Altered Bacterial Profiles in Saliva from Adults with Caries Lesions: A Case-Cohort Study

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Key Words

16S rRNA · Bacteria · Biomarker · Caries · Human Oral Microbe Identification Microarray · Saliva

Abstract

The aim of this study was to learn whether presence of caries in an adult population was associated with a salivary bacterial profile different from that of individuals without untreated caries. Stimulated saliva samples from 621 participants of the Danish Health Examination Survey were analyzed using the Human Oral Microbe Identification Microarray technology. Samples from 174 individuals with dental caries and 447 from a control cohort were compared using frequency and levels of identified bacterial taxa/clusters as endpoints. Differences at taxon/cluster level were analyzed using Mann-Whitney's test with Benjamini-Hochberg correction for multiple comparisons. Principal component analysis was used to visualize bacterial community profiles. A reduced bacterial diversity was observed in samples from subjects with dental

caries. Five bacterial taxa (*Veillonella parvula*, *Veillonella atypica*, *Megasphaera micronuciformis*, *Fusobacterium periodontium* and *Achromobacter xylosoxidans*) and one bacterial cluster (*Leptotrichia* sp. clones C3MKM102 and GT018_ot417/462) were less frequently found in the caries group (adjusted p value <0.01) while two bacterial taxa (*Solobacterium moorei* and *Streptococcus salivarius*) and three bacterial clusters (*Streptococcus parasanguinis* I and II and sp. clone BE024_ot057/411/721, *Streptococcus parasanguinis* I and II and *sinensis*_ot411/721/767, *Streptococcus salivarius* and sp. clone FO042_ot067/755) were present at significantly higher levels (adjusted p value <0.01). The principal component analysis displayed a marked difference in the bacterial community profiles between groups. Presence of manifest caries was associated with a reduced diversity and an altered salivary bacterial community profile. Our data support recent theories that ecological stress-induced changes of commensal microbial communities are involved in the shift from oral health to tooth decay.

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Dental caries, one of the world's most prevalent diseases, is a biofilm-mediated condition resulting from a complex interaction between the commensal microbiota, host susceptibility and environmental factors, such as diet [Selwitz et al., 2007; Wade, 2013]. During the past decades, the understanding of the role of oral bacteria in the caries process has changed from the specific plaque hypothesis, mainly focusing on the role of *Streptococcus mutans* [Loesche, 1986], to the ecological plaque hypothesis [Marsh, 2010; Nyvad et al., 2013]. Today, caries is considered to be due to endogenous bacteria, and the concept is that ecological stress may shift the microbial community composition and favor growth of aciduric bacteria from a state of mutualism towards parasitism in the oral ecosystem [Takahashi and Nyvad, 2011].

The rapid development of advanced molecular methods has provided important insights to the oral microbiome and complex biofilm compositions. More than 700 oral bacterial species have been identified, of which only 50% are cultivable with traditional agar-based methods [Dewhirst et al., 2010]. The bacterial profiles related to caries have been investigated with various molecular techniques [Preza et al., 2009; Luo et al., 2012; Tanner et al., 2012; Torlakovic et al., 2012]. Most studies analyzed samples collected directly from caries lesions. However, being easy and inexpensive to collect, saliva is an almost ideal biological secretion for studies of the oral microbial profile in health and disease [Baum et al., 2011]. The bacterial composition of saliva in oral health has been examined in previous studies [Nasidze et al., 2009; Bik et al., 2010], but the question whether or not dental caries is characterized by detectable changes in the bacterial profile of saliva has been addressed mostly in studies of young populations [Ling et al., 2010; Crielaard et al., 2011; Luo et al., 2012; Yang et al., 2012]. Thus, the objective of this study was to analyze whether presence of dental caries in a large adult population was associated with a different salivary bacterial profile when compared to individuals without untreated caries.

Subjects and Methods

Study Population

The Danish Health Examination Survey (DANHES) was carried out in 13 Danish municipalities during 2008 and 2009 [Eriksson et al., 2011]. A total of 76,484 people completed a questionnaire concerning general health and lifestyle, of whom 18,065 underwent a thorough medical examination and 4,402 an oral examination (mean age 54 years, range 18–96 years), including a second questionnaire regarding self-perceived oral health. In addition, a sample of stimulated whole saliva was collected. In this study, sa-

Table 1. Background characteristics of the two study groups

	Control cohort	Caries group
Number of participants	447	174
Mean age, years (range)	55.0 (53.8–56.3)	50.0 (47.5–52.5)
Men/women	40/60%	52/48%
Smokers	34 (7.6%)	28 (16.0%)
With periodontitis	21 (4.7%)	10 (5.7%)
With caries (≥ 3 DS)	30 (6.7%)	174 (100%)
DMFT, mean (SD)	19.0 (6.8)	19.0 (7.6)
DMFS, mean (SD)	62.0 (29.9)	61.0 (35.0)
DT, mean (SD)	0.6 (1.1)	3.7 (1.7)
DS, mean (SD)	0.6 (1.3)	4.6 (2.2)

liva samples from 174 patients with caries lesions were analyzed and compared with a control cohort of 447 samples. The study population of the oral part of DANHES is described elsewhere [Kongstad et al., 2013] and the characteristics of the present material are shown in table 1. All participants received written information and signed an informed consent before enrolment in the study. The regional ethical committee approved the study (H-C-2007-0118), and the study was reported to the Danish Data Authority (2007-41-15-67). This study is part of a group of studies conducted on the data from DANHES, including analysis of saliva samples investigating how periodontitis and different lifestyle are related to the bacterial profile of saliva.

Study Design

The present study employed a cross-sectional case-cohort design. Thus, a sub-cohort (control cohort) was randomly selected from the entire cohort without any regards to dental status. This design was chosen due to the high caries experience in the original cohort and in order to facilitate comparison of the caries group with the general population, thereby minimizing the risk of selection and information bias [Le Polain de Waroux et al., 2012].

Sample Selection

A flow chart of sample selection is presented in figure 1. From the total of 4,402 samples, 600 were randomly selected as control cohort. Thereafter, 205 samples from patients with dental caries, defined as manifest caries lesions on ≥ 3 surfaces, were identified from the remaining DANHES cohort of 3,802 samples. From the total of 805 samples selected, 148 samples were excluded because of insufficient amount of saliva. 36 of the remaining samples contained too little bacterial DNA for Human Oral Microbe Identification Microarray (HOMIM) analysis and were excluded after a polymerase chain reaction (PCR) quality control step. Thus, the final material consisted of a dental caries group comprising 174 cases and a control cohort of 447 subjects. Further background data of the two groups are presented in table 1.

Oral Examination and Registration of Dental Caries

The oral examination was carried out in either a mobile dental setting or at fully equipped local dental clinics. Six specially trained dental professionals (three dental hygienists and three dental assistants) performed the procedure in pairs; the hygienist carried

Fig. 1. Flow chart of sample selection and group establishment.

out the examination and the assistant scored the registrations. Caries was scored according to Moller and Poulsen [1973] as a clinically manifest lesion present on surface and tooth levels and expressed as DMFS (decayed, missed and filled surfaces) and DMFT (decayed, missed and filled teeth). No discrimination was made as to whether the lesion was primary or secondary. Furthermore, no information from bitewing radiographs was available. The dental hygienists were calibrated on two occasions during the data collection with inter- and intra-examiner kappa coefficients between 0.85 and 0.93 [Kongstad et al., 2013].

Collection of Saliva Samples

Prior to stimulated whole saliva collection, the participants were asked to rinse their mouth and drink half a glass of water. A 1-gram piece of paraffin wax was handed out and the subjects were

asked to chew for 4 min. Saliva secreted during the last 3 min was collected in a graded plastic cup. Saliva was then transferred to plastic tubes in aliquots of 0.5 ml and immediately stored at -80°C until further processing.

DNA Isolation and HOMIM Analysis

DNA isolation was performed according to the manufacturer's instruction given in protocol: Pathogen_Universal_200 (Roche, Mannheim, Germany). The laboratory procedures of the HOMIM technique have been described in detail by Colombo et al. [2009]. In brief, HOMIM is a high-throughput molecular technique using 16S rRNA-based reverse-capture oligonucleotide probes for simultaneous identification of around 300 bacterial species. At first, a quality control PCR was performed using universal forward (NF1: CCA CRG TTY CAT YMT GGC) and re-

verse (1541R: RRA GGA GGT GWT CCA DCC/1492R: GDT AYC TYG TTA CGA CTT) primers (custom-made primers, Sigma-Aldrich, St. Louis, Mo., USA). PCR products were analyzed on a 1% agarose gel, and compared with negative and positive control samples. As a second step of quality control, DNA concentration was analyzed using NanoDrop 8000 Spectrophotometer, assuring that only samples with a DNA concentration >10 ng/μl were further analyzed (Thermo Scientific, Waltham, Mass., USA). For incorporation of fluorescent Cy3-dCTP, a second nested PCR was performed using another forward (9F: GRD TTY GAT YMT GGC TCA G) (Sigma-Aldrich) and the same 1492R reverse primer. Using DNA-DNA hybridization, oligonucleotide probes (18–20 bases long) printed on an aldehyde-coated glass slide captured the fluorescent-labeled PCR products. After hybridization, an Axon 4000B scanner was used for scanning of slides and collection of crude data. Further analysis of data was performed using the Genepix 6 software (Molecular Devices, Sunnydale, Calif., USA). Each bacterial taxon/cluster was represented by a specific area on the microarray and the presence/absence was based on the mean value of duplicate spots. Data were normalized to positive and negative controls, and a semi-quantitative HOMIM value from 1 to 5 was calculated for each taxon/cluster. The mean fluorescent intensity had to be at least two times higher than the background of the microarray for positive probe identification. All probes were thoroughly validated and probes identified with cross-reactions and/or missing reaction were removed from the analysis. Further analysis and generation of microbial profiles were performed using an HOMIM online tool (<http://bioinformatics.forsyth.org/homim/>).

Statistical Analysis

The non-parametric Mann-Whitney test was used for comparison of hybridization patterns and the background characteristics were processed with χ^2 tests. For these analyses, a p value <0.05 was considered statistically significant. Using Mann-Whitney's test with Benjamini-Hochberg correction [Hochberg and Benjamini, 1990] for multiple comparisons, differences between the groups with respect to taxon/cluster levels were analyzed using frequency (presence/absence) and levels (mean HOMIM value) of each taxon/cluster as parameters. Adjusted p values <0.01 were considered statistically significant. To investigate mathematical patterns in this n-dimensional dataset, microbial profiles were displayed graphically using principal component analysis. MeV 4.8.1 [Saeed et al., 2006] and the GraphPad Prism software (GraphPad, San Diego, Calif., USA) were used for statistical analysis.

Results

Background Characteristics

Mean age and gender distribution differed significantly ($p < 0.05$) between the caries cases and the control cohort, as shown in table 1. Furthermore, smoking was more frequent in the caries group ($p < 0.05$). The mean DMFT and DMFS values, representing the cumulative caries experience, were almost identical in the two groups. However, the DT and DS values, illustrating

the present caries activity, were more than 6 times higher in the caries group than in the control cohort ($p < 0.05$). Seven percent ($n = 30$) of the control cohort exhibited ≥ 3 decayed tooth surfaces. A small proportion of participants in both the caries group and the control cohort were diagnosed with periodontitis (5.7 and 4.7%, respectively).

General Findings

A reduced microbial diversity was observed in the caries group. Positive hybridization with the target of 116 probes (68 bacterial taxa and 48 bacterial clusters) and 146 probes (98 bacterial taxa and 48 bacterial clusters) was identified in the caries group and the control cohort, respectively ($p < 0.05$). The mean number of hits identified per sample was 20.6 (SD 8.8) in the caries group and 23.2 (SD 9.0) in the control cohort ($p < 0.05$). *Firmicutes* was the dominant bacterial phylum, constituting around 40% of the total probe signal in both groups, while *Actinobacteria*, *Proteobacteria* and *Bacteroidetes* accounted for approximately 10% each. The predominant bacterial taxa and clusters (present in more than 50% of the samples) were similar in both groups and were dominated by *Streptococcus* species. *S. mutans* was observed only sporadically in both groups. A complete list of probes identified is presented as supplementary material (www.karger.com/doi/10.1159/000357502).

Taxon and Cluster Findings

Five bacterial taxa (*Fusobacterium periodontium*, *Veillonella atypica*, *Megasphaera micronuciformis*, *Achromobacter xylosoxidans* and *Veillonella parvula*) and one bacterial cluster (*Leptotrichia* sp. clones C3MKM102 and GT018_ot417/462) were significantly less frequently detected in the caries group than in the control cohort ($p < 0.01$; fig. 2). On the other hand, two bacterial taxa (*Streptococcus salivarius* and *Solobacterium moorei*) and 3 bacterial clusters (*Streptococcus parasanguinis* I and II and sp. clone BE024_ot057/411/721, *Streptococcus parasanguinis* I and II and *sinensis*_ot411/721/767, *S. salivarius* and sp. clone FO042_ot067/755) were found at higher levels in samples from the subjects with dental caries ($p < 0.01$; fig. 3). In addition, four bacterial taxa (*F. periodontium*, *V. atypica*, *M. micronuciformis* and *A. xylosoxidans*) and one bacterial cluster (*Leptotrichia* sp. clones C3MKM102 and GT018_ot417/462) were present at higher levels in the control cohort ($p < 0.01$; fig. 3). Five out of six taxa/clusters identified more frequently in samples from the control cohort were also present at higher levels. To check the in-

Fig. 2. Taxa and clusters less frequently identified in saliva from the caries group. Binary values (presence/absence) of each taxon/cluster were compared between the caries group and the control cohort. The bars represent the percentage of each taxon/cluster of the total samples. White bars = Caries group; black bars = control cohort. * Adjusted p value <0.01; ** adjusted p value <1.0 × 10⁻⁵.

Fig. 3. Taxa and clusters identified at significantly different levels (mean HOMIM value) in the caries group and the control cohort. The mean HOMIM values and standard deviation of each taxon/cluster are displayed. White bars = Caries group; black bars = control cohort. * Adjusted p value <0.01; ** adjusted p value <1.0 × 10⁻⁴.

fluence of the case-cohort design, the 30 subjects with caries in the control cohort were compared with the caries group and no significant differences were observed at taxon/cluster level. In addition, different age and gender groups were compared to check the influence of these parameters. No difference was observed based on age and gender (data not shown).

Bacterial Community Profiles

Principal component analysis was performed for visualization of bacterial community profiles using levels (mean HOMIM value) of each identified taxon/cluster as

parameter of investigation (fig. 4). Two-dimensional bacterial community profiles were displayed, using component 1 and component 3 accounting for 26.3% of the variation within the total sample cohort. Principal component analysis revealed markedly different bacterial community profiles between the caries group and the control cohort, showing clustering of samples from caries patients and random spreading of the control cohort samples. In addition, the 30 caries samples from the control cohort were observed to cluster with the caries samples from the caries group.

Fig. 4. Principal component analysis exploring patterns in the dataset in mean HOMIM values (level). Two-dimensional presentation of samples from caries patients, $n = 174$ (blue), healthy controls, $n = 417$ (red), and caries samples from the control cohort, $n = 30$ (green), characterized by principal component 1 and 3 accounting for 26.3% of the total variation of the dataset.

Discussion

The main and novel finding of this study was that the salivary bacterial profiles differed significantly between individuals with and without untreated caries lesions in a large population of adult Danes, all characterized by a high experience of previous caries. A reduced diversity and an increased prevalence of acidogenic and aciduric streptococci were evident in the untreated caries group. Similar differences have been disclosed before, but mainly in caries-active vs. caries-free infants, children or adolescents [Li et al., 2007; Aas et al., 2008; Luo et al., 2012; Tanner et al., 2012]. Our findings, based on the HOMIM technology, are in line with the concept that an ecological stress-induced imbalance of the resident microflora is involved in the caries process [Takahashi and Nyvad, 2011], and apparently, this seems true also in healthy adults with prior significant caries experience, i.e. a history of more than 60 filled tooth surfaces.

The case-cohort design employed here is, in contrast to the more common case-control setup, sometimes misjudged since also the control cohort includes cases. However, with the control cohort mimicking the general population, possible differences between the groups are more likely to be obscured than overestimated, which indicates that the results obtained here are reli-

able and with high external validity, a fact underlined by the separate analysis of the 30 'cases' within the control cohort. Furthermore, the fact that the control cohort was approximately 2.5 times larger than the caries group substantially increased the power of the study. Thus, the possible influence of the differences in gender distribution, age and smoking habits between the groups was minimized. Obviously, the cross-sectional nature of this project did not allow for any causative statements. Therefore, the question whether an altered bacterial profile foregoes lesion development or whether it is a result of active caries processes could not be addressed. It should however be emphasized that the participants of this study were regularly attending dental care and had a high level of oral hygiene habits [Kongstad et al., 2013]. It is therefore very likely that the 'cases', exhibiting at least three decayed surfaces, truly were in a caries-active phase. Being the only major difference between the groups, the characteristics associated with manifest untreated caries were therefore believed to account for the altered salivary profiles observed.

In this study, we sampled whole saliva as a biomarker for the caries process. Saliva can be easily and rapidly collected and could ideally facilitate early identification of caries-prone individuals and provide dental professionals with targeted tools for preventive regimens of high-risk individuals. On the other hand, it is important to point out that findings from saliva cannot directly be compared with supragingival plaque samples from selected sites. For example, we found the acidogenic bacteria *S. salivarius* and *S. parasanguinis* to be positively associated with caries patients, while the same species were shown under-abundant in samples collected from caries lesions [Becker et al., 2002; Corby et al., 2005]. It is however important to underline that the HOMIM technique is only able to recognize bacterial taxa/clusters that have a target probe present on the microarray. As a consequence, the complete oral microbiome is not unveiled. Furthermore, the oligonucleotide probes on the microarray are composed of 18–20 bases, and therefore all members of some genera, e.g. *Streptococcus* and *Prevotella*, may not be identified at species level. Another possible limitation of this study was that the stimulated saliva samples were not collected at a standardized time of day and potential confounders associated with food intake and toothbrushing cannot be excluded. Furthermore, since no information from radiographs was available, the prevalence of caries was likely somewhat underestimated in both groups.

The number of positive identifications per sample was found to be higher in the control cohort than in the caries group, suggesting a reduced diversity in caries-related biofilms. This was in agreement with Li et al. [2007], but conflicting with the findings of Yang et al. [2012]. Likewise, a similar phylogenetic distribution as shown in both study groups has been unveiled in previous trials [Ames et al., 2012; Luo et al., 2012; Segata et al., 2012]. The genera *Streptococcus* and *Veillonella* constituted the predominant bacterial profile, which was in accordance with earlier findings from saliva of adults [Nasidze et al., 2009; Segata et al., 2012] and from Dutch [Crielaard et al., 2011] but not Chinese children [Luo et al., 2012]. One interesting study performed by Crielaard et al. [2011] demonstrated that the relative abundance of *Veillonellaceae* in saliva increased from deciduous to permanent dentition. Thus, it is possible that factors such as age, diet and life-style can affect oral bacterial community structures. The final answer to the question whether caries-active individuals exhibit a greater [Luo et al., 2012; Yang et al., 2012] or smaller [Li et al., 2007] microbial diversity than caries-free subjects is yet to be determined.

In concordance with a previous report [Yang et al., 2012], major differences were observed at the bacterial taxon/cluster level between the caries group and the control cohort, indicating that dental caries is associated with an alteration of the bacterial profile of saliva distinct from that of oral health in quantitative rather than qualitative terms. Interestingly, *S. moorei*, *S. parasanguinis* and *S. salivarius* have previously been found to be associated with orthodontically induced white spot lesions [Torlakovic et al., 2012]. Furthermore, *F. periodontium* has been identified to be more associated with oral health than with dental caries [Torlakovic et al., 2012], which is in accordance with this study. *S. mutans* and *Lactobacillus* species have traditionally been associated with dental caries, but in

concert with earlier studies [Luo et al., 2012; Yang et al., 2012], these bacteria were found only sporadically in the groups studied here, supporting the assumption that saliva is not a reservoir for these putative caries pathogens and that their role in the caries process may have been overestimated.

In conclusion, within the limitations of this study, our findings suggest that presence of manifest untreated caries is associated with reduced diversity and an altered bacterial community profile in saliva. Our data support the view that ecological stress-induced changes of the commensal bacterial communities are involved in the caries process. Population-based longitudinal studies are however required to reveal whether screening of bacterial profiles in saliva can be used as an early biomarker and predictor of future caries development.

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Disclosure Statement

The authors have no conflicts of interest to declare.

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