12-30-2015

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Cristiane Machado Mengatto

Leonardo Marchini
University of Iowa

Luciano Angelo de Souza Bernardes

Please see article for additional authors.
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Cristiane Machado Mengatto 1*, Leonardo Marchini 2, Luciano Angelo de Souza Bernardes 3, Sabrina Carvalho Gomes 1, Alessandro Moura Silva 4, Célia Marisa Rizzatti-Barbosa 5

1Department of Conservative Dentistry, Federal University of Rio Grande do Sul, School of Dentistry, Porto Alegre, Rio Grande do Sul, Brazil
2Department of Preventive and Community Dentistry, University of Iowa, College of Dentistry, Iowa City, Iowa, USA
3Department of Science and Technology, State University of Santa Cruz, School of Computer Science, Ilheus, Bahia, Brazil
4Department of Dental Materials and Prosthodontics, State University Julio de Mesquita Filho, School of Dentistry, Sao Jose dos Campos, Sao Paulo, Brazil
5Department of Prosthodontics and Periodontology, State University of Campinas, Piracicaba Dental School, Piracicaba, Sao Paulo, Brazil

INTRODUCTION

There has been a longstanding controversy over the influence of wearing removable partial denture (RPD) on oral health. While many studies report that the RPD alters neither caries prevalence nor periodontal status in the long-term others argue that there is a detrimental effect of RPD on abutment teeth. Furthermore, the literature agrees that dentures, both partial and complete, are considered reservoirs for various microorganism species, particularly opportunistic pathogens associated with systemic and oral diseases, such as caries, dentoalveolar abscesses, noma lesions, endocarditis, aphthous ulcers, denture stomatitis, and periodontal disease. For example, caries-associated bacteria, such as Streptococcus mutans and Bifidobacteria spp., and periodontal pathogens were identified in the complete denture plaque and oral cavity of patients even after the extraction of all teeth. However, no study has ever investigated the microorganisms adhering to the surface of the RPD framework. Thus, the microbial species adhering to...
the inner surface of the cobalt-chromium alloy clasp remain unknown.

Culture-dependent efforts were made to analyze changes in oral bacterial composition of denture wearers. However, these studies have been limited by the difficulties of *in vitro* growth techniques. Advances in molecular techniques have allowed the identification of many species of oral microorganisms, including those that cannot be cultivated. A recent *in vitro* study showed that potentially pathogenic microorganisms from human saliva adhere more largely at framework alloys than at acrylic resin surface and that cobalt-chromium alloy has a unique pattern of microorganism biofilm formation compared to other investigated surfaces. Other authors evaluated the microbial diversity of supragingival plaque of RPD wearers, using denaturing gradient gel electrophoresis (DGGE), a culture-independent method. They observed that health-associated genera tended to decrease, whereas the disease-associated species including *Streptococcus mutans* tended to increase. Thus, it is possible that the oral environment may be altered by RPD treatments.

To the best of our knowledge, no study determined bacterial diversity on RPD framework *in vivo* through culture-independent methods. Therefore, the objectives of this preliminary descriptive study are 1) to characterize bacterial community adhered to the RPD clasps and 2) identify changes in bacterial diversity at two time points: 1 week and 4 months after RPD placement. Our hypothesis is that the bacterial diversity on the RPD framework would change over time.

**MATERIALS AND METHODS**

The dental records of 130 partially dentate patients referred to the Piracicaba School of Dentistry, State University of Campinas, Sao Paulo, Brazil, were assessed. Individuals were eligible if they were completely edentulous at the maxilla and used previous complete denture and partially edentate at the mandible with no previous RPD (n=54). Twenty eight individuals did not respond to the telephone contact. The remaining (n=26) were clinically examined and included if they were systemically healthy, without periodontitis (clinical attachment loss no more than 3 mm with no bleeding on probing), no smokers, with no presence of restorations, active caries, and crowns, no need of pre-prosthetic surgery, no prescription of systemic antibiotics or chemical plaque control in the last six months before the study commencement. Only patients without mandibular posterior teeth and at least 6 mandibular teeth were to be included in order to standardize RPD framework design.

A total of five women (60 to 74 years, mean age 67.6) composed the sample. The study was approved by the institutional ethical review board and the subjects signed informed consent.

During a pre-experimental period (approximately 45 days before RPD delivery) supragingival scaling and polishing were performed and oral hygiene instructions (OHI) were given. The OHI included how to properly floss and how to brush the RPD and the teeth with soft toothbrush and specific toothpaste (Colgate Triple Action, Colgate Palmolive Industry Com., Sao Paulo, Brazil). Subjects were instructed to remove the dentures for cleaning after each meal and before sleeping. Fig. 1 shows the fluxogram of the study.

The clinical procedures to fabricate maxillary complete dentures and mandibular RPDs were performed by a single professional and followed a strict protocol for the production and placement of the dentures. The cobalt-chromium RPD design was standardized and fabricated by the same dental technician. The clasps used were T-bar (Roach) retainers for direct abutment teeth (canines). The clasps were about 1 mm distant from the gingival edge. The denture bases and/or retainers were connected by a lingual plate that was seated on cingulum rests.

At t₀ (day 0, baseline), the new maxillary complete denture and mandibular RPD were delivered to patients. Oral hygiene was checked and instructions were repeated at the delivery appointment. Patients were oriented to abstain from any form of chemical control during the study. They also received reinstructions on how to perform tooth brushing as well as denture cleaning.

Biofilm was sampled after 1 week (t₁) and 4 months (t₂)...

**Fig. 1.** Timeline of the study after enrolment process, interventions, and assessments performed on participants. (RPD) removable partial denture; (OHI) oral hygiene instructions.
of RPD delivery. All subjects consumed no food or beverages 30 minutes prior to the biofilm collection. First, the RPD was removed from the patient’s mouth, washed with saline solution for 10 seconds, and left dry on paper towel for 1 minute. Then, the biofilm adhered on the inner surface of the T-bar clasps was collected with sterile curettes, pooled in a test tube containing 100 μL of DNA extraction solution, and stored at -20°C. rDNA extraction and ethanol precipitation steps followed a previously described protocol. Concentrated rDNA was suspended in 20 μL 10 mM Tris-HCl pH 7.5, 1 mM EDTA solution and PCR-amplified by using universally conserved primers D88 and E94.16 Briefly, 1 μL of each sample was added to 49 μL of reaction mixture containing 5 μL 10 × PCR Buffer (Promega Corporation, Madison, WI, USA), 1.25 unit Taq DNA Polymerase (Promega Corporation), 20 pmol of each primer, and 0.2 mM of each deoxyribonucleotides. PCR amplification was performed in a DNA thermal cycler (PTC-100, MJ Research, Watertown, MA, USA), as reported previously.15 The PCR products were purified by using spin columns (Wizard Clean-up System, Promega Corporation). A total of 3 μL of the purified PCR products were cloned by using the plasmid vector (TOPO TA Cloning® Kit for Sequencing, Invitrogen Life Technologies, Carlsbad, CA). Transformation used chemically competent cells (One Shot® TOP10 Chemically Competent E. coli, Invitrogen Life Technologies). The next steps for cloning and plasmid DNA purification followed previously described procedures.15 Purified plasmid DNA was sequenced using primer D88.16 Sequencing reactions were performed in a DNA thermal cycler (MJ Research PTC-100, MJ Research, Inc., Waltham, MA, USA) using DYEnamic ET Terminator Cycle Sequencing Kit (Amersham Biosciences Corp., Piscataway, NJ, USA). Once chain termination was complete, DNA sequencing was carried out in an ABI Prism 3100 Genetic Analyzer (Life Technologies, Carlsbad CA, USA). The sequencing protocol was 95°C for 30 seconds, followed by 40 cycles of 95°C, 20 seconds; 50°C, 15 seconds, and 60°C, 2 minutes; with a final hold step at 4°C. The resultant 250 sequences presenting 499 or more nucleotides of good quality, which was ascertained by inspecting the chromatograms, were pooled and grouped into two 16S rDNA clone libraries, named t1 and t2. Chimeric sequences were identified using Bellerophon (http://foo.maths.uq.edu.au/huber/bellerophon.pl) and discarded. A total of 180 reminiscent sequences, including 84 from t1 and 96 from t2, were compared with sequences of known phylotypes from GenBank (http://www.ncbi.nlm.nih.gov) using the BLASTn algorithm. Sequence similarity of 98% or greater was used as the definition of a species-level cluster. Clones that were less than 98% similar to the closest known organisms were supposed to represent novel phylotypes.16 Contiguous sequences were assembled with the Phred/Phrap/Consed software package (http://www.phrap.org), aligned with the CLUSTAL W software. Phylogenetic trees were constructed with MEGA 5 software, according to the calculation of a Jukes and Cantor distance matrix using the neighbor-joining method. Bootstrap confidence values for branching nodes were inferred by generating 1,000 resampling trees. The percentage of coverage was calculated by Good’s method with the formula \( \left[ 1 - \left( \frac{n}{N} \right) \right] \times 100 \), where n is the number of phylotypes in a sample represented by one clone (singletons) and N is the total number of sequences in that sample. The computer program J-LIBSHUFF (http://whitman.myweb.uga.edu/libshuff.html) was used to statistically compare the clone libraries between time points \( P \leq .05 \). Chao28 and ACE29 (Abundance-based Coverage Estimator) statistics were calculated using MOTHUR (www.mothur.org). The Chao and ACE estimators are statistical approaches used to estimate the species diversity and richness of microbial communities. Chao calculation considers the number of observed species, the number of singletons (species captured once), and the number of doubletons (species captured twice).28 The observed species (Sobs) represent the microorganisms collected and identified. The ACE calculation considers the data from all species with fewer than 10 individuals, rather than just singletons and doubletons.29 Bacteria diversity was considered the dependent variable and time was considered independent variable. The sequences representing novel phylotypes were deposited at GenBank under the following accession numbers KF715649 to KF715662.

RESULTS

Phylogenetic trees are shown in Fig. 2 and Fig. 3 and reveal 25 known phylotypes for t1 and 17 known phylotypes for t2. The library t1 also included 3 novel phylotypes while t2 had 11 novel phylotypes. The phylotypes accounted for 86% of t1 and 80% of t2 cloned sequences, according to Good’s population coverage. The J-Libshuff analysis indicated that library t1 is contained in t2 \((P = .05)\), but t1 differed from t2 \((P = .004)\). So, community structure of the first library can be considered a subset of the second. When OTU (operational taxonomic unit) definition of 0.02 is considered, the t2 shared approximately 40% of its membership (11 phylotypes) with those of t1, while 17 phylotypes were exclusive of t1 and 17 were unique to t2.

The sequences were assigned to five phyla: Firmicutes (82% of t1 and 60% of t2), Actinobacteria (5% of t1; 10% of t2), Bacteroidetes (2% of t1; 6% of t2), Proteobacteria (10% of t1; 15% of t2) and Fusobacteria (1% of t1; 8% of t2) (Fig. 2 and Fig. 3). The major phyla were different between the libraries: the Firmicutes were decreased, while the Proteobacteria as well as other minor phyla were increased in t2. Sequences with more than 98% homology to Genbank represented 13 genera in t1 and 9 genera in t2. Seven genera were shared between t1 and t2 libraries (Veillonella, Streptococcus, Capnoeytophaga, Fusobacterium, Haemophilus, Neisseria, and Lautropia). The Collector’s curves, estimated by Chao and ACE, represent that the species richness of the two libraries differs; it was highest in t1 but lower in t2 (Fig. 4).
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Fig. 2. Phylogenetic tree with the highest-scored BLAST search results from 84 clones of library t1. The right side of the Fig. shows grayscale bars that represent the distribution of phylotypes among 5 phyla. The scale bar represents evolutionary distance (5% nucleotide sequence divergence). The organism names in bold letters mark the new phylotypes identified in the project. Final codes correspond to GenBank accession numbers.

Fig. 3. Phylogenetic tree with the highest-scored BLAST search results from 96 clones of library t2. The right side of the Fig. shows grayscale bars that represent the distribution of phylotypes among 5 phyla. The scale bar represents evolutionary distance (5% nucleotide sequence divergence). The organism names in bold letters mark the new phylotypes identified in the project. Final codes correspond to GenBank accession numbers.
DISCUSSION

The present study revealed that the RPD may act as a reservoir of bacteria species, many of them putative pathogens mainly associated with periodontal disease and caries.

The literature has posed that RPD may be associated with increased risk of dental caries and periodontal disease. However, the bacterial pathogens of such association are not clearly described. Even though more than 700 bacterial species have been identified in distinct oral habitats such as saliva, supra and subgingival biofilms, tongue, hard and soft palate in healthy or diseased conditions, few studies have aimed at evaluating the bacterial composition and shift related to RPD use. To the best of our knowledge, this pilot study is the first to attend this subject by means of culture-independent molecular methods. The advantages of such methods are highlighted in the literature and the present results illustrate them. Additionally, this is the first clinical study which described the diversity of biofilms adhered to the Co-Cr framework in contact to the enamel tooth surface. Most previous studies about the accumulation of biofilm in prosthesis wearers have based their observations on the acrylic denture base biofilm presence and/or composition. However, it is considered that the bacterial species adhered to the Co-Cr surfaces may differ from that of acrylic resin surfaces. It was shown not only several species colonizing the RPD clasps but also an expressive shift of microbial species over time. The libraries population contains a small number of dominant species (Streptococcus sanguinis and Veillonella parvula) but presents a large number of unique phylotypes, i.e., a high degree of richness. Streptococcus was the most abundant genus in both libraries, comprising about a half of the clones ($t_1 = 56\%$; $t_2 = 49\%$). Other studies showed that 63 to 86% of the initial colonizing bacteria in dental plaque were Streptococcus along with some Veillonella and Actinomyces. The present study did not find Actinomyces genus in $t_1$, but it was present in $t_2$.

Most Streptococcus of the $t_1$ fell in 3 species ($S. sanguinis$, $S. mitis$, and $S. oralis$). The second library ($t_2$) had more varied species of Streptococcus ($S. sanguinis$, $S. oralis$, $S. mitis$, $S. gordonii$, $S. infantis$, and $S. pneumonia$) some of which may be considered opportunistic pathogens. Previous studies reported that certain Streptococcus species, including $S. sanguinis$, $S. mitis$, $S. oralis$, and $S. gordonii$ act as primary colonizers in bacterial adhesion to tooth surfaces. Initial adhesion involving Streptococcus and surfaces with similar hydrophobic properties would be facilitated on metal alloys, particularly for small coccus (1 μm), like $S. oralis$. Actinomyces, which presents larger cells (2 to 3 μm) compared to Streptococcus, need greater surface defects for retention or depend on Streptococcus first colonizers when the metal surface is polished. No sequence representative of $S. mutans$ was found. The present results agree with previous literature, reporting that $S. mutans$, shown to be less hydrophobic than $S. sanguinis$ and $S. oralis$, is less likely to adhere to alloy surfaces.

Rarefaction curves show that the $t_1$ library represents a small part of the overall population and $t_2$ library seems to be a development of $t_1$ community. The different genus found on the present study indicates a complex microbial community on the metal clasp surface, formed by bacteria of Actinomyces, Yellow, Green, Purple, and Orange periodontal complexes. Moreover, the reduction of cocci over time (56% vs. 46%) may be indicative of a more mature plaque in $t_2$. In $t_2$, bacterial species diversity is also increased.
compared to \( t_1 \) (\( P = .004 \)). Fusobacteria filum was represented more in \( t_2 \). Although Fusobacterium spp. have been identified in supragingival plaque of healthy individuals, they have been typically associated with periodontal diseases. The present results demonstrated that Firmicutes were reduced, while Fusobacteria were increased in \( t_2 \) compared to \( t_1 \), which suggests that the RPD clasps is a reservoir of bacteria that shift from non-maleficent to potentially-disease-related bacteria over time, as reported previously. Whether oral and abutment tooth conditions change from health to periodontal disease will require further investigation.

Many clones were considered novel phylotypes. In \( t_1 \), two sequences exhibited low similarity to \( S. sanguinis \) (93.3% and 96.8%) and one clone had \( C. ochracea \) as closest relative (96.8%). In \( t_2 \), 4 sequences displayed very low identity with \( L. goodfellowii \) (89.6%, 90.2%, 92.1%, and 91.1%), commonly isolated from healthy oral cavities. Two novel phylotypes had 91.0% and 91.6% identity with \( C. casei \). Although \( C. casei \) can be typically isolated from dental plaque, it is not associated with oral diseases. Other novel phylotypes in \( t_2 \) exhibited 96.3% similarity to \( E. corrodens \) and less than 91.5% identity to \( C. morbi \). \( C. morbi \) resides in the oral cavity and is associated with primary endodontic infections and endocarditis. One clone of \( t_1 \) had 96.4% identity to \( S. sputigena \), which is typically found in the oral cavity in congregation to \( F. nucleatum \) and may be associated with aggressive periodontitis. Other novel phylotype had 95.7% identity to its closest relative \( A. odontolyticus \), which has been isolated from deep dental caries and is an opportunistic pathogen of immunocompromised patients, involved in bacteremia and pneumonia. Obviously, the physiological and clinical relevance of the novel phylotypes detected in this study remain unclear. At one extreme, some of the phylotypes may be transient residents. On the other extreme, these organisms may be critical for maintenance of ecosystem stability and oral health or may be occasional pathogens.

Although 16S rDNA sequencing represents a broad-range analysis, data should be interpreted with caution. A different definition for OTU could provide different results. Additionally, it cannot be inferred that bacteria that have not been detected are not present on the clasp surface. Note that some species may be present but below the limit of detection, and thus may be poorly represented in the clone libraries.

In the present study oral hygiene instructions (OHI) were given up to day zero. In this sense, it is conceivable that the absence of OHI between \( t_1 \) and \( t_2 \) might have resulted not only in biofilm growth and maturation per se, observed herein, but also in an inflammatory response, even subclinical, that, in turn, also can influence the biofilm composition. This retro-filling process is described at the literature. Bearing this two-way influence in mind, the results herein show that RPD wearers retain mature biofilm at clasps favoring the maintenance of an environment adequate to the maturation of the biofilm as a whole and the long-term impact to the gingival status. On the other hand, it should be noted that the present study established careful oral hygiene instructions and training for subjects up to \( t_1 \). This situation simulates what usually occurs in the dental office, where patients receive oral hygiene instructions during RPD fabrication and adjustment appointments, but the next follow-up appointment takes a long time to be scheduled. The result of this delay is that dentures are used for longer time than they are supposed to be, and the patients lose the ability to perform plaque control. Previous studies reported that a maintenance interval longer than 6 months was significant predictor for positive red complex bacteria in RPD wearers. The results of the present study demonstrate that, after 4 months, a complex and mature biofilm is formed on RPD clasp surfaces. The establishment of more frequent follow-up appointments for RPD wearers may be a valuable strategy to reduce potential pathogen accumulation on RPD clasps, possibly favoring the gingival condition along time.

CONCLUSION

This study concluded that the RPD clasps harbors a highly diverse bacterial population and the bacterial community on the RPD metal clasp develops into a complex mature community, including putative periodontal and opportunistic pathogens.

ORCID

Cristiane Machado Mengatto  http://orcid.org/0000-0002-1059-8419
Leonardo Marchini  http://orcid.org/0000-0003-1291-6684
Célia Marisa Rizzatti-Barbosa  http://orcid.org/0000-0002-8747-0034

ACKNOWLEDGEMENTS

The authors thank Professor Francisco G. Nobrega for advice and access to laboratory facilities and supplies. The authors are also grateful to the Center for Computational Biotechnology and Biotechnological Information Management (NBMGI) of the State University of Santa Cruz (UESC) for computational support.

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