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

Hamzah Abdulrahman Salman*1 R. Senthilkumar2

1Department of Medical Laboratory Techniques, Al-Esraa University College, Baghdad, Iraq.
2Department of Microbiology, J.J. College of Arts and Science, Pudukkottai, affiliated to Bharathidasan University, Tamil Nadu, India.

*Corresponding author: hamza.alayash@gmail.com*, micrsk13@rediffmail.com.
ORCID ID: https://orcid.org/0000-0001-7060-9995* https://orcid.org/0000-0001-8672-2263

Received 23/5/2019, Accepted 13/9/2020, Published 1/12/2020

This work is licensed under a Creative Commons Attribution 4.0 International License.

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 ATCC 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′-IIICGICICICATCGGC-3′, REP2I: 5′-ICGICTTATCIGGCCCTAC-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 Vasilie (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
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.
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* were 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 republication 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.

References:

1. Billings M, Hoflreiter B, Papapanou PN, Mitnik GL, Kocher T, Dye BA. Age-dependent distribution of periodontitis in two countries: Findings from NHANES 2009 to 2014 and SHIP-TREND 2008 to 2012. J Clin periodontal. 2018;45:S130-148.
2. López R, Smith PC, Göstemeyer G, Schwendicke FJ. Ageing, dental caries and periodontal diseases. J Clin Periodontal. 2017;44:S145-S152.
3. Philip N, Suneja B, Walsh LJ. Ecological approaches to dental caries prevention: paradigm shift or shibboleth?. Caries res. 2018;52:153-65.
4. Costa S, Martins C, Pinto M, Vasconcelos M, Abreu M. Socioeconomic factors and caries in people between 19 and 60 years of age: an update of a systematic review and meta-analysis of observational studies. Int J Environ Res Public Health. 2018;15:1775.

5. Frencken JE, Sharma P, Stenhous L, Green D, Laverty D, Dietrich T. Global epidemiology of dental caries and severe periodontitis – a comprehensive review. J Clin Periodontal. 2017;44:S94-S105.

6. Bagramian RA, Garcia-Godoy F, Volpe AR. The global increase in dental caries. A pending public health crisis. Am J Dent. 2009;22:3-8.

7. Baker JL, Edlund A. Exploiting the Oral Microbiome to Prevent Tooth Decay: Has Evolution Already Provided the Best Tools?. Front Microbiol. 2019;9:3323.

8. Salman HA, Senthilkumar R, Imran K, Selvam KP. Isolation and typing of Streptococcus mutans and Streptococcus sobrinus from caries-active subjects. Contemp Clin Dent. 2017;8:587-93.

9. Saraithong P, Pattanaporn K, Chen Z, Khongkunthian S, Laohapensang P, Chhun N, et al. Streptococcus mutans and Streptococcus sobrinus colonization and caries experience in 3-and 5-year-old Thai children. Clin Oral Investig. 2015;19:1955-64.

10. Oda Y, Hayashi F, Okada MJBOH. Longitudinal study of dental caries incidence associated with Streptococcus mutans and Streptococcus sobrinus in patients with intellectual disabilities. BMC Oral Health. 2015;2;15:102.

11. Salman HA, Senthilkumar R, Mahmood BS, Imran K. Detection and characterization of Streptococcus downei, a rare bacterial species of mutans streptococci from caries-active patients. Indian J Dent Res. 2019;30:579-82.

12. Cheon K, Moser SA, Wiener HW, Whiddon J, Momeni SS, Ruby JD, et al. Characteristics of Streptococcus mutans genotypes and dental caries in children. Eur J Oral Sci. 2013;121:1148-155.

13. Braga MP, Piovesan A, Valarini N, Maciel SM, Andrade FBd, Poli-Frederico RC. Genotypic diversity and virulence factors of Streptococcus mutans in caries-free and caries-active individuals. Braz Arch Biol Technol. 2013;56:241-8.

14. Bedoya-Correa CM, Rodríguez RJ, Parada-Sanchez MT. Genomic and phenotypic diversity of Streptococcus mutans. J Oral Biosci. 2019;61:22-31.

15. Mineyama R, Yoshino S, Maeda NJ. DNA fingerprinting of isolates of Streptococcus mutans by pulsed-field gel electrophoresis. Microbiol Research. 2007;162:244-9.

16. Valdez RMA, Duque C, Caiaffa KS, dos Santos VR, de Aguair Loesch ML, Colombo NH, et al. Genotypic diversity and phenotypic traits of Streptococcus mutans isolates and their relation to severity of early childhood caries. BMC Oral Health. 2017;17:115.

17. Momeni SS, Whiddon J, Moser SA, Cheon K, Ruby JD, Childers NK. Comparative genotyping of Streptococcus mutans by repetitive extragenic palindromic polymerase chain reaction and multilocus sequence typing. Mol Oral Microbiol. 2013;2818-27.

18. Moser SA, Mitchell SC, Ruby JD, Momeni S, Osgood RC, Whiddon J, et al. Repetitive extragenic palindromic PCR for study of Streptococcus mutans diversity and transmission in human populations. J Clin Microbiol. 2010;48:599-602.

19. Versalovic J, Koeth T, Lupski RJ. Distribution of repetitive DNA sequences in ucbacteria and application to finerpipering of bacterial enomes. Nucleic Acids Res. 1991;19:6823-31.

20. Fakruddin M, Mannan B, Shahnawej K, Mazumdar RM, Chowdhury A, Hossain N. Identification and characterization of microorganisms: DNA-fingerprinting methods. Songklanakarin J Scien Technol. 2013;35:397-404.

21. Stern MJ, Ames GF-L, Smith NH, Robinson EC, Higgins CF. Repetitive extragenic palindromic sequences: a major component of the bacterial genome. Cell. 1984;37:1015-26.

22. Healy M, Huong J, Bittner T, Lising M, Frye S, Raza S, et al. Microbial DNA typing by automated repetitive-sequence-based PCR. J Clin Microbiol. 2005;43:199-207.

23. Momeni SS, Whiddon J, Cheon K, Ghazal T, Moser SA, Childers NK. Genetic Diversity and Evidence for Transmission of Streptococcus mutans by DiversiLab rep-PCR. J Microbiol Methods. 2016;128:108-117.

24. Salman HA, Kumar RS, Babu NC, Imran K. First detection and characterization of Streptococcus dentapri from caries active subject. J Clin Diagn Res. 2017;11:DM01-3.

25. Versalovic J, Schneider M, De Bruijn FJ, Lupski JR. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. Methods Mol Cell Biol. 1994;5:25-40.

26. Pavel AB, Vasile CI. PyElph - a software tool for gel images analysis and phylogenetics. BMC Bioinformatics. 2012;13:9.

27. Okada T, Takada K, Fujita K, Ikemi T, Osgood RC, Childers NK, et al. Differentiation of banding patterns between Streptococcus mutans and Streptococcus sobrinus isolates in rep-PCR using ERIC primer. J Oral Microbiol. 2011;3:7190.

28. Alam S, Brailsford SR, Whiley RA, Beighton D. PCR-based methods for genotyping viridans group streptococci. J Clin Microbiol. 1999;37:2772-6.

29. Claridge IIIJ, Osting C, Jalali M, Osborne J, Waddington M. Genotypic and phenotypic characterization of “Streptococcus milleri” group isolates from a veterans administration hospital population. J Clin Microbiol. 1999;37:3681-7.

30. Cheon K, Moser SA, Whiddon J, Osgood RC, Momeni S, Ruby JD, et al. Genetic diversity of plaque mutants streptococci with rep-PCR. J Dent Res. 2011; 90:331-35.

31. Whiley RA, Russell RR, Hardie J, Beighton D. Streptococcus downei sp. nov. for strains previously described as Streptococcus mutans serotype h. Int J Syst Bacteriol. 1988;38:25-9.
التنوع الوراثي لانواع البكتريا (mutans streptococci) في تنوع الأسنان

R. سينذكلمار

حمزة عبد الرحمن سلمان

قسم تقنيات المختبرات الطبية، كلية الدراسات الجامعية، بغداد، العراق.
قسم الأحياء الدقيقة، ج. كلية الآداب والعلوم، بودوكوتاي، منتسبة إلى جامعة بهاراتيداسان، تاميل نادو، الهند.

الخلاصة:
المكورات العقدية هي مجموعة من البكتيريا التي توجد بالفم والمسببة لتسوس الأسنان. يتكون مرض تسوس الأسنان 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 أنواعاً رئيسية في تسوس الأسنان، باستخدام طريقة البلمرة المتسلسل PCR والبادئ REP primer. تم استخدام الحمض النووي للسلالات S. mutans = (عدد 10) ، S. sobrinus = (عدد 5)، S. downei = (عدد 4) ومعارفها بمتطلباتها ولكن المعزولة سابقاً من الأشخاص المعانيين بتسوس الأسنان. تعرّض الحمض النووي للسلالات S. mutans و S. sobrinus و S. downei للمجاور الأشري (dendrogram phylogenetic) باستخدام REP primer. واظهرت النتائج أيضاً وجود قطعة مميزة من المادة الوراثية المضاعفة بحجم 480 bp لكل عزلات البكتريا S. sobrinus، في حين أن S. downei S. mutans نمط جزيئي مختلف عند النواة، يتضمن رفعIGG في حالة REP-PCR باستخدام REP primer. هذه الدراسة توضح التنوع الجزيئي لكل من S. downei S. mutans، بالإضافة إلى ذلك، فإن REP primer يعتبر رفعIGG في حالة REP-PCR باستخدام REP primer. هذه الدراسة توضح أن REP primer يندرج من S. downei S. mutans S. sobrinus في حالة REP-PCR.

الكلمات المفتاحية: البكتيريا المسببة للتسوس، التنوع الجزيئي، Streptococcus