotyping and/or genotyping methods suggest that the mother is the major primary source of infection for children who carry *S. mutans* and/or *S. sobrinus* strains [18]. Zhan [20] observed non-maternal transmission is more prevalent than maternal in children with severe early dental caries. However, detection of genotypes that are not found in children's mothers or other family members indicates that *S. mutans* and/or *S. sobrinus* may also be acquired from other sources [10].

S. mutans matching genotypes in children from unrelated families and attending the same day nursery showed evidence for horizontal transmission reported in Brazilian [10] Japanese [19] and Chinese children [16]. Study was also done for the children older than 4 year (school going 5-7 age group) and showed similar result [2]. Present study hypothesized that *S. mutans* could be laterally transmitted among school children with prolonged

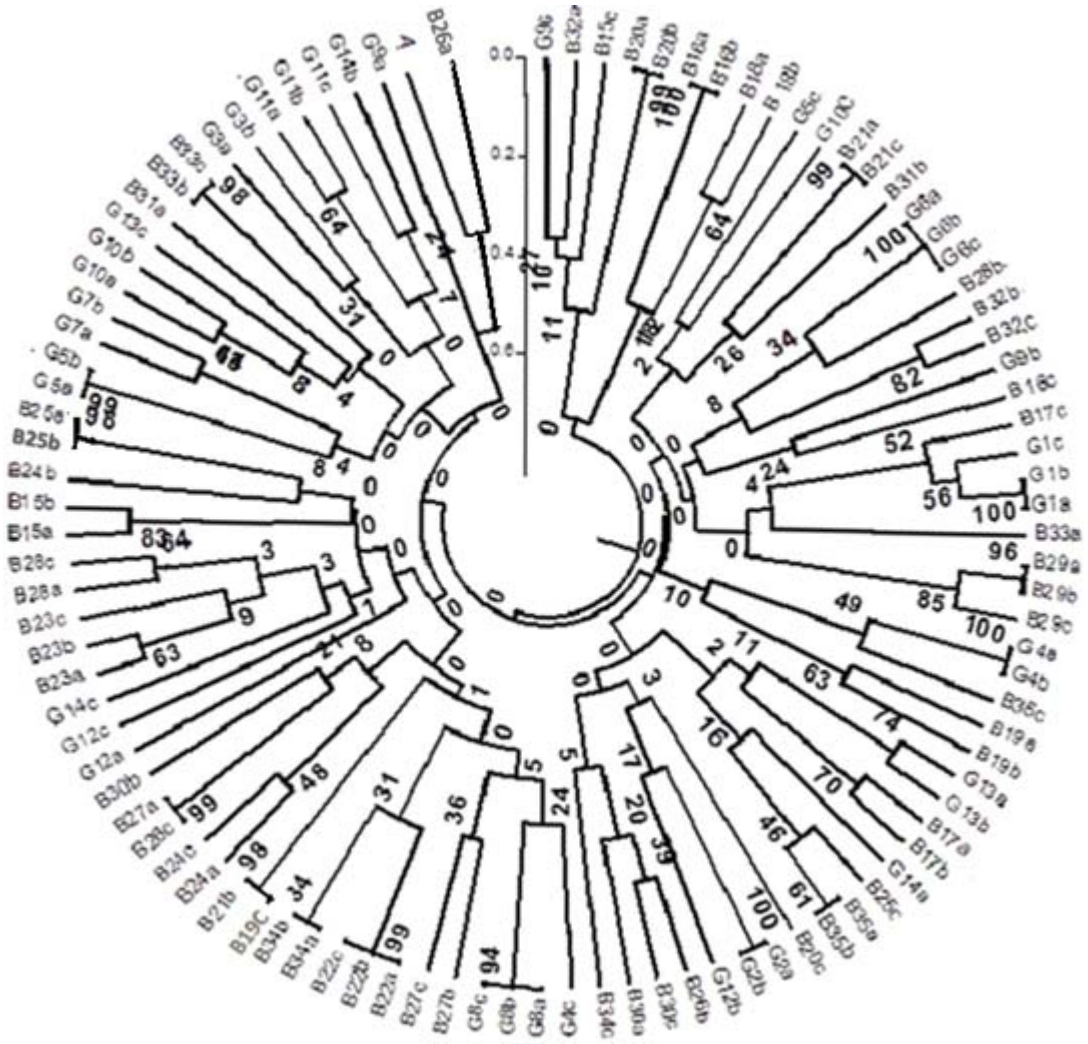

Fig. 3. Dendrogram (Group A) representing the genomic similarities obtained based on RAPD. Model: Jaccard's similarity coefficient, Methods: UPGMA Neighbor-Hood joining, Data Replication method: Bootstrap, number of replicates: 100. Software: PAST

exposure to an environment that favors the spread of infectious agents, having same mid-day meal and shorter periods of contact with mothers, hence horizontal transmission is suggested from the present study.

Conclusions

To our knowledge, this is the first report in India reported *S. mutans* matching genotypes in children from unrelated families that had no contact. Our study favors the horizontal transmission through the sharing the food and pacifiers. Since dental caries can potentially be prevented by interfering with transmission of *S. mutans*, this study can further direct the development of caries-preventive programs worldwide. It could also be recommended that several preventive programs could be beneficial to prevent the intra-familial transmission by directing antibacterial measures at highly colonized children.

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