Isolation and Genetic Characterization of Mutans Streptococci Associated with Dental Caries in Rural Field Practice of a Dental Institution: In vivo Study

Abstract

Background: *Streptococcus mutans* is well-known causative microorganism in the development of dental caries because they drop the plaque pH and produce acids from carbohydrates and survive in the acidic environment. It is now evident that knowledge of the bacteria enforces empirical approach to therapy, then specific antimicrobial therapy that might allow more conservative treatment options. Over the past few decades, there has been a remarkable increase in the prevalence rate of dental caries among children and the elders. Genotypic methods help in the detection and manipulation of nucleic acids which allows microbial genes to be examined directly. Aim: The aim of this study is to isolate and characterize *S. mutans* from rural population and to obtain genomic DNA and screen DNA band pattern. Methodology: A total of 80 plaque samples were collected from the buccal surfaces of maxillary and lingual surfaces of mandibular first molar with carious teeth in patients at a rural outreach center in Chikkaballapur district, Karnataka. Among these, 48 clinical isolates of *S. mutans* were recovered. Further, genomic DNA was extracted from all the positively isolated strains including the standard strain (microbial type culture collection 497), and stored at 4°C in tris EDTA buffer (TE). To analyze the molecular heterogeneity of the clinical strains, polymerase chain reaction (PCR), and restriction fragment length polymorphism was performed using restriction enzymes Hind III and Hae III. Using agarose gel electrophoresis, genomic DNA band pattern was analyzed. Results: Statistically significant difference was seen in the “dex” gene collected from sample DNA and standard DNA in three different parameters (*S. mutans* 497). Conclusion: Genomic DNA of *S. mutans* was successfully isolated from the rural population. Dex gene was successfully amplified using PCR. Hae III enzymes successfully digested PCR amplicons and the fragments exhibited visible heterogeneity.

Keywords: Dental caries, dental plaque, polymerase chain reaction, restriction fragment length polymorphism, *Streptococcus mutans*

Introduction

Dental caries is the most communal among chronic infectious diseases in the world, characterized by dissolution and destruction of calcified tissues of the tooth structure. The disintegration of the enamel matrix by organic acids, particularly lactic acid delivered by *Streptococcus mutans*, is essential in caries development.[1]

The human oral microbiome consists of various microorganisms that colonize on an array of surfaces as biofilms. Dental plaque is a dynamic and enormous complex oral biofilm ecosystem that consists of no <800 bacterial species.[2]

It has been observed that only a few specific species, such as *S. mutans* and *Streptococcus sobrinus*, are dynamically involved in the carious process.[3] The acid and extracellular polymers produced from carbohydrate metabolism by these bacteria and the consequent decrease in environmental pH is responsible for the demineralization of tooth surfaces.[4]

Mutans produces both water-dissolvable and water-insoluble glucan from sucrose which plays a major role in plaque formation as they contribute to the union of this species both to tooth surface and microbes.[5]

The aggregate sum of glucan present is influenced by the activity of the glucosyltransferase and dexA gene, an extracellular dextranase which separates glucan to isomaltosaccharides by cleaving 1,6-a-linkages.[6] Dextranase possibly

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change glucan by altering the proportion of 1,6-a-to 1, 3-a-linked chains and hence decline solubility in water, and may also provide 1,6-a-rich fragments to prime further glucan synthesis.[7]

Dextranase from *S. mutans* converts dextran to isomaltosaccharides predominantly isomaltotriose, isomaltotetraose, and isomaltopentaose, which in turn increases the infectious activity.[8] However, there is little evidence proving the extracellular *dexA* dextranase and the intracellular *dexB* dextran glucosidase work in a planned catabolic pathway. The exact significance of extracellular dextranase in the ecology of *S. mutans* is unclear.[9]

Molecular methods have added new and energizing dimensions to examine and comprehend medical microbiology, microbial pathogenicity, and host-bacterial interactions. They have overcome the drawbacks of cultivation methods.[10] The field of molecular biology has provided alternatives to phenotypic-based strategies. The detection and manipulation of DNA and RNA, allows microbial gene to be inspected specifically. Among all molecular diagnostic methods, polymerase chain reaction (PCR) is gold standard.[11]

Studies done in various countries have indicated some heterogeneity in the study samples. Heterogeneity of *S. mutans* in Bengaluru urban population has been studied. There is limited literature on the isolation and characterization of genomic DNA of *S. mutans* in rural population. The study focuses on the isolation and genetic characterization of *S. mutans* associated with dental caries in rural population of Kaiwara, Chikballapur District, Karnataka.

**Methodology**

This clinical study was conducted at a rural outreach center as well as the department of biotechnology of an institution in Bengaluru, India. Permission was obtained from the Institutional Ethical Board, and prior consent was taken from the patients attending the Out Patient Department.

**Sample collection**

A total of 80 plaque samples were collected from carious buccal surface of maxillary first molars or lingual surface of mandibular first molar teeth by using sterile toothpick. Forty-eight of these samples could be isolated and identified as *S. mutans* positive, which were used for the study and compared with the standard microbial type culture collection (MTCC) 497. (Patients with any significant medical history, systemic illness, and with any significant oral pathological state or developmental disturbances were excluded from the study). The collected samples were transported to the biotechnology laboratory in phosphate buffer saline solution as the transport media.

Using a sterile cotton swab, the samples were inoculated into plates-containing brain heart infusion agar, and the plates were kept at room temperature for 24 hours. They were then transferred to the CO₂ incubator and incubated for 48 hours.

**Isolation and identification of Streptococcus mutans**

By using a sterile loop, a single colony was selected from the growth media and cultured into Mitis salivarius agar plates [Figure 1]. The isolates were identified as *S. mutans* and then were sub cultured and maintained until further study.

**Revival of standard strain**

Lyophilized *S. mutans* culture was procured from the MTCC and Gene Bank MTCC, Chandigarh, namely MTCC 497. The lyophilized culture was revived using Todd Hewitt (TH) broth. The standard strain was periodically sub cultured and maintained at TH slants.

**Extraction of genomic DNA**

Extraction of genomic DNA from *S. mutans* was carried out. The overnight cultures of the isolates and standard in TH broth was used for the DNA extraction. A volume of 1.10 ml of culture was centrifuged at 5000 rpm to obtain a pellet and then the pellet was suspended in 0.2 ml H₂O. Equal volume of 0.15 M NaCl was added, and vortex mixed till pellet dissolved. It was centrifuged at 12000 rpm for 10 min at 4°C and supernatant was discarded. A volume of 1 ml of Tris Buffer1 was added, and vortex mixed till pellet dissolved. It was centrifuged at 12000 rpm for 10 min at 4°C and supernatant was discarded. After this, 0.2 ml of Tris Buffer2 was added and pellet was dissolved using vortex or tapping. Mixture was centrifuged at 12000 rpm for 10 min at 4°C and supernatant was retained, and the samples were incubated at 56°C water bath for 15 min. After incubation, 0.1 ml of 5NaCl was added and vortex mixed, centrifuged at 12000 rpm for 10 min, and the supernatant was transferred to fresh tube. To this fresh tube, 0.03 ml of sodium acetate (3M, pH 5.2) was added and 0.6 ml of ice-cold absolute ethanol and mixed slowly, without vortex mixer. The samples were incubated for 30 min at −20°C. It was centrifuged at 12000 rpm for 30 min at 4°C. Supernatant was discarded. About 1 ml of 70% ethanol was added. It was centrifuged at 12000 rpm.
for 20 min at 4°C and supernatant was discarded. The pellet was dried overnight at room temperature. Finally, 0.03–0.06 ml of TE buffer was added and pellet was reconstituted. The reconstituted genomic DNA was stored at -20°C till further use.

Polymerase chain reaction-restriction fragment length polymorphism

The genomic DNA obtained from standard and isolates were used for amplification. The N and C terminals regions of dex gene were subjected to amplification by PCR [Figure 2]. The oligonucleotide primers (Sigma Aldrich) are designed to be complementary to the ends of the sequences to be amplified. The forward primer sequence ATTCSGCTTAGAAATTAA and the reverse primer sequence GCAAGTTCTTCAGCTTGTTTT was derived from the dex gene [Figure 3]. This was followed by restriction fragment length polymorphism (RFLP). It is a variation in the DNA sequence of a genome that can be detected by breaking the DNA into pieces with restriction enzymes and analyzing the size of the resulting fragments by gel electrophoresis. RFLPs involves fragmenting a sample of DNA by restriction enzymes [Figure 4], which can recognize and cut DNA wherever a specific short sequence occurs, in a process known as restriction digestion. The resulting DNA fragments are then separated by length through polyacrylamide gel electrophoresis.

The vials containing the amplified DNA and the restriction enzymes were placed at 37°C for 4 h. The reaction was arrested by using gel loading buffer containing ethylenediaminetetraacetic acid, which chelates Mg ions, which are required for enzyme activity. Demonstration of genomic DNA was done by using agarose gel electrophoresis by the following method in which 50 ml of 0.8% agarose solution containing 2 µl ethidium bromide maintained at 50°C, then poured it into the casting gel tank. A suitable well-forming comb was placed about 1 cm from the cathode, without teeth of comb touching the glass plate. The gel was allowed to set for 1 h. Single-strength tris acetate EDTA (TAE) buffer poured into tank until the gel was submerged, electrodes were connected to power supply with the cathode. DNA samples (5–20 µl) were added and standard were taken in gel loading dye solution with a micropipette. Then, power was turned on and run at 50V for one to two hours. Then, the gel was transferred on to a thick sheet and placed on UV transilluminator and viewed under UV light (300 nm). Nucleic acid on the gel appears orange due to the fluorescence of bound ethidium bromide.

Results

The results revealed that there is heterogeneity seen in dex gene, supported by results obtained from digital gel analysis which shows a significant variation in the size of the gene, migration and relative mobility as in comparison with the standard DNA MTCC 497 [Table 1].

Discussion

Dental caries is one of the most common infectious diseases, and S. mutans is always associated with this. It has been implied as a cariogenic bacteria conclusively. Yet, there is an uncertain complexity that exists in the bacterial biofilm. Studies have revealed the multifaceted nature of the bacterial biofilm associated with dental caries initiation. The relationship of dental plaque with dental caries has been substantiated by using molecular analysis in urban population. However, seldom molecular studies have been carried out to understand
the association of dental plaque and dental caries in rural population. A detailed knowledge of the bacterial biofilm will help to deliver a specific antimicrobial therapy. That will allow a more conservative approach rather than an empirical approach. S. mutans has a strong association with the initiation of dental caries. These Gram-positive bacteria tend to colonize the mouth, especially the tooth surfaces. To know the percentage of the bacterial group in the plaque, Gibbons and associates performed a conventional analysis which showed 27.9% prevalence of S. mutans in dental plaque. Thus in our study, dental plaque was chosen as the source for collecting the S. mutans. Mitis salivarius agar and bacitracin is preferred for isolation of the Streptococcus species, because of its selective and differential properties. Genomic DNA isolation was carried out using standard protocols. The isolated DNA was then used for further analysis. Yoo et al. have done a similar protocol in the Korean population.

After this, PCR was carried out to increase the copies of dex gene as described by Rabeah et al. RFLP is used for the detection of variation in the DNA sequence. This is done by analysing the size of the resultant fragments, achieved by gel electrophoresis. In our study, restriction enzymes Hind III and Hae III were used specifically for the resection of amplified genomic sequence of dex gene. There was no restriction seen with Hind III enzyme, whereas single band was seen with Hae III restriction enzyme under UV light. Hae III restriction enzyme can be used for rapid identification of different species of mutans streptococci as suggested by Bretthall et al.

In our study, the dex gene bands were analysed using digital gel documentation and analysis software (Digi DAS) to reveal the genomic DNA band pattern. The results of which showed statistically significant variation in migration rate, relative mobility rate, and estimated size of the gene when compared to the standard DNA MTCC 497. The single band shown by Hae III restriction enzyme under UV light implies that there is heterogeneity in dex gene. We observed that the heterogeneity of the sample strains obtained from Kaiwara population may be due to high fluoride content in water (Kaiwara is present in high fluoride geographical zone), less exposure to highly refined food (consumption of more fibrous food) and also the dry weather in the region may probably would have contributed to this variation.

**Limitations of our study**

1. A better understanding of dex gene would require larger sample size; hence, further studies have to be carried out to substantiate the claim
2. For accurate analysis, gene cloning and molecular sequencing of S. mutans should be done. This would augment the evidence.

**Conclusion**

Within the limitations of our study, the fragments of isolated DNA when compared with standard strain showed visible heterogeneity regarding molecular weight, migration rate, and relative mobility.

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**Table 1: Migration, relative mobility, and size of standard DNA (Group 1) and Isolated DNA (Group 2) using unpaired t-test**

| Group | Migration (Pixel) | Relative mobility | Size |
|-------|------------------|------------------|------|
| Group 1 Mean | 119 | 0.832 | 600 |
| SD | 0.00 | 0.00 | 0.00 |
| Group 2 Mean | 126.225 | 0.868 | 284.7560 |
| SD | 6.353 | 0.033 | 207.027 |

SD=Standard deviation
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Conflicts of interest

There are no conflicts of interest.

References

1. Roberson M, Harald O, Heymann H, Edward J, Swift JR. Sturdevants Art and Science of Operative Dentistry. 4th ed. St. Louis: Mosby; 2001. p. 66-7.
2. Krzysciak W, Jurczak A, Koscienliak D, Bystrowska B, Skalniak A. The virulence of Streptococcus mutans and the ability to form biofilms. Eur J Clin Microbiol Infect Dis 2014;33:499-515.
3. Aas JA, Griffin 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.
4. Takahashi N, Nyvad B. The role of bacteria in the caries process: Ecological perspectives. J Dent Res 2011;90:294-303.
5. Ebisu TS, Misaki A. The structure of water-insoluble glucans of cariogenic Streptococcus mutans, formed in the absence and presence of dextranase. Carbohydr Res 1984;38:374-81.
6. Roll G. A preliminary report on the interaction between concanavalina and soluble extracellular polysaccharides produced by mutans and sanguis streptococci. Arch Oral Biol 1971;38:967-969.
7. Colby SM, Whiting GC, Tao L, Russell RR. Insertional inactivation of the Streptococcus mutans dexA (dextranase) gene results in altered adherence and dextran catabolism. Microbiology 1995;141 (Pt 11):2929-36.
8. Nyvad B, Crielaard W, Mira A, Takahashi N, Beighton D. Dental caries from a molecular microbiological perspective. Caries Res 2013;47:89-102.
9. Nayak M, Hedge MN, Nanda Kishore KJ. Molecular diagnostic methods in endodontics. Endodontology 2006;18:35-42.
10. Sreenivasa Murthy BV, Narayana IH, ShamaRao HN. Molecular analysis of Streptococcus mutans associated with dental caries in Bangalore urban population. Rajiv Gandhi Univ Health Sci J Dent Sci 2013;5:87-92.
11. Jiang Q, Yu M, Min Z, Yi A, Chen D, Zhang Q, et al. AP-PCR detection of Streptococcus mutans and Streptococcus sobrinus in caries-free and caries-active subjects. Mol Cell Biochem 2012;365:159-64.
12. Seghatoleslami S, Ohlsson L, Hamberg K, Carlsson P, Ericson D, Ljunggren L. Quantitative detection of Streptococcus mutans from salivary FTA<sup>TM</sup> elute cards and real-time polymerase chain reaction. Am J Mol Biol 2013;3:148-52.
13. Chhour KL, Nadkarni MA, Byun R, Martin FE, Jacques NA, Hunter N, et al. Molecular analysis of microbial diversity in advanced caries. J Clin Microbiol 2005;43:843-9.
14. 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.
15. Mejare B, Edwardsson S. Streptococcus milleri (Guthof); an indigenous organism of the human oral cavity. Arch Oral Biol 1975;20:757-62.
16. Carlson J. A numerical taxonomic study of human oral streptococci. Odontol Revy 1968;19:137-60.
17. Drucker DB, Melville TH. The classification of some oral streptococci of human or rat origin. Arch Oral Biol 1971;16:845-53.
18. Yoo SY, Park SJ, Jeong DK, Kim KW, Lim SH, Lee SH, et al. Isolation and characterization of the mutans streptococci from the dental plaques in Koreans. J Microbiol 2007;45:246-55.
19. Hildebrandt GH, Bretz WA. Comparison of culture media and chairside assays for enumerating mutans streptococci. J Appl Microbiol 2006;100:1339-47.
20. Brathall D. Immunodiffusion studies on the serological specificity of streptococci resembling Streptococcus mutans. Odontol Revy 1969;20:231-43.