Predominance of non-Streptococcus mutans bacteria in dental biofilm and its relation to caries progression

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Abstract

This study aims to assess differences in biofilm bacterial composition between patients with low and high caries. Patients without a medical problem and with no history of antibiotic use, mouth wash or fluoride application in the previous 3 months were recruited. Caries was recorded at cavitation level; score was calculated by a national mean (dmft of 4.8 and DMFT of 2.7). Pooled biofilm samples were collected from mesial, distal, buccal, lingual, and occlusal surfaces. Based on caries experience, individuals were classified into low and high caries and both groups were compared regarding bacteria identified using 16S rRNA gene sequencing, and molecular phylogenetic analysis of the isolates was performed. A total of twenty seven randomly selected samples with low (n = 13) and high (n = 14) caries. Identification of oral bacteria was performed using 16S rRNA sequence, Rothia mucilaginosa and R. aeria were identified in low caries individuals, while R. dentocariosa was detected in high caries individuals. Two Streptococcus spp. were identified only in low caries S. salivarius and S. gordoni whereas S. sanguinis, S. mitis, S. siniensis, S. rubneri, S. vestibularis, S. crista and S. massiliensis were identified only in individuals with high caries. This study revealed the absence of R. mucilaginosus in the high caries subjects and its coexistence with the low caries subjects. Streptococcus mutans was insignificant contributor of caries among samples, while, Streptococcus sanguinis was the main constituent of high caries Saudi patients.

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1. Introduction

The oral microbiome is one of the most diverse among bacterial habitats in various parts of the human body and across populations. It exists in a state of balance and ecosystem (Gupta et al., 2017). Disturbance in this balance and genetic factors cause oral diseases including caries which is one of the commonest diseases worldwide and the most frequent cause of dental pain and tooth loss (Alyousef et al., 2017; Featherstone, 2004; Kilian, 2018). Streptococcus mutans were linked to caries because of their virulence which is determined to a large extent by their interaction with other less virulent bacteria in the oral microbiome (Meric et al., 2020). However, up to 20% of people with caries do not have S. mutans (Gross et al., 2012; Johansson et al., 2016; Krishnan et al.,
Prevalence of various combinations of bacteria are risk factor for increased childhood caries (Meriç et al., 2020). Variations in the constituents of the oral microbiome were attributed to genetic, dietary, oral hygiene (Gupta et al., 2017; Willis et al., 2018) and environmental factors such as water composition and others (Willis et al., 2018; Rosier et al., 2021). Variation was also reported between populations and countries (Gupta et al., 2017; Sarkar et al., 2017; Yamashita and Takeshita, 2017). Because of these differences, it is important to assess the bacterial constituents of dental biofilm in different populations so that preventive strategies can be tailored to each community.

Most studies describing the oral microbiome were conducted in western countries with no studies in any of the 22 countries in the World Health Organization Eastern Mediterranean Region (Gupta et al., 2017). The present study aimed to assess differences in the composition of biofilm microbiome between Saudis with low and high caries for risk assessment and management of caries by targeting bacteria implicated in caries causation in this population. This focused targeting reduces the risk of side effects and provides cost-effective solution to the problem of high caries levels in the country. The hypothesis of this study was that the biofilm microbiome in low and high caries Saudis would be similar and that the most abundant bacteria in their biofilm would be like that identified in the biofilm of other populations in previous studies.

2. Materials and methods

2.1. Participants and sample collection

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Imam Abdulrahman Bin Faisal University’s Institutional Review Board (IRB-2018-02-124). Participants were recruited from visitors of an oral health campaign conducted by Imam Abdulrahman Bin Faisal University, Saudi Arabia in March 2018 upon receiving signed informed consent from the study participants above 16 years, or a parent and/or legal guardian for study participants under the age of 16. Participants were included if they fit the following criteria: patients without a medical problem and with no history of antibiotic use, mouthwash or fluoride application in the previous 3 months because these conditions may vary at the individual level.

Data was collected using a questionnaire, a clinical oral examination and pooled biofilm samples. For each participant, the questionnaire recorded gender, age, whether the participant brushed twice daily or not, used fluoridated toothpaste or not, date of last dental visit and intake of frequency of sugary snacks intake. Full dental examination was performed by a trained examiner. Examination was carried out under natural light, using a standard size 4 mirror, explorer and UNC periodontal probe #5.

Caries was recorded at cavitation level, using the World Health Organization criteria (Sarkar et al., 2017) and dmft/DMFT (decayed, missing, and filled teeth) score was calculated. Al Agili (Al Agili, 2013) reported a national mean dmft of 4.6 and DMFT of 2.7. We dichotomized caries levels into below the mean national level and above it: respectively low caries (dmft < 5 and DMFT < 3) and high caries (dmft ≥ 5 and DMFT ≥ 3). Pooled biofilm samples were collected from mesial, distal, buccal, lingual, and occlusal surfaces of the present molars whether primary or permanent or mixed using sterile curette. Samples were placed in collection tubes containing transport medium (sterile phosphate-buffered saline), stored at −80 °C and transported to Microbiological Lab for further processing.

2.2. Isolation of bacteria and 16S rRNA gene sequencing

Samples were serially diluted in 0.05 M potassium phosphate buffer and bacterial organisms were isolated on two culture media; blood agar and mitis salivarius agar, culture plates were incubated at 37 °C for 48 h (Köhler et al., 1974; Schmidt et al., 1974). 16S rRNA was used for bacterial identification. Colony PCR was used to amplify the 16S rRNA gene of the isolated and pure culture of the bacterial organisms using: absolute master mix 2 (Molecine ON, Auckland, Newzeland), forward primer, reverse primer (Applied Biosystems, Life Technologies Corporation, USA, Primers will be available on request), distilled water and a loop full of colony with the annealing temperature of 56 °C for 75 s in MyCyclerTM (Bio- rad, USA). All the amplified products were purified using PCR Purification Kit (Qiagen, Germany). BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Life Technologies Corporation, USA) was used to cycle sequence the purified amplicons and electrophoresed in Genetic Analyzer 3500 (Applied Biosystems, Life Technologies Corporation, USA) using POP 7. Sequencing Analysis Software Version 5.4 (Applied Biosystems, Life Technologies Corporation, USA) was used to confirm the absence of background noise. EzTaxon tool and expanded Human Oral Microbiome Database (eHOMD) were used to identify the 16S rRNA similarity of the isolated organisms with the 64,329 sequences of various bacteria (Chun et al., 2007). MAFFT version (Katoh and Standley, 2013) was used for the manual verification of the sequence similarity, and evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016; 2016).

3. Results

A total of 27 individuals (age 10.33 ± 3.00 years) were included in the study. Thirteen individuals (age 11.3 ± 2.94 years) with low caries and fourteen (age 9.64 ± 2.95 years) with high caries. None of them had regular dental care or used preventive modalities to control caries and the most recent dental visit for any of them was before a year. All of them consumed sugary snacks at least once per day. Identification of oral bacteria was performed using 16S rRNA sequence. Rothia mucilaginosa and R. aeria were identified in low caries individuals, whereas seven species; S. sanguinis, S. mitis, S. sibiricus, S. gordonii, S. massiliensis, S. oralis and S. san- guinis were identified only in individuals with high caries (Figs. 1, 2). Streptococcus parasanguinis and Streptococcus oralis are present in both high and low caries subjects. Irrespective of age Rothia mucilaginosa observed in low caries patients (Fig. 2). Representatives of seven sequences were submitted to GenBank of National Center for Biotechnology Information with the following accession numbers: S. salivarius (HM424526), S. sanguinis (MH974112), S. oralis (MK253273), R. aeria (MH973721), and R. mucilaginosa (MH973726), (MH981965), (MK253275).

Molecular phylogenetic analysis of Rothia mucilaginosa (MP7) and Rothia aeria (MP6) isolates were performed by maximum likelihood method, the evolutionary history was inferred by using the Maximum Likelihood method and the tree was drawn to scale, with branch lengths measured in the number of substitutions per site (Fig. 3).

4. Discussion

The present study showed that there were differences in the biofilm bacteria between patients with low and high caries. Rothia mucilaginosa was identified in low caries individuals and
Streptococcus sanguinis was identified in high caries individuals. In the present study, *R. mucilaginosa* and *Streptococcus* species had the greatest abundance in the biofilm microbiome representing the culturable bacteria. *R. mucilaginosa* is a primary oral colonizer that may be resistant to penicillin (Tomczak et al., 2013) while, *R. aeria* is a rare colonizer related to invasive infection (Aas et al., 2005). A recent study by Karabudak and his colleagues using next generation sequencing of buccal microbiome revealed the significant increase in the presence of *R. mucilaginosa* in a smoker group (Karabudak et al., 2019) another study reported the abundance of *R. mucilaginosa* among patients with severe aplastic anemia (Ames et al., 2019). However, *Rothia* species colonize the tongue and teeth surfaces and were identified as part of the oral microbiome of healthy individuals (Gross et al., 2012). The high prevalence of *R. mucilaginosa* in our study disagrees with Willis et al. (Willis et al., 2018) who reported that *Rothia* was found in only 5.5% of Spanish adolescents.

*Rothia* species are part of the normal flora residing in the respiratory tract and oral cavity (Rosier et al., 2021; Elkattawy et al., 2021). Our findings showed an abundance of *R. mucilaginosa* and *S. salivarius* in low caries individuals. Our study concur with Hurley et al. (Hurley et al., 2019) who reported greater abundance of *R. mucilaginosa* in the microbiome of caries free saliva than caries active saliva and both had greater abundance than caries active dentine in children from Ireland. It also agrees with Richards et al. (Richards et al., 2017) who reported that *R. aeria* was more common in caries free than caries active adolescents from Sweden and Romania. Our study showed greater abundance of *S. sanguinis* in high caries individuals and this agrees with Hurley et al (Hurley et al., 2019), Diaz-Garrido et al. (Diaz-Garrido et al., 2020) and Richards et al (Richards et al., 2017) who both reported greater abundance of *S. sanguinis* in caries free than caries active children. Meric and colleagues reported similar frequencies of *S. mutans* and *S. sanguinis* among caries and caries free groups of subjects (Meric et al., 2020). Furthermore, *S. sanguinis* was reported for the pathogenesis of caries (Herdiyati et al., 2021; Kurnia et al., 2020), and infective endocarditis (Martini et al., 2020). In the pre-
sent study, *S. mutans* was not identified in any of the 40 individuals. Richards et al. (Richards et al., 2017) reported that *S. mutans* occurred in caries active children from Sweden and Romania. Yet, *S. mutans* is found as a part of a diverse microbial community and not even present in all children with caries (Featherstone, 2004). Previous reports showed that oral disease is not due to an isolated organism such as *S. mutans* causing caries, but is more polymicrobial in nature (Nyvad et al., 2013).

Two *Streptococcus* species were identified in both low and high caries individuals: *S. oralis*, *S. parasanguinis*. A study by Gross et al. reported that *S. parasanguinis* was remarkably linked to caries active but was not a dominant member of the community but level of *S. oralis* reduced or disappear as caries advanced (Featherstone, 2004). Our findings concur with this study as *S. oralis* was found in ratio of 3:2 with low caries to high caries samples, respectively.

Our study is limited by the small sample size although we were able to detect differences in oral microbiome between individuals with low and high caries. Previous studies using sophisticated techniques were also based on small sample sizes (Aas et al., 2005; Ren et al., 2017; Ribeiro et al., 2017). The higher sensitivity and precision of molecular-based technologies may have reduced the need for larger sample sizes that were used in previous studies based on culturing (Gross et al., 2012). The 16S rRNA allows the identification of bacterial taxa (Gross et al., 2012). One of the strengths of our study was that it included participants at different ages sharing the same cultural, dietary and oral hygiene backgrounds thus allowing us to study the oral microbiome differences between caries active and caries free individuals unconfounded by these factors. Previous studies mostly assessed the oral microbiome in one age group (Hurley et al., 2019; Richards et al.,

![Molecular phylogenetic analysis of two isolates, *Rothia mucilaginosa* (MP7) and *Rothia aeria* (MP6) by maximum likelihood method. The yellow highlights indicate the query sequence by evolutionary analyses using MEGA7.](image-url)
Further studies are needed to collect and analyse site-specific biofilm with its bacterial constituents and to include participants from different locations in Saudi Arabia so that regional variations in biofilm constituents can be analyzed. This allows better profiling of the oral microbiome of Saudis and differentiating bacteria in low and high caries microorganisms so that preventive strategies can be tailored to them.

5. Conclusion

This study revealed the absence of R. mucilaginosa and Streptococcus salivarius in the high caries subjects and its coexistence with the low caries subjects. The main constituent of high caries bacteria differed from that reported in other studies. Streptococcus sanguinis and Streptococcus mutans are insignificant contributor of caries among our samples. This study shed light on other bacteria that associated with low and high dental caries and further studies are crucial to study the genetic analysis of these bacteria.

6. Ethics and dissemination

The study was conducted in accordance with the Declaration of Helsinki and was approved by the Imam Abdulrahman Bin Faisal University’s Institutional Review Board (IRB-2018-02-124).

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Data availability

It is not applicable. This study was only the primary research, and further study has been in progress.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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