Cariogenic microflora and pH in superficial and deep layers of occlusal carious lesions - A metagenomic analysis

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Abstract

Background: Streptococcus mutans and Lactobacilli were the primary microorganisms that cause dental caries. However, current molecular microbiology advancements have suggested the possible roles of other microorganisms in causation of carious lesions. Aims and Objectives: The aim is to explore the complete bacterial profile and pH in superficial and deep layer of carious dentinal lesion in reversible pulpitis patient. Materials and Methods: A total of 12 patients with occlusal cavitated lesion were chosen for this study. The carious dentinal sample was collected. The samples were subjected to DNA extraction quantification with 16S rRNA amplification and pH measurement by suspending the carious sample into 0.9% of NaCl solution. Results: The results showed higher number of Actinobacteria followed by Firmicutes, Fusobacteria, Bacteroidetes, and Spirochaetes. The superficial layer was found to be acidic pH. Conclusion: There are more bacteria in the superficial carious layer than in the deep layer, with a fold difference of 2.8%.

Keywords
Bacterial population, deep dentinal carious layer, metagenomic analysis, superficial carious layer

Introduction
Dental caries is a disease that is characterized by the localized destruction of susceptible dental hard tissue by acidic products from bacterial fermentation of dietary carbohydrate. Various studies continue to prove the association between the oral microbiota and the changes in the oral environment. [1,2] Traditional culture techniques have shown that Streptococcus mutans is the chief pathogen associated with caries in addition to Lactobacillus spp. and Actinomyces spp. [3-6] Recent technology, using 16S rRNA gene, has supported the theory of "mixed/nonspecific microbial hypothesis" where diverse array of bacteria have been identified in caries initiation and progression. This includes S. mutans, non-mutans Streptococci and members of the genera Actinomyces, Bifidobacterium, L. bacillus, Propionibacterium, Veillonella, Selenomonas, and Atopobium are associated with different stages of carious lesions. [7-10]

Low environmental pH was due to shift of an acid-tolerant and acid-producing consortium of bacteria, which altered the balance of remineralization to demineralization, thus forming the carious lesions. [11] The size and thickness of the lesion was also correlated with the initial pH. [12,13] The etiology of caries being multifactorial, there is evidence of carious lesions developing in the absence of S. mutans. With the advent of new technology in the world of molecular microbiology, it has been proven that numerous novel bacteria other than S. mutans have been isolated from the carious dentin. [14] Thus, the aim of the study was to explore the complete bacterial profile and pH of the superficial layer and deep layer of carious dentinal lesion through metagenomics.

Materials and Methods
This study had undergone the institutional review board and the institutional ethical committee (Approval code IGIDSIRB2014 NDP03PGVSCDE). Written consents were obtained from all participants in this study.

The sample size was obtained as 12 depending on the rationale given for pilot study by Julious 2005. [15] Patients of age group between 18 and 35 years, with moderate and high caries risk, diagnosis of reversible pulpitis were included in this study. Written informed consent was obtained. Preoperative radiograph was taken to assess the depth of the carious lesion and the remaining dentine thickness of 1.5 mm as shown in Figure 1. Under rubber dam isolation carious dentine samples
were excavated with a sterile sharp discoid excavator. Around 3 to 4 layers of 1 mm thick dentin was excavated, starting from the periphery of the cavity then progress to the sound dentin. The layers ranged from layer 1, representing the most superficial layer of the lesion, to layer 4, which was the deepest layer of the lesion as shown in Figure 2. All sample collections were performed by a single calibrated dentist. If excavation of caries cannot be done in layer and at the time of excavation if cusp gets fractured, these samples were excluded.

**pH measurement**

The carious sample were suspended in 0.9% of NaCl solution and measured in ion-sensitive field-effect transistor electrode.

**DNA isolation**

The lysed bacterial cells were processed for DNA extraction with silica DNA capture columns as per manufacturer’s protocol (QIAamp DNA Mini Kit, Cat# 51304).

**16S rRNA amplification and sequencing**

Polymerase chain reaction amplification of the 16S rRNA gene hypervariable region V6 was performed on 10 mg of total DNA extracted from each of the sample with a pool of six degenerate forward and reverse primers, which detect all the bacteria present in any given sample as described by Junemann et al.\(^{16}\) The diversity is calculated as the 16S rRNA operational taxonomic units and recorded the read count.

**Results**

16S rRNA gene sequencing was carried out on superficial and deep layer of carious samples of 12 patients. In this study, a total of 35 distinct genera were present at abundance. *Actinobacteria* dominated and accounted for the majority at 63.3% of the total identified genera followed by *Firmicutes* at 32%, *Fusobacteria* at 3.8%, *Bacteroidetes* at 0.6%, and *Spirochaetes* at 0.2% Majority of bacteria in the superficial layer and deep layer were from *Actinobacteria* followed by *Firmicutes* as shown in Graph 1. The pooled read counts of all bacteria in superficial layer were at least 2.8 fold more than those in deep layer as shown in Graph 2. In this study, a total of 13 bacterial genera identified from Phylum *Actinobacteria*, higher read counts for *Olsenella* (56629) and acidogenic *Atopobium* (26917) were identified in the superficial layer followed by *Propionibacterium*. In deep layer, acidogenic *Parascardovia* (34350) and *Actinomyces* (13868) showed higher read count. A total of 17 bacterial genera were identified.
from phylum Firmicutes. Acidogenic Lactobacillus (30553), Streptococcus (14452), and Granulicatella (14429) showed the higher read count in superficial layer. In Phylum Fusobacteria showed increased bacterial count with presence of three bacterial genera, namely, Fusobacterium, Leptotrichia, and Streptococcus, in the superficial layer. Phylum Spirochaetes and Phylum Bacteroidetes showed the presence of only one genus, which showed increase in read count in the superficial layer. The pH of superficial zone was found to be significantly more acidic than the deepest areas of the lesion as shown in Graph 3.

Discussion

Bacterial levels in deep and superficial layers may show significant changes in their population as the deeper layers tend to be more acidic due to bacterial activity in the active caries zones. These sites are likely to undergo shifts in the pH with acidogenic or aciduric bacterial by-products. In this study, 16S rRNA sequencing-based metagenomic analysis was employed to determine the bacterial profile of superficial and deep layers of caries and association with pH. The bacterial flora was more in the superficial carious layer than the deep layer with the fold of 2.8 times for this study. The DNA extraction was done with a QIAamp DNA mini kit, with primers and quantification was done.[19,16] In this study, a total of 35 distinct genera were evident. Among them, 63.3% of Actinobacteria were predominant, followed by Firmicutes at 32%, Fusobacteria at 3.8%, Bacteroidetes at 0.6%, and Spirochaetes at 0.2% which was in accordance to a study by Dewhirst et al.[1]

Among the phylum Actinobacteria, the bacterial genus Olsenella showed the highest level of read counts at 25.5%. In this study, 9 samples showed higher level of Olsenella. Another study conducted by Chhour et al. in 2005 showed the presence of Olsenella in two samples.[20] Parascardovia and Scardovia belong to the family Bifidobacteriaceae, which was found to be 17.3% in this study. Mantzourani et al. demonstrated that Scardovia, and Parascardovia were associated with cavitated caries lesions, together with S. mutans, Lactobacilli, and yeasts, indicating that the acidic environment of the lesions provided a suitable habitat for the proliferation of these aciduric microorganisms.[21]

Atopobium was found to be next in the higher level of incidence with 13.5% in the phylum Actinobacteria. The distribution of acidogenic Atopobium is more in the superficial layer than the deep layer at 26917 and 2980 read counts respectively. Atopobium has been reported to be present in varying stages of the disease including early colonization of dental tissues. [22,24] Actinomyces is an acidogenic bacteria with 8.3% of total population from phylum Actinobacteria. Similar study have shown that microflora of clinically sound enamel surfaces contains mainly non-mutans Streptococci and Actinomyces, in which acidification is mild and infrequent.[25] Propionibacterium is an acidogenic bacteria with a prevalence of 7.3% of total population from phylum Actinobacteria. Total pooled count in the superficial layer was 12660, and deep layer was found to be 3949. Propionibacterium is commonly associated with advanced caries lesion and with low pH.[24,26,27] Firmicutes which are mostly acidogenic in nature accounted for the second most abundant phylum following Actinobacteria. Among the 17 Firmicutes identified, the genus Lactobacillus was predominant with 33.7% of the total population from phylum Firmicutes. The Superficial carious layer showed more prevalence than the deep layer. The dominant phyla in the dentine caries lesions usually include Firmicutes with Lactobacillus accounting for nearly 40% of the total general.[28] The occurrence of Lactobacillus in saliva is common in high caries patient. Lactobacillus metabolizes dietary sugars immediately and produces acid which is cause decalcification of teeth.[21,17,29,30] In the deep dental lesions, acid producing Lactobacillus spp are found to be predominant. In the molecular perspective, both S. mutans and Lactobacillus spp sometimes are at very low levels or even go undetected suggesting that initiation and progression of caries cannot be solely attributed to these traditionally causative bacteria.[31,32] In this study, five patients showed very minimal prevalence of Lactobacillus both in the superficial and deep layer. These data indicate that the Lactobacillus are not absolute requisite for the development of carious lesions. Nonetheless, they may potently contribute to the demineralization of the teeth once lesions are established. Streptococcus an acidogenic Firmicutes constituted 13.3% of population, superficial layer expressed more concentration of S. mutans than the deep layer. In this study, four patients showed very less concentration of S. mutans. For many years, acidogenic S. mutans was considered as the causative organism for dental caries.[33] Recent molecular study using second-generation sequencing and metagenomic techniques has discovered an extraordinary ecosystem where S. mutans accounted for only 0.7-1.6% of carious lesions.[33,35] However, some recent studies indicate that the relationship between S. mutans, and caries is not absolute high proportions of S. mutans may persist on tooth surfaces without lesion development, and caries can develop in the complete absence of S. mutans.[34,35] Veillonella are an acidogenic Firmicutes with 5.8% of the total population. In this study, 5 patients showed complete absence of this genus in the deep layer. Veillonella have been shown to be predominant at all stages of caries progression and has coaggregation property with S. mutans.[36,37] The pH analysis revealed a more acidic superficial
layer which coincided with its greater number of acidogenic bacteria compared to the deep layer. This difference in acidity can be directly correlated with the acidogenic potential of the bacteria. The pH of superficial layer ranges from 4.9 to 5.8; in the deep layer, it was present in the range of 6.1-6.9. Newly detected organisms, Scardovia and Propionibacteria, were found to exist in deeper dental samples of this study which might lead to a hypothesis in which these bacteria along with known pathogens such as *Lactobacilli* may coexist and mediate caries progression. Another bacteria *Kocuria*, around 3.4%, was found to be present in the samples, which was evident in the deeper layer. This finding is in accordance with the recent evidence of newly detected cariogenic organisms in 16s RNA amplification technique and sequencing. Current literature reveals no studies which have clearly demarcated the existence or absence of certain bacteria, both known and novel, both pathogenic and commensals in designated samples such as caries of plaque. This has led to newer questions in which future studies are warranted.

**Conclusion**

There are more bacteria in the superficial carious layer than in the deep layer, with a fold difference of 2.8%. The superficial layer of the caries is more acidic compared to the deeper layer, at the range of 4.7-5.9 and 6.1-6.9.

**References**

30. Xiao C, Ran S, Huang Z, Liang J. Bacterial diversity and community structure of supragingival plaques in adults with