Original Article

Relative presence of *Streptococcus mutans, Veillonella atypica,* and *Granulicatella adiacens* in biofilm of complete dentures

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Abstract

Aims and Objective: Oral biofilms in denture wearers are populated with a large number of bacteria, a few of which have been associated with medical conditions such as sepsis and infective endocarditis (IE). The present study was designed to investigate the relative presence of pathogenic bacteria in biofilms of denture wearers specifically those that are associated with IE.

Methods: Biofilm samples from 88 denture wearers were collected and processed to extract total genomic DNA. Eight of these samples were subjected to 16S rRNA gene sequencing analysis to first identify the general bacterial occurrence pattern. This was followed by species-specific quantitative polymerase chain reaction (qPCR) on entire batch of 88 samples to quantify the relative copy numbers of IE-associated pathogens.

Results: 16S rRNA gene analysis of eight biofilm samples identified bacteria from *Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes,* and *Fusobacteria* species. Interestingly, *Streptococcus mutans, Veillonella atypica,* and *Granulicatella adiacens* from *Firmicutes,* all known to be associated with early-onset sepsis and IE was present in five of eight biofilm samples. The other three samples carried bacteria from genus *Proteobacteria* with *Neisseria flava* and *Neisseria mucosa*, which are known to be commensals, as dominant species. Species-specific qPCR of *S. mutans V. atypica,* and *G. adiacens* on 88 biofilm DNA samples identified the presence of *S. mutans* in 83%, *V. atypica* in 79%, and *G. adiacens* in 76% of samples.

Conclusion: The findings from the present study demonstrate co-occurrence of *S. mutans, V. atypica,* and *G. adiacens* in a majority of denture wearers, which is clinically significant as elderly patients with compromised immune system are more prone to develop IE. To the best of our knowledge, the co-occurrence of *S. mutans, V. atypica,* and *G. adiacens* is being reported for the first time in biofilms of denture wearers.

Keywords: Denture biofilm, denture biofilm bacteria, *Granulicatella adiacens, Streptococcus mutans, Veillonella atypica*

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INTRODUCTION

Microbial inhabitants of oral cavity are increasingly being recognized for their role in systemic diseases.[1,2] Denture wearers are highly susceptible to microbial diseases, especially under immunocompromised conditions as biofilms on denture surfaces have been found to serve as reservoirs for pathogenic microbes. Some of the microbial diseases that have been reported in denture wearers include denture-related mucosal tissue inflammation (denture stomatitis), atherosclerosis, infective endocarditis (IE), peritonitis, and ulcerative colitis.[3-9] Of the above diseases, atherosclerosis and IE are the chief cause of concern as mortality associated due to cardiovascular diseases (CVDs) in India accounts to up to 53% among those affected.[10] Despite the existence of compelling evidence of association between oral microorganisms and systemic diseases, only a limited knowledge is available on the extent of microbial flora in denture wearers. An idea on the type of pathogenic bacteria colonizing the denture biofilms along with its relative presence can serve as an indicator for the clinicians to administer an appropriate antibiotic regimen in the elderly denture wearers with IE. Based on this proposed outcome, the present study was designed with the following purpose: (1) to find the list of bacteria-colonizing denture biofilm, specifically those pathogenic bacteria that is associated with IE and then (2) quantify the identified pathogens to understand its relative presence.

METHODS

Patient samples and DNA extraction

The study was approved by Institution Ethics Committee, and informed consent was obtained from all participants after explaining the purpose of the study. Eighty-eight complete denture wearers visiting outpatient department of the institute, who had been wearing denture for at least 2–5 years but not exceeding 5 years were included in the study based on the following criteria: (1) age of patients should be in the range of 50–70 years, (2) be nonsmokers, (3) followed a vegetarian diet, (4) had not taken antibiotics for at least 3 months before to the date of sampling, and (5) should not have other hormone-related metabolic conditions such as diabetes. For biofilm sample collection, the mucosal surfaces of both upper and lower complete dentures were scraped gently with a swab and dispersed into a 1.5 ml microfuge tube containing lysis buffer (Cat #740235.50, Macherey Nagel) and were then processed for DNA extraction in accordance with manufacturer's protocol.

16S rRNA gene amplification, direct sequencing, and BLASTN analysis

16S rRNA gene analysis technique uses a pair of universal primers that are designed to anneal conserved regions flanking variable regions of 16S rRNA gene.[11] As the conserved regions are shared among diverse species of bacteria, the universal primer pair amplifies almost all known bacterial species in any given sample. In the present study, 50 ng of total genomic DNA from each of the sample was amplified with 16S universal primers: sense, 5'-AGAGTTTGATCCTGGCTCAG-3' and antisense, 5'-ATTACCGCGGCTGCTGG-3' (Eurofins India Pvt Ltd., Bangalore) corresponding to *Escherichia coli* nucleotide positions 8-534, under the following conditions: after an initial denaturation for 5 min at 95°C, the samples were subjected to 35 cycles of 1 min at 94°C, 1 min at 48°C, and 1 min at 72°C, with a final extension for 10 min at 72°C. The resultant 526 base pair amplicon was sequenced with forward primer and compared with reference bacterial gene sequences deposited in public database (ncbi.nlm.nih.gov) using BLASTN program. The BLASTN program compares the bacterial sequence being analyzed with that of reference bacterial sequence present in the public database and gives a list of a positive match. Since the nucleotide sequences of variable regions are species-specific, only those bacteria present in a given sample will produce a positive match. The stringency of the BLASTN analysis can be increased by inclusion of a few of parameters. In the present study, the stringency of analysis was increased by including the following parameters in the BLASTN program: (1) that the query score should be ≥400 bp, (2) the homology of analyzed sequence with that of reference bacterial sequence must be ≥90%, and (3) the query coverage should be ≥90%.

Species-specific polymerase chain reaction

Nearly 50 ng of total genomic DNA from each of the sample was amplified with the following species-specific primers (Eurofins India Pvt Ltd., Bangalore). Primers for *Streptococcus mutans* were sense, 5'-ACTACACTTTCGGGTGGCTTGG-3' and antisense, 5'-CAGTATAAGCGCC AGTTTCATC-3'.[12] Primers for *Veillonella atypica* were sense, 5'-TCTCTTTGGGAAGAATTAGAACGC-3' antisense, 5-GTGTAACAAGGGAG TACGGACC-3'.[13] Primers for *Granulicatella adiacens* were sense, 5'-GGTTTATCCTTAGAAAGGAGGT-3' and antisense, 5'-GAGCATTCGGTTGGGCA CTCTAG-3'.[14] Species-specific real-time quantitative polymerase chain reaction (qPCR) with SYBR green dye was performed as follows: after an initial denaturation for 5 min at 95°C, the samples were subjected to forty cycles at 94°C for 10 s, 55°C for 30 s, and 72°C for 20 s.

RESULTS

Bacterial flora in denture biofilm

To first identify the occurrence of bacterial flora in denture biofilms, eight DNA samples selected at random were first subjected to 16S rRNA gene sequencing. Only eight samples were sequenced as the resultant data were expected serve as an overall representative of bacterial presence. Primers for V1 to V3 hypervariable region corresponding to positions 8-534 of *E. coli* were selected as this region identifies bacterial amplicons at the genus and species level unambiguously.[15] Sequence analysis identified bacteria from five different phyla, namely, *Firmicutes, Actinobacteria, Proteobacteria, Bacteroidetes,* and *Fusobacteria* [Table 1]. A majority of bacterial species identified during analysis were from *Firmicutes* phyla.

Species specific real-time quantitative polymerase chain reaction

Three bacteria with known pathogenic role in early-onset sepsis and IE, namely, *S. mutans, V. atypica,* and *G. adiacens* were identified in five out of eight biofilm samples. As the presence of these pathogens constitute risk in the elderly immunocompromised patients, we performed real-time qPCR to specifically identify the relative presence of these three bacteria in all biofilm samples. To do so, SYBR green-based qPCR amplification protocol with species-specific primers for *S. mutans, V. atypica,* and *G. adiacens* was performed. Standards with predetermined copy numbers were included in each run so as to estimate the copy number of each bacterium in and among the samples. The cutoff value for the entire detection protocol was established to be between 15 and 25 cycles. Any deviation from this cutoff region, especially when the cutoff value exceeds 25 cycles, was considered as false-positive amplification and was excluded from further analysis. A representative image of qPCR amplification curves is shown in Figure 1. Analysis of the qPCR data under this stringent condition indicated the presence of *S. mutans, V. atypica,* and *G. adiacens* in 73of 88 (83%), 70/88 (79%), and 67/88 (76%) of biofilm samples, respectively. Thirty seven (42%) samples showed co-occurrence of *S. mutans, V. atypica,* and *G. adiacens*, whereas 18 samples (21%) carried *S. mutans* and *V. atypica* 16 samples (18%) carried *S. mutans* and *G. adiacens*, and 14 samples (16%) carried *V. atypica* and *G. adiacens*. Presence of *S. mutans* or *V. atypica* alone was observed in one and two samples, respectively. However, unlike *S. mutans* and *V. atypica, G. adiacns* did not occur in an independent manner.

Table 1: Bacterial species identified in denture biofilms by BLASTN analysis of V1–V3 16S rRNA gene region

Phylum	Species	Number of samples with bacteria
Firmicutes	*Streptococcus mutans*	5
	Streptococcus mitis	2
	Streptococcus sanguinis	2
	Veillonella atypica	5
	Veillonella parvula	3
	Granulicatella adiacens	5
	Granulicatella elegans	1
	Selenomonas sputigena	1
Actinobacteria	*Actinomyces naeslundii*	2
	Rothia dentocariosa	1
Proteobacteria	*Neisseria flava*	6
	Neisseria mucosa	8
	Neisseria oralis	7
Bacteroidetes	*Prevotella intermedia*	2
Fusobacteria	*Leptotrichia buccalis*	1

Bacterial copy number analysis

To determine the hierarchical pattern of occurrence within the samples, the copy number of three pathogenic bacteria in each of the sample was analyzed. When samples carrying all three bacteria were analyzed, a higher presence of *S. mutans* followed by *V. atypica* was found. The cumulative copy number of *S. mutans* was 4.9-fold greater than *V. atypica* and 17.5-fold greater than *G. adiacens*. The cumulative copy number of *V. atypica* was 3.6-fold higher than *G. adiacens* [Figure 2].

To understand whether the predominant presence of two bacteria only influenced its copy numbers, the cumulative copy number of each bacterium was compared in the absence of third bacteria. In the absence of *S. mutans,* the cumulative copy number of *V. atypica* was 5.8-fold higher than *G. adiacens*. In the absence of *V. atypica,* the cumulative copy number of *S. mutans* was 8.9-fold higher than *G. adiacens.* Moreover, in the absence of *G. adiacens,* the cumulative copy number of *S. mutans* was 2.5-fold higher than *V. atypica* [Figure 2].

DISCUSSION

Bacteria from oral microbial flora are associated with a variety of systemic diseases,[3-9] of which IE is considered as the major cause for mortality among patients with CVDs.[10] Several bacterial species have been identified in the IE-associated cardiac tissues including *Streptococcus, Staphylococcus, Veillonella, Pseudomonas, Salmonella,* and *Granulicatella* in independent studies.[16,17] Interestingly, some of these bacteria have also been confirmed to be simultaneously present in the oral cavity of patients.[18-21] In the present study, we first investigated the oral bacterial flora in eight denture biofilms by 16S rRNA gene sequencing technique to first identify most commonly occurring IE-associated pathogens. 16S rRNA gene

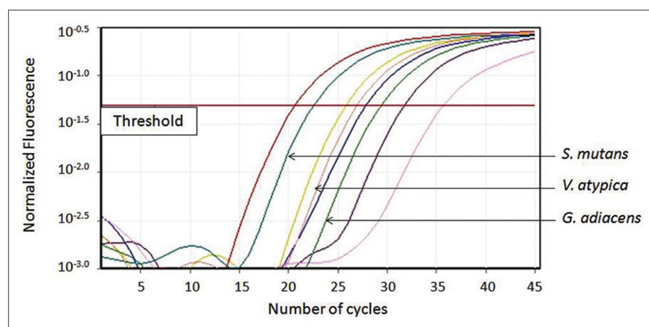

Figure 1: Representative image of real time PCR of *Streptococcus mutans*, *Veillonella atypica*, and *Granulicatella adiacens* detected with species-specific primer in sample 1

sequencing analysis is a powerful technique that is capable of identifying all bacteria present in a given sample at genus and species level and hence was used.[11] The technique has also been used earlier to identify the bacterial presence in biofilms of denture stomatitis patients, wherein several species of bacteria including *Streptococcus, Lactobacillus, Veillonella,* and *Selenomonas* were found.[22] Unlike the above technique, microbiological investigations often provide a limited knowledge on the extent of a bacteriological profile in any given sample as they require multiple culture medium and conditions. Nevertheless, it serves as the most accessible and inexpensive technology, which has indeed brought forth several interesting findings. For example, the existence of several species of *Candida* in denture biofilm was identified by simple microbiological staining protocol.[23] Similarly, bacterial species from *Diphtheroid, E. coli,* and *Micrococcus* genus were found by culture-based protocols in biofilms of partial dentures.[24] However, due to its inherent limitation, 16S rRNA gene sequencing analysis is gaining more attention in clinical research. We performed the 16S rRNA gene sequencing analysis only on a limited sample size as a way to identify commonly occurring pathogenic bacteria. The technique was not extended on the entire sample size due to inherent complexity and economics of the protocol, both of which served as a major limiting factor. Nevertheless, this approach indeed identified the presence of three IE-associated pathogenic bacteria, *S. mutans, V. atypica,* and *G. adiacens* in five out of eight samples. *Staphylococcus aureus*, which accounts for >60% of all forms of IE,[16] was not detected during this analysis. It should be noted, however, that *S. aureus*-associated IE pathogenesis occurs more commonly due to invasive medical procedures and old age but not the status of dentition.[16] Besides *S. aureus,* several other bacteria also known to be associated with IE were not detected during 16S rRNA gene analysis. It is possible that those samples that were not subjected to 16S rRNA analysis may have carried one or more of IE-associated pathogens, which indicates the requirement of a higher sample size.

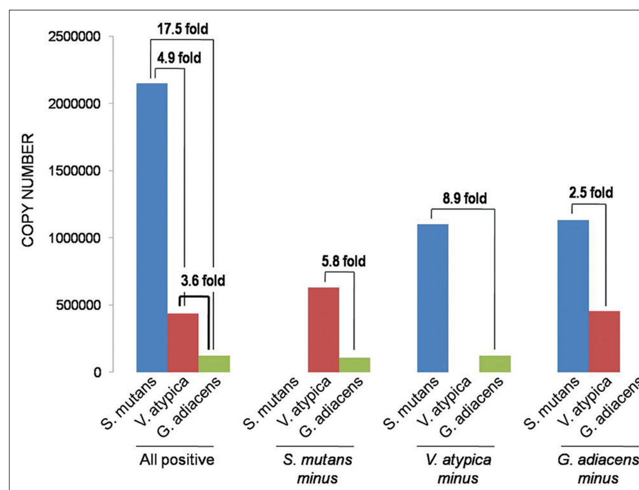

Figure 2: Graphical representation of cumulative copy numbers of *Streptococcus mutans*, *Veillonella atypica*, and *Granulicatella adiacens*

To understand the overall relative presence of *S. mutans, V. atypica,* and *G. adiacens,* 88 biofilm DNA samples were analyzed by qPCR with species-specific primers. This analysis identified the presence of *S. mutans, V. atypica,* and *G. adiacens*, both in an independent and overlapping manner in many of the samples. Interestingly, comparison of the cumulative copy numbers of two bacteria in the absence of third bacteria revealed a selective dominance pattern. For example, in the absence of *S. mutans*, *V. atypica* displayed dominance. Similarly, in the absence of *V. atypica*, *S. mutans* displayed dominance. However, in the absence of *G. adiacens,* the fold difference between the two dominant species (*S. mutans* and *V. atypica*) was less. This clearly indicated how *S. mutans* and *V. atypica* can act as codominant species in biofilm microflora in an otherwise competitive environment within the biofilm.

While the finding revealed an interesting pattern of bacterial co-occurrence, it also sheds light into their probable mechanistic role in biofilm formation, as both *S. mutans* and *V. atypica* are known early colonizers of oral biofilms. It is important to note that biofilms serve as niche for bacterial colonization and hence is capable of aggravating an existing clinical condition such as native valve endocarditis.[25] Other bacteria among those detected by 16S rRNA gene analysis are also capable of forming and establishing biofilms.[26] For example, *Streptococcus sanguinis, Rothia dentocariosa* and species of *Neisseria* act as early colonizers of biofilm community along with *S. mutans*.[27] Both *S. sanguinis and R. dentocariosa* have also been associated with IE.[28] However, they were not included on the extended samples as the study was designed to investigate most commonly present IE-associated pathogens. The finding of *S. mutans, V. atypica,* and *G. adiacens* in the denture wearers without antibiotic cover for at least 3 months before the date of

sampling reflects an unbiased higher overall occurrence pattern. Although all of the above bacteria have been found to be associated with IE independently,[18-20] none of the studies have confirmed their co-occurrence pattern. The present study is the first to identify its co-occurrence, which highlights the importance of an inclusive analysis of bacterial signatures in patients with IE so as to administer an effective antibiotic treatment regimen. Thus, the findings of the present study gains clinical significance that necessitates an improved maintenance of dentures so as to prevent denture-induced inflammatory events, which may otherwise serve as a port of entry for IE-associated bacterial pathogens.

CONCLUSION

The 16S rRNA gene-based sequence analysis in combination with quantitative real-time PCR has identified for the first time the co-occurrence of *S. mutans, V. atypica,* and *G. adiacens* along with its relative presence in biofilms of complete dentures, which demonstrated the presence of co-operative existence mechanism within the biofilm environment. Hierarchical distribution of the three bacteria indicated the dominance of *S. mutans* followed by *V. atypica* in the biofilms. As all the three bacterial species are known to be strongly associated with IE, the data from the present study highlight for the requirement of increased awareness among the elderly for better maintenance of their complete dentures.

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Conflicts of interest

There are no conflicts of interest.

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