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ORIGINAL ARTICLE

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%.

Introduction

Dental caries is a disease that is characterized by the localized destruction of susceptible dental hard tissue by acidic by 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.

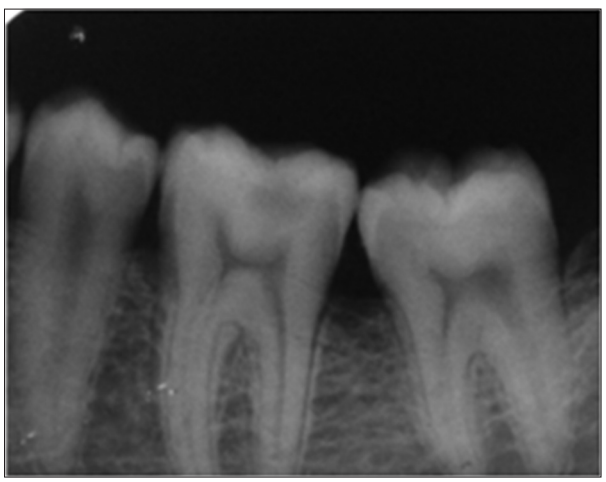


Figure 1: Radiographic assessment for depth of the lesion

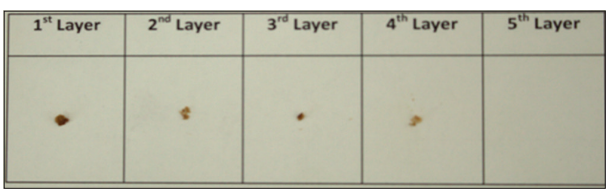
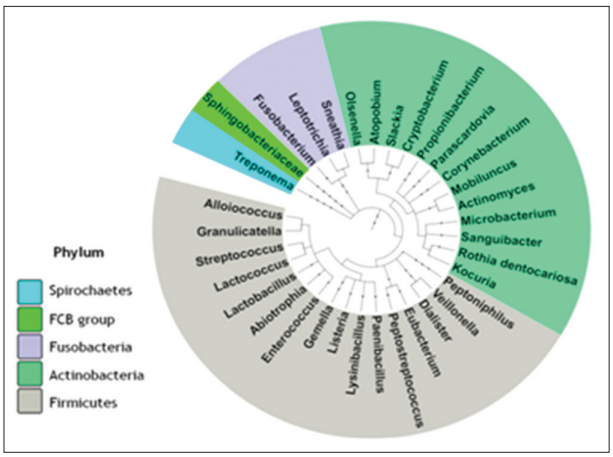


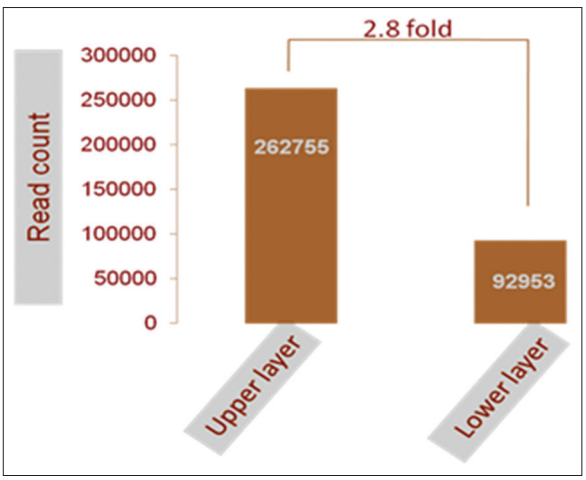
Figure 2: Represents the superficial layer and deep layer of the lesion

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



Graph 1: Phylogenetic representation of the bacterial genus indicates higher prevalence of *Firmicutes* and *Actinobacteria*

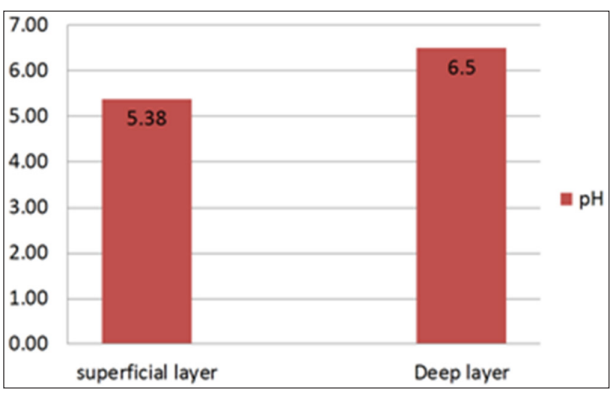


Graph 2: Comparison of pooled read counts of bacteria between superficial and deeper caries layers

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 *Sneathia*, 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.[17-19] 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]



Graph 3: Comparison of pH of carious dentine between superficial layer and deep layer-superficial layer is more acidic

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.[2,22-24] *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.[2,17,29,30] In the deep dentinal lesions, acid producing *Lactobacillus* spp are found to be predominant. In the molecular perspective, both *S. mutans* and *Lactobaccillus* 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 *Lactobacilli* 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.[14] 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.[10,33] 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*.[9,36] 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 Propniobacteria, were found to exist in deeper dentinal 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.[17] 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.

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