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# Genotypic Variations of Mutans Streptococci Isolated from Dental Caries by REP-PCR

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## Abstract:

Mutans streptococci (MS) are a group of oral bacteria considered as the main cariogenic organisms. MS consists of several species of genus *Streptococcus* which are sharing similar phenotypes and genotypes. The aim of this study is to determine the genetic diversity of the core species of clinical strains of *Streptococcus mutans*, *Streptococcus sobrinus* and *Streptococcus downei* by using repitative extragenic palindromic (REP) primer. The DNA of the clinical strains of *S. mutans* (n=10), *S. sobrinus* (n=05) and *S. downei* (n=04) have been employed in the present study, which have been previously isolated from caries active subjects. The DNA of the clinical and reference strains was subjected to PCR amplification using REP primer. The phylogenetic dendrogram is constructed from the REP PCR banding profile by neighbour-joining method using PyElph 1.4 software. The size of the DNA amplicons generated by using REP primer were *S. mutans* (1500 bp to 250 bp), *S. sobrinus* (6000 bp to 250 bp) and *S. downei* (5000 bp to 400 bp). The results present common band at 480 bp in all the clinical strains of *S. sobrinus*. The current study is the first to demonstrate the genetic variety of *S. sobrinus* and *S. downei* by using REP primer. REP-PCR have been found to be a powerful method to study the molecular diversity of *S. mutans*, *S. sobrinus* and *S. downei*. Additionally, further studies are suggested to analyze the species specific bands and also to find the possibility to produce a new specific primer for *S. sobrinus*.

**Key words:** Cariogenic bacteria, Molecular diversity, Neighbour-joining method, PyElph software, *Streptococcus downei*.

## Introduction:

Dental caries is ubiquitous and pandemic disease affecting all the age groups of humans. However, due to the fact that dental caries is cumulative process, infected individuals are increased with ageing (1-3). Among adult population, dental caries demonstrating a higher level of severity affecting 5 to 10 teeth per individual (4,5). An extensive and comprehensive National Health Survey performed throughout India revealed that 80 % of the population in the age group 35-44 years old affected by dental caries (6).
Mutans streptococci (MS), *Streptococcus mutans* and *Streptococcus sobrinus*, are the principal causative agents of the formation of dental caries (7,8). *S. mutans* is considered as the solitary pioneer cariogenic determinant (7,9). Recent studies validate that the conjoined action of *S. mutans* and *S. sobrinus* have intensified the process of oral caries (10). Even though the incident of isolation of *S. downei* is infrequent, latest report confirmed the isolation of *S. downei* from caries active subjects (11).

The majority of people harbor MS in their oral cavities; nevertheless, not all acquire dental caries. This leads to the theory that these bacteria are genetically diverse and possess variable virulence prospective. Genetic diversity referred as the number of genotypes present within an organism. The genotyping can illuminate the phenotypic diversity in microorganisms, such as antibiotic resistance, geographic dissemination, host specificity, pathogenicity, transmission and virulence factors (8,12). The genetic diversity can

also study the heterogeneity of the MS (13,14) and the possibility to design a vaccine.

Many genotypic tools were used in favor of studying the genetic diversity of MS, e.g. pulse field gel electrophoresis (15), arbitrarily-primed PCR (16), multi-locus sequence typing (17) and repetitive elements based PCR (rep-PCR) (18). Rep-PCR amplifying repetitive elements in genomic bacteria to generate genetic markers (19). Repetitive DNA elements are non-coding genes generally located in eubacteria. One of the main types of rep-PCR is repetitive extragenic palindromic (REP) (20). REP consists of 33-40 bp of conserved palindromic sequences (21). REP-PCR produces a highly sensitive, specific and steady gene profile out of nanogram DNA to amplify many unique bands (22,23).

In the previous published studies, we have detected strains of *S. mutans*, *S. sobrinus* and *S. downei* isolated from dental caries active subjects aged between 35 to 44 years (8,11). Insight to this context, the objective of the existing study was focused to investigate the genetic diversity of those species using REP-PCR.

## Materials and Methods:

### Bacterial Isolation

Ten clinical strains of *S. mutans* (H5, H17, H18, H19, H20, H23, H26, H35, H36 and H37), five clinical strains of *S. sobrinus* (H16, H21, H29, H43 and H65) and four clinical strains of *S. downei* (H45, H47, H50 and H62) were obtained from our previous studies (8,11).

### Molecular Identification

All the clinical strains were identified previously (8,11) at species level based on 16S rDNA sequencing and their GenBank accession numbers are KP975169, KP975180, KP975181, KP975182, KP975183, KP975185, KP975188, KP975195, KP975196 and KP975197 for *S. mutans*, KP975179, KP975184, KP975191, KP975203 and KP975213 for *S. sobrinus* and KP975204, KP975205, KP975206 and KP975211 for *S. downei*, respectively (8,11). *S. mutans* ATCC 25175, *S. mutans* MTCC 497 and *S. sobrinus* ATCC 33478 were used as reference strains.

### DNA amplification of *S. mutans*, *S. sobrinus* and *S. downei*

The extraction and purification of the DNA have been performed by cetyl trimethyl ammonium bromide method as previously described (24). The DNA amplification was conducted according to the methodology explained by Versalovic *et al* (25). Amplification was carried out in 25 µl of reaction mixture containing: 5 µl 5x Gitschier buffer [83 mM (NH4)2SO4, 335 mM Tris-HCL (pH 8.8), 33.5 mM MgCl2, 32.5 µl EDTA and 150 mM β-mercapto-ethanol], 10 % DMSO, 160 µg/ml BSA, 1.25 mM of each dNTPs, 0.3 µg/ml of each REP primer (REP1R: 5′-IIIICGICGICATCIGGC-3′, REP2I: 5′-ICGICTTATCIGGCCTAC-3′) (Sigma-Genosys, UK), 2 U of DNA polymerase and 50 ng of the each DNA of reference and clinical samples. DNA amplifications were performed in the thermal PCR cycler (G-Storm, UK) using 30 cycles PCR with the following conditions: initial denaturation at 95 ºC for 7 min, initiation 94 ºC for 1 min, annealing 52 ºC for 1 min, extension 65 ºC for 8 min and final extension 65 ºC for 16 min. The final PCR products were resolved in 1.5 % agarose in 1X TAE buffer at 4 ºC for 16 h at 55 V. The PCR genomic fragments were visualized under a UV transilluminator (BioBee, India) followed by digital capturing of the picture using gel documentation system.

### Phylogenetic analysis

Phylogenetic analysis based on REP-PCR results was constructed by the neighbour-joining method using PyElph 1.4 software as described by Pavel and Vasile (26). The banding patterns of the clinical strains were also evaluated.

## Results:

The genetic banding pattern of *S. mutans* and *S. sobrinus* by REP primer is shown in Fig. 1, while the genetic banding pattern of *S. downei* is shown in Fig. 2.

The banding pattern of REP primer in both the reference and clinical strains of *S. mutans* demonstrated bands ranged from molecular size 1500 bp to 250 bp, while for *S. sobrinus* from 6000 bp to 250 bp. The banding pattern of REP-PCR in the clinical strains of *S. downei* demonstrated bands between 5000 bp to 400 bp. REP primer demonstrated its ability to generate bands for all the tested species which can refer as a strong tool for genetic diversity.

Figure 1 revealed the presence of common bands in the strains of *S. mutans* and *S. sobrinus* at the molecular weights 1500 bp, 1300 bp, 1100 bp, 880 bp, 750 bp and 250 bp. All the clinical strains of *S. sobrinus* have characteristic band at molecular weights 6000 bp, 5000 bp, 4000 bp and 480 bp. While, clinical and reference strains of *S. mutans* lacked bands at the same molecular weights. All the strains of *S. downei* showed the presence of monomorphic bands at molecular weights 1500, 1400 and 1100 bp (Fig. 2).

The data suggest that these monomorphic bands can be further analyzed and used as a species specific primers for *S. sobrinus* and *S. downei*. The results also revealed the absence of bands at different

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molecular weights for strains of *S. mutans*, *S. sobrinus* and *S. downei*.

The results of the phylogenetic dendrogram inferred from REP-PCR banding pattern using the neighbour-joining method is presented in Fig. 3 for *S. mutans* and *S. sobrinus*, and in Fig. 4 for *S. downei*. Two groups of *S. mutans* were clustered with the strains of *S. sobrinus*. Strains number H5 and H17 of *S. mutans* were genetically close to *S. mutans* ATCC 25175 and *S. mutans* MTCC 497.

While, reference strain of *S. sobrinus* is clustered with other strains of the same species. In *S. downei*, the genetic distance of strain number H62 was far than the rest of the other strains of the same species.

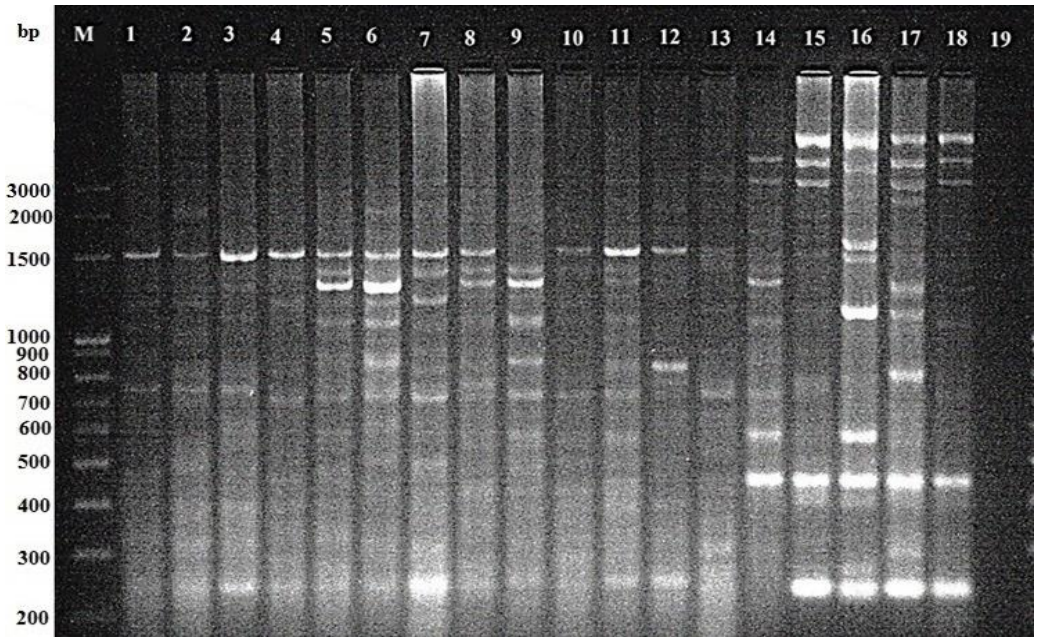


**Figure 1. REP-PCR banding pattern of *S. mutans* and *S. sobrinus*. M: DNA ladder, Lane 1: *S. mutans* ATCC 25175, Lane 2: *S. mutans* MTCC 497, Lane 3: *S. mutans* H5, Lane 4: *S. mutans* H17, Lane 5: *S. mutans* H18, Lane 6: *S. mutans* H19, Lane 7: *S. mutans* H20, Lane 8: *S. mutans* H23, Lane 9: *S. mutans* H26, Lane 10: *S. mutans* H35, Lane 11: *S. mutans* H36, Lane 12: *S. mutans* H37, Lane 13: *S. sobrinus* ATCC 33478, Lane 14: *S. sobrinus* H16, Lane 15: *S. sobrinus* H21, Lane 16: *S. sobrinus* H29, Lane 17: *S. sobrinus* H43, Lane 18: *S. sobrinus* H65 and Lane 19: negative control.**

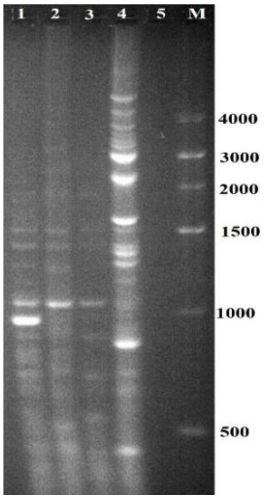


**Figure 2. REP-PCR banding pattern of *S. downei*. Lane 1: *S. downei* H45, Lane 2: *S. downei* H47, Lane 3: *S. downei* H50, Lane 4: *S. downei* H62, Lane 5: negative control, M: DNA ladder.**

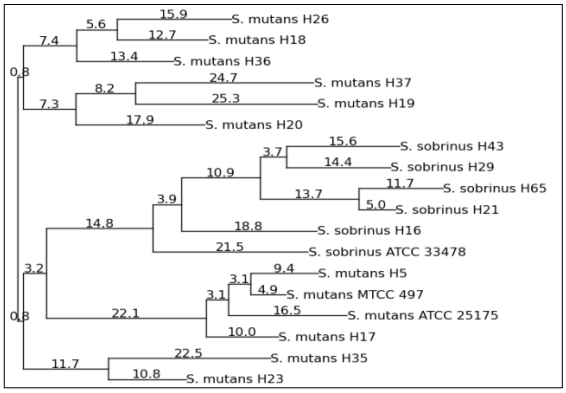


**Figure 3. Phylogenetic dendrogram based on REP-PCR results showing the relatedness between *S. mutans* and *S. sobrinus* strains subjected to analysis. The dendrogram constructed by neighbour-joining method using PyElph 1.4 software. The genetic distances are demonstrated above the branches.**

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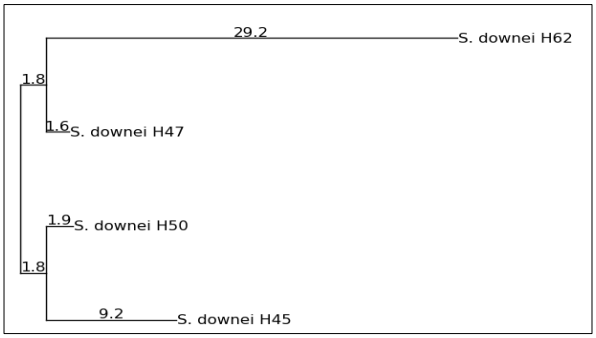


**Figure 4. Phylogenetic dendrogram based on REP-PCR results showing the relatedness between *S. downei* strains subjected to analysis.**

The dendrogram constructed by neighbour-joining method using PyElph 1.4 software. The genetic distances are demonstrated above the branches.

## Discussion

Due to the fact that *S. mutans* and *S. sobrinus* are the prime agents of dental caries (7,8), the genetic diversity of the representative clinical strains of *S. mutans* and *S. sobrinus* was studied using rep-PCR with REP primer. However, clinical strains of *S. downei* were recently isolated from caries active subjects (11). As per literatures survey, this is the first study to determine the genetic diversity of *S. sobrinus* and *S. downei* by using REP primer.

REP-primer employed in the present study generated bands for all the strains of *S. mutans*, *S. sobrinus* and *S. downei* (Figs. 1 and 2). This is in contrary to the previous report stating that REP primer did not produce any amplicons with *S. mutans* and *S. sobrinus* (27). The reason for this might be due to the variation of the primers employed or the heterogeneity of the strains. Moreover, no indication of banding patterns among alpha hemolytic streptococci was observed by using REP primer (28). However, our results are in agreement with the previous reports stating that rep-PCR with different primers i.e. REP, ERIC, SERE, and BOX are reproducible, diverse among strains and appropriate for strain-typing (22,29).

Among the bands generated by REP primer, bands at 1500 bp, 1300 bp, 1100 bp and 250 bp were common in both *S. mutans* and *S. sobrinus*. Moreover, similar bands were observed between *S. mutans*, *S. sobrinus* and *S. downei* at molecular weights 1500 bp, 1400 bp and 1100 bp (Figs. 1 and 2).

All the clinical strains of *S. sobrinus* had a specific bands at 6000 bp, 5000 bp, 4000 bp and 480 bp. Thus, these bands could be further studied to generate species-specific primer for identification of *S. sobrinus*. Recent studies reported the genotypic, diversity, commonality, and stability of the *S. mutans* using REP-PCR (30,12). In few strains, bands were missing; the reason for this may be attributed to different serotypes. The present results indicate that the clinical strains of both *S. mutans* and *S. sobrinus* were more diverse compared with the reference strains (Fig. 1).

The correlation among species was well presumed from the banding pattern dendrogram. Phylogenetic trees reconstructed by the neighbour-joining method, established the genetic settlement of representative strains of MS. The reference strains of *S. mutans* and *S. sobrinus* are assembled with its own strains (Fig. 3). Clinical strains of *S. downei* H45 and H50 are genetically related and clustered together with strains no. H47 and H62 (Fig. 4). The genetic distance of strain number H62 was attributed to the variation in gene composition among the members of MS. Genetically, MS species are closely related to each other, in particular, *S. sobrinus*, *S. downei* and *S. mutans* (8,31).

## Conclusion:

The findings presented herein show the usefulness of REP-PCR to study the diversity and genotypic of *S. mutans*, *S. sobrinus* and *S. downei*. However, the present study recommend more research to find the possibility to produce genetic markers for MS species.

## Author's declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in Al-Esraa University College.

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# التنوع الوراثي لانواع البكتريا (mutans streptococci) المعزولة من تسوس الاسنان عن طريق -REP PCR

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**الخلاصة:**

المكورات العقدية هي مجموعة من البكتيريا التي توجد بالفم والمسببة لتسوس الاسنان. يتكون مرض تسوس الاسنان من عدة أنواع من جنس *Streptococcus* التي تتقاسم أنماط وراثية مماثلة. الهدف من هذه الدراسة هو تحديد التنوع الوراثي للسلالات *Streptococcus mutans*, *Streptococcus sobrinus* و*Streptococcus downei* ، التي تعتبر الأنواع الرئيسية المسببة لتسوس الاسنان ، باستخدام طريقة البلمرة المتسلسل PCR والبادئ REP. تم استخدام الحمض النووي للسلالات *S. mutans* (عدد = 10) ، *S. sobrinus* (عدد = 5) و *S. downei* (عدد = 4) ومقارنتها بمثيلاتها المعزولة سابقا من الاشخاص المصابين بتسوس الاسنان. تعرض الحمض النووي للسلالات السريرية والنموذجية لتضخيم PCR باستخدام REP primer . شيدت الشجرة التطويرية (dendrogram phylogenetic) لهذة الاصناف من خلال حساب عدد القطع الطفرية المتكونة من البلمرة المتسلسلة عن طريق neighbour-joining method باستخدام برنامج PyElph 1.4. واظهرت النتائج ايضا ان حجم قطع المادة الوراثية التي تمت مضاعفتها في الجل المنتج (amolicons) هي : *S. mutans* (1500 bp إلى 250 bp) ، *S. sobrinus* (6000 bp إلى 250 bp) و *S. downei* (5000 bp إلى 400 bp). واظهرت النتائج ايضا وجود قطعة مميزة من المادة الوراثية المضاعفة بحجم 480 bp لكل عزلات البكتريا *S. sobrinus* بخلاف الانواع الاخرى. الدراسة الحالية هي الأولى التي توضح التنوع الوراثي لكل من *S. sobrinus* و *S. downei* باستخدام REP primer. هذة الدراسة اثبتت ان REP-PCR لذلك تعتبر هذة الدراسة وسيلة قوية لدراسة التنوع الجزيئي لل S. mutans، *S. sobrinus* و *S. downei*. بالإضافة إلى ذلك ، يُقترح إجراء مزيد من الدراسات لتحليل النطاقات الخاصة بالتفرقة بين الانواع وأيضًا لإيجاد إمكانية إنتاج بادئات جديد للكشف عن *S. sobrinus* و *S. downei*.

**الكلمات المفتاحية:** البكتيريا المسببة للتسوس ، التنوع الجزيئي ، *Streptococcus*، neighbour-joining method ، برنامج PyElph.