Original research

Genotypic characterization of Streptococcus mutans in child-mother pair—A PCR based study

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ABSTRACT

Objectives: At present, there is lack of evidence in literature regarding the transmission of Streptococcus Mutans from a caries-free mother to child by genetic methods, so the present study aimed to identify the genetic characterization of Streptococcus Mutans strains isolated from caries-free and caries active children and their mother being caries-free in both the groups and also to identify the genetic patterns of Streptococcus Mutans between caries-free and caries-active individuals.

Methods: Twenty child-mother pair were selected and divided into 2 groups and the mothers being caries-free in both the groups. Saliva samples were collected using a sterile tube, followed by microbial culture of Streptococcus mutans, DNA isolation and PCR amplification. The molecular weights of each band were converted into a binary data and data were entered into SPSS software version 20.0 to generate similarity dendrograms.

Results: Amplified products of Streptococcus Mutans demonstrated a same genetic distance between the mother-child pair, indicative of a closely related species. Dendrogram interpretation represented a greater genetic polymorphism of Streptococcus Mutans between caries-free and caries-active children. **Conclusions:** Identical genetic distance between child-mother pair showed that, these Streptococcus Mutans were closely related and could have vertically transmitted from their mothers. Different genotypic pattern between caries-free and caries active subjects showed a genetic polymorphism among the Streptococcus Mutans strains.

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1. Introduction

Mutans Streptococci (MS) make up a major component of oral microflora and considered as one of the important etiological agent in initiation of dental caries.¹ Among MS, Streptococcus mutans (S. mutans) appears to be a predominant bacteria in children with Early childhood caries.² Studies have proved that prevalence of S. mutans is high even in individuals with little or no caries activity. Two major factors in the streptococcus mutans namely, virulence factor and the dependence on caries promoting factors, discriminate the differences in the level of caries experience between the caries active and caries free groups.³

The importance of high level of mother to child vertical transmission of S.mutans have been addressed in several microbiological and genomic studies and the studies also have proved that, isolated strains of S.mutans from the mother-child pair exhibited similar bacterial profile and DNA patterns.^{4–6} Pregnant mothers with high level of S.mutans count can transmit the bacteria to their child and the transmission is high during the period of “window of infectivity” of 19–31 months through direct contact between the child and the mother.⁷ S.mutans possess both common and unique genetic loci and these genetic diversity varies among different species of S.mutans.⁸ It is imperative to determine the unique DNA pattern of S.mutans to classify the virulent group of streptococci.

Studies on mother-child transmission of S.mutans provided a conflicting results, few suggested a positive transmission of S. mutans from mother to child^{9,10} and few had reported a contradictory results.^{11,12} Studies on S.mutans between caries-active and caries-free children by PCR (Polymerase chain reaction)

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have demonstrated that caries-active children have greater genotypic diversity of *S. mutans*, compared to caries-free children.¹³

Since no studies in literature had evaluated the transmission of *S. mutans* from a caries-free mother to child by genetic methods, the present study aimed to identify the genetic characterization of *S. mutans* strains isolated from caries-free and caries active children born to caries-free mothers. The secondary objective is to identify the genetic patterns between caries-free and caries-active individuals.

2. Materials and methods

2.1. Ethical consideration

Ethical approval for this study was obtained from the University Ethics and Research Committee of Saveetha Dental College and Hospitals (SRB/SRTG). Written informed consent was obtained from the parents of participating children. Only children who agreed to participate were enrolled in the study.

2.2. Subject recruitment

Twenty child-mother pair were selected and divided into 2 groups and the mothers being caries-free in both the groups. Group I comprised of children between 3–6 years of age with early childhood caries, whereas group II comprised of caries-free children between 3–6 years of age. Treatment was provided to the participating children who had active caries lesion at the Paediatric Dental Clinic of the Saveetha University.

2.3. Inclusion criteria

- Children between 3–6 years of age.
- No systemic illness.
- No intake of antibiotics for past 3 months.

2.4. Exclusion criteria

- Child's parents failed to give consent for the study.
- Child who required emergency dental management.

2.5. Intraoral examination

Intraoral examination was carried out by a single, qualified Paediatric dentist and the examiner was calibrated prior to the study. Ten children with early childhood caries who were not a part of the present study were examined by the examiner and the dmft and pufa index was recorded. Intra examiner reliability score was >0.89 according to kappa statistics. Following the calibration of the examiner, intraoral examination for the present study was done using a standardized examination tool (Mouth mirror and WHO dental explorer) under artificial light. Dental caries was examined using WHO criteria and scored according to dmft index.¹⁴ Clinical consequence of untreated dental caries was evaluated visually by pufa index without using probe.¹⁵ The examiner was also trained and calibrated for assessing dmft/pufa scores. The absence of caries in mothers and children belonging to caries-free groups was also confirmed by clinical examination.

2.6. Sample collection

Children and their mothers were instructed to relax for 5 min and to swallow all saliva present in their mouths before starting saliva collection. The subjects were instructed to spit all the saliva they produce into a graduated test tube. The unstimulated whole

saliva collected for 5 min was then measured by volume and expressed as millilitres per minute (ml/min).¹⁶

2.7. *Streptococcus mutans* cultivation and isolation

The samples were transferred to the microbiology lab of Saveetha Dental College immediately for bacterial culture. The samples were diluted in 1:40 and 10 µl of the sample was transferred to Mitis-Sanguis agar (Difco). The samples were spread evenly all over the plate and incubated at 37 °C for 48 h in an atmosphere of 10% CO₂. Isolation of *S. mutans* was done by observing its colony morphology and the number of colony forming units in each plate. The tests were done in triplicate to minimise the test error. A few colonies from the cultures were randomly selected for DNA isolation and PCR amplification.

2.8. Genomic DNA extraction of *Streptococcus mutans* and purification

Genomic DNA preparation, isolation and purification were done according to Chassy and Giuffrida.¹⁷ 1.5 ml of randomly selected bacterial culture of each participant were transferred to a micro centrifuge tube and spun at 10,000 rpm for 2 min at 4 °C. The supernatant was decanted and the pellet was re-suspended in 467 µl of TE (Tris EDTA) buffer by repeated pipetting. 30 µl of 10% SDS (Sodium lauryl sulphate) and 3 µl of 20 mg/ml of Proteinase K was added to the sample and incubated for 1 hr at 37 °C. Equal volumes of Phenol: Chloroform (24:1) was added and mixed gently by inverting the tubes until the phase was completely mixed. The tubes were spun at 12,000 rpm for 10 min at 4 °C. The cellular RNA was removed. The upper aqueous layer was transferred to a new tube and an equal volume of Chloroform was added. The samples were mixed by gently inverting the tubes and spun at 12,000 rpm for 10 min at 4 °C. The upper aqueous phase was transferred to a new tube and 1/10th volume of 3 M sodium acetate was added. Double the volume of 95% ice cold ethanol was added and mixed by inversion until the DNA was precipitated. The tube was spun for 10 min at 12,000 rpm at 4 °C and the supernatant was discarded. The pellet was washed with 0.2 ml of 70% ethanol and tube was spun as before. 70% ethanol was discarded and the pellet was air dried. The DNA was then suspended in TE buffer and run on 0.8% agarose gel to confirm the quality of DNA by agarose gel electrophoresis and viewed under Ultra violet light. The gel pictures of extracted DNA were picturized using digital camera (Canon EOS 7000 DZ SLR). Purification of DNA was calculated by the ratio of absorbance at 260 and 280 nm Ultra violet light and the value was found to be 1.8. The same procedure was followed for all the cultures.

2.9. PCR amplification and visualization of amplified products

The extracted DNA from the isolates of *S. mutans* were proceeded for PCR amplification. There were 2 random primers screened for amplification of genomic DNA of *S. mutans* namely, OPA 13 (Operon Technologies Inc., California) as described in previous studies and OPN6 showed DNA amplification. The sequence of OPA 13 primer is 5'CAGTATAAGCGCCAAGTTTCATC3' and OPN6 is 5'ACTGAACGAC3'. The PCR amplification was carried out using an Eppendorf Master Cycler Personnel Thermo cycler (Mastercycler[®] nexusgradient) using the following program: Initial denaturation at 95 °C for 5 min, followed by denaturation at 95 °C–45 s, annealing at 59 °C for 1 min, followed by extension at 72 °C for 1 min and final extension at 72 °C for 7 min. Gel casting unit was prepared to view the amplified products and sealed with a tape to form a mould. The comb was then positioned parallel to the

Fig. 1. Qualitative estimation of DNA of *Streptococcus mutans* by gel electrophoresis.

open edge of the frame. Agarose powder was dissolved and ethidium bromide was added and poured into the gel casting unit and the gel was visualized under ultra violet light illumination by staining with 0.5 μ g of Ethidium bromide/mL. A 1Kb DNA ladder was also loaded along with the samples as a molecular marker. The gels were picturized using a digital imaging system. (Canon Digital science).

2.10. Analysis of PCR amplified data

The molecular weights of each band were converted into a binary data and data were entered into SPSS software version 20.0. The dendrogram for the dissimilarity matrix was developed using Squared Euclidean Distance (SED), which estimates the pair wise differences in amplification products. Genetic distances among the

Fig. 2. Amplification band obtained from purified DNA of *Streptococcus mutans* using the OPA13 primer.

clusters were determined using UPGMA (Unweighted Pair-Group Method with Arithmetical Average) hierarchical method.

3. Results

Clear and unambiguous bands were taken for analysis and the faint fragments were not scored. DNA finger prints of tested *S. mutans* with reference strain by electrophoresis were depicted in Fig. 1.

A total of 80 amplicons were obtained from the selected 2 primers, but OPA 13 primer provided a clear, unambiguous band for all the selected samples. So the DNA finger prints of OPA 13 primer were taken into account. Fig. 2 demonstrates the overall fingerprints of PCR amplification with primer OPA 13. The 1-kb ladder markers are in base pairs (bp) and the marked size amplicons indicate a distinct characteristic of *S. mutans*. Of the 20 amplified products of caries-active child and their corresponding

Fig. 3. (a) & (b) Comparison of genetic distance and similarity matrix between caries active child and their corresponding caries-free mothers using UPGMA analysis.

Fig. 4. (a) & (b) Comparison of genetic distance and similarity matrix between caries free child and their corresponding caries-free mothers using UPGMA analysis.

caries-free mothers, 14 amplicons shared a same genetic distance. Fig. 3(a) & (b) illustrates the genetic distance and similarity matrix in Group I. Among the 10 child-mother pair in group I, 7 pairs exhibited identical genetic distance and similarity in DNA patterns.

Of the 20 amplified products of caries-free child and their corresponding caries-free mothers, 18 amplified products shared a same genetic distance. Fig. 4(a) & (b) illustrates the genetic distance and similarity matrix in Group II. Out of 10 pairs in Group II, 9 pairs exhibited high similarity in genetic distance. Most of the observed genetic distances were zero, indicating closely related species.

Fig. 5 interpretes the genetic polymorphism in DNA patterns between caries-free and caries-active children constructed using Squared Euclidean Distance (SED). The Dendrogram represents a greater genetic polymorphism between caries-free and caries-active children, where the caries-active children exhibited more clonal types than the caries-free children.

4. Discussion

Early childhood caries is a virulent form of dental caries in children and several studies have stated the importance of vertical transmission of *S.mutans* from mother to child.^{9,10} But there exist a paucity in literature regarding the transmission of *S.mutans* from caries-free mothers. So the present study was conducted on 20 Child-mother pair to compare the transmitted *S.mutans* DNA patterns between the mother- child pair. The mother being totally caries-free in both the groups, whereas, children in Group I had

active caries lesion and Group II children were completely caries-free.

Mothers were carefully chosen without any active carious lesion to investigate, whether caries-free mothers with significantly low *S.mutans* count have an ability to vertically transmit to their child. Children between 3–6 years were selected, because it is difficult to collect saliva in children less than 3 years, as they lack spitting ability. Saliva was taken as a sample for DNA analysis, as it is a valuable tool for DNA isolation and PCR analysis.¹⁸

The DNA extraction and isolation was done based on the technique explained by Chassy et al.¹⁷ The DNA finger prints of *S.mutans* obtained by gel electrophoresis was depicted in Fig. 1. The DNA finger prints of the tested *S.mutans* were highly specific with the reference strains of *S.mutans*. Though the PCR amplifications were carried out with 2 screened primers namely, OPA 13 and OPN 6, the amplified bands of OPN 6 was found to be ambiguous. So the data analysis was forwarded with the amplicons obtained with OPA 13 as described previously in various studies.^{13,19} Fig. 2 demonstrated the PCR amplification with OPA 13 as described in materials and methods and the marked size amplicons indicate a distinct characteristic of *S.mutans*.

In the current study, the genetic distance was compared between child-mother pair in both the groups (Fig. 3(a)&(b) and Fig. 4(a)&(b)). On comparing the genetic distance between caries-active child and their corresponding caries-free mother (Fig. 3(a) & (b)), out of 10 child-mother pair, 7 pairs exhibited same genetic distance. However, 2 caries- active children and their corresponding caries free mothers presented with a divergence in genetic

Fig. 5. Dendrographic interpretation of genetic polymorphism in DNA patterns between caries-free and caries-active children.
*CFC (Caries free child), *CAC(Caries-active child).

distance (Fig. 3(a) and (b)), indicative of genetic diversity. The similarity of genetic distance between the 7 child-mother pair indicates a high chance of vertical transmission from their mothers. Though these children carry a similar *S.mutans* of their caries-free mothers, development of caries in these children may be due to other risk factors such as behavioural, biological, snacking frequency, duration and frequency of breast or bottle feeding, type and the amount of tooth paste.^{20,21} These risk factors play an imperative role in the initiation of dental caries. In addition, there was a difference in genetic distance in 3 child-mother pair. This variation in genetic distance may be due to horizontal mode of *S.mutans* transmission from other family members, care givers or from peers¹¹ and this may be responsible for variation in genetic distance between the mother-child pair and the presence of caries in children.

On comparing genetic distance between caries-free child and their corresponding mother, an identical genetic distance of zero ($d=0$) was observed between 9 child-mother pair. This similarity in genetic distance between child-mother pair implies that, the *S. mutans* are closely related to each other and there was a high chance of vertical transmission from the mother. This result supports the study done by Tahmourespour et al, who had stated that isolates from caries free subjects were presented with genetic distance of zero ($d=0.00$) with no divergence.²² One child-mother pair presented with divergence of 0.375 observed in child and 0.439 observed in mother. This variation in genetic distance in child indicates, that the transmission could have occurred through horizontal mode of transmission.¹¹

Divergences in clonal types were present in both caries-active and caries-free subjects, but the caries-active children exhibited 6 different clonal types, whereas, the caries-free subjects presented only with 3 clonal types (Fig. 5). This supports the findings of Napigoma et al, and Pieralisi et al. The authors had concluded that, the caries-active children exhibited more genotypes than the caries-free individuals.^{13,23} A research done by Braga et al on genotypic diversity had stated that, the caries-free subjects possess more than one amplotype, which is in agreement with the present study, where the caries-free mothers and the caries-free children in the present study also represented genotypic diversity with more than 1 amplotype but lesser than the caries-active subjects.²⁴

Streptococcus mutans along with *Streptococcus sobrinus* increase the risk of caries development,²⁵ in the present study we had isolated only *Streptococcus mutans* strains for PCR analysis. This may be a limitation of this study, where the isolation of *Streptococcus Sobrinus* would have added further diagnostic values.

In the present study, identical genetic distance was evident between the mother-child pair, clearly signifies that these *S. mutans* are closely related and could have transmitted vertically from the mother. Most of isolates of caries-active and caries-free children presented a genetic distance of zero, though they were isolated from non-related individuals. This explains the fact that, genetic distance does not signifies the virulence of *S.mutans* in caries activity and there are several other factors determines the virulence of *S.mutans* in promoting caries in children. Genetic diversity between caries-active and caries-free individuals signifies different genotypes exist between them and caries-active individuals possess more genotypic patterns.

5. Conclusion

- Identical genetic distance between child-mother pair signifies that, these *S.mutans* are closely related and could have vertically transmitted from their mothers.
- Identical genetic distance between child-mother pair demonstrates that, all *S.mutans* with identical genetic distance are not equally virulent in promoting dental caries.
- Different genotypic pattern between caries-free and caries active subjects indicates a genetic polymorphism among the *S.mutans* strains.

Conflict of interest

The authors declare that there are no conflicts of interest.

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