Biofilm formation of salivary microbiota on dental restorative materials analyzed by denaturing gradient gel electrophoresis and sequencing

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The microbial diversity of biofilms formed on the surfaces of amalgam, glass-ionomer cement, and resin composite was analyzed by denaturing gradient gel electrophoresis (DGGE). The V2-V3 region of salivary microbial 16S rDNA gene sequences of planktonic and biofilm bacteria, after 1 day and 1 week of incubation, was amplified by polymerase chain reaction (PCR) and analyzed by DGGE. The amounts of strongly adherent phylotypes after 1 day and 1 week on the three dental restorative materials were more than those on hydroxyapatite. Streptococcus salivarius was detected in both loosely adherent and strong adherent groups of all 1-day samples. At 1 week, the amounts of loosely adherent and strongly adherent phylotypes present on the three restorative materials ranked in this ascending order: glass-ionomer cement < resin composite < amalgam. Results of DGGE analysis suggested that glass-ionomer cement was the best material of choice in terms of suppressing bacterial phylotypes in biofilms.

Keywords: Dental restorative materials, Biofilm, Denaturing gradient gel electrophoresis, Microbial diversity

INTRODUCTION

Approximately 700 highly diverse bacterial species inhabit the human oral cavity1). Interaction between oral bacteria and solid surfaces, such as enamel, restorative materials, implants and orthodontic appliances, often results in the attachment and coaggregation of microorganisms and subsequent biofilm formation2). The mechanisms involved in the process of bacterial adherence to solid surfaces in the oral cavity are very complicated3). The accumulation of dental biofilms plays a key role in the development of caries, periodontitis, and other oral diseases4,5). Dental restorative materials may also promote bacterial aggregation that potentially leads to caries development6).

Denaturing gradient gel electrophoresis (DGGE) is a promising technique that differentiates bacterial species based on the size separation of bacterial DNA fragments using electrophoresis7). When double-stranded DNA molecules are run in polyacrylamide gel containing a linear gradient of DNA denaturants (urea and formamide), DNA fragments of the same length but with different base-pair sequences will stop migrating at different positions in the gel8). DGGE has been widely employed to examine the microbial populations found in subgingival plaque9-11), endodontic infections12), and dental plaque microbiome. It has been proposed that DGGE could be a useful tool for identifying the profiles of microbial pathogens implicated in periodontal diseases and caries, which then aids in the diagnosis and risk assessment of these patients13).

In the present study, biofilms were grown on the surfaces of three dental restorative materials in vitro. We hypothesized that DGGE technique would detect population diversity among the biofilms formed on these different dental restorative materials. After the saliva-coated dental restorative materials were incubated for 1 day and 1 week, microbial diversity in saliva was determined using molecular biotechnology which did not depend on traditional colony count technique.

MATERIALS AND METHODS

Preparation of dental restorative material blocks

Three dental restorative materials were chosen for this study, viz. amalgam, glass-ionomer cement, and resin composite (Table 1). Blocks of these dental restorative materials were prepared according to manufacturers’ instructions, using cylindrical molds of 5 mm diameter and 5 mm thickness.

Amalgam was prepared by mixing each amalgam capsule using an amalgamator (ST-B type, AT&M, Beijing, China) at 4,500 oscillations/min for 20 s. Glass-ionomer blocks were prepared by mixing glass-ionomer powder and liquid at a ratio of 1:5:1 (wt/vol) within 1 min, using plastic spatula and mixing pad provided by the manufacturer and set with vaseline covering the surfaces. Surface polishing under water was not
Table 1 Dental restorative materials used in this study

<table>
<thead>
<tr>
<th>Names</th>
<th>Lot no.</th>
<th>Manufacturers</th>
<th>Chemical compositions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amalgam</td>
<td>071220</td>
<td>AT&amp;M, Beijing, China</td>
<td>Hg&amp; powder: 43% Ag, 32% Sn, 25% Cu (w/w) in one 600 mg capsule (Hg and powder at a weight ratio of 1:1)</td>
</tr>
<tr>
<td>Glass-ionomer cement</td>
<td>200806</td>
<td>Shanghai Medical Instruments Co., Ltd., Shanghai, China</td>
<td>Powder: silica, alumina, aluminium fluoride, calcium fluoride and aluminium phosphate Liquid: polyacrylic acid and maleic acid</td>
</tr>
<tr>
<td>Resin composite (Ceram•X duo D4)</td>
<td>0707000739</td>
<td>Dentsply DeTrey, Konstanz, Germany</td>
<td>Methacrylate modified polysiloxane, dimethacrylate resin, fluorescence pigment, UV stabilizer, stabilizer, camphorquinone, ethyl-4(dimethylamino)benzoate, barium-aluminum-borosilicate glass, methacrylate functionalized silicon dioxide nano filler, iron oxide pigments and titanium oxide pigments and aluminum sulfosilicate pigments according to shade</td>
</tr>
</tbody>
</table>

performed until complete set after 24 h. These glass-ionomer blocks were stored in water to prevent desiccation. To prepare resin composite blocks, each 2-mm increment layer was polymerized for 40 s using a conventional halogen curing light (Elipar™ 2500, 3M ESPE, St. Paul, MN, USA) with an output intensity of 550 mW/cm². After polymerization and hardening, each block was polished with a series of silicon carbide (SiC) abrasive papers (#360, #600, P1000, and P2000, Panda, Beijing, China) until the surface was smooth. To remove the unpolymerized monomers, resin composite blocks were immersed in distilled water for 2 days at room temperature until use.

Hydroxyapatite is the main component of tooth enamel. Therefore, hydroxyapatite (HA; [Ca₁₀(PO₄)₆(OH)₂]) disks of >99% purity (Department of Dental Materials, Sichuan University) were used as controls in this study.

For each material, their prepared blocks were gently wiped with 75% ethanol and washed with distilled water. Their moist surfaces were sterilized by ultraviolet (UV) radiation (1.2×10⁵ μJ/cm²) for 1 h. Unstimulated whole saliva samples from four healthy subjects were obtained in a similar manner to that described above to determine salivary microbiota. Pooled saliva sample was mixed with sterilized brain heart infusion (BHI) broth (Difco, Beckton Dickinson, Sparks, MD, USA) complemented with 1% glucose and 1% sucrose at a ratio of 1:3 and briefly centrifuged at 2,600 rpm for 10 min to remove debris containing exfoliated oral epithelial cells. Supernatant containing salivary microbiota was collected.

Coating with saliva
The research protocol of this study was approved by the Ethics Committee of Health Science Center, Peking University. Informed consent was obtained from all subjects involved in this study. Four healthy 24–26-year-old volunteers who had not consumed any antibiotics in the six months prior to the study were enrolled. One milliliter of unstimulated whole saliva was collected at 10:00 am from each subject, pooled together at equal proportions, and then added with the same volume of phosphate-buffered saline (PBS; pH 7.0, Sinopharm, Beijing, China). Sample was mixed by vortexing and centrifugation at 12,000 rpm for 10 min, and the supernatant was collected.

Dental restorative material blocks and HA disks were completely immersed in the supernatant at 37°C overnight until each was well coated with a moist surface.

Biofilm formation on restorative materials
Saliva-coated blocks and disks were sterilized by UV radiation (1.2×10⁵ μJ/cm²) for 1 h. Unstimulated whole saliva samples from four healthy subjects were obtained in a similar manner to that described above to determine salivary microbiota. Pooled saliva sample was mixed with sterilized brain heart infusion (BHI) broth (Difco, Beckton Dickinson, Sparks, MD, USA) complemented with 1% glucose and 1% sucrose at a ratio of 1:3 and briefly centrifuged at 2,600 rpm for 10 min to remove debris containing exfoliated oral epithelial cells. Supernatant containing salivary microbiota was collected.

Blocks of three different restorative materials and HA disks were placed in 24-well polystyrene tissue culture plates (Biousing Biotech Co. Ltd., Wuxi, Jiangsu, China) at two blocks/disks per well and incubated with 2 mL of supernatant in each well. The 24-well plates were incubated under anaerobic conditions (N₂ 85%, H₂ 10%, CO₂ 5%) at 37°C for 1 day and 1 week. BHI broth was replenished every 48 h in the 1-week group as previously described¹⁴). Before broth replenishment, the supernatant containing planktonic bacteria was separately collected to avoid disturbing the biofilm. This was followed by adding 2 mL of fresh BHI broth to each well.

On the other hand, two blocks of each material and two HA disks in BHI broth without the salivary bacterial inoculum were used as negative controls for microscopic Gram stain examination in detecting bacterial contaminants.

DNA extraction
After 1 day and 1 week of incubation, supernatant
samples from the different wells containing planktonic bacteria were carefully collected to avoid disturbing the biofilms formed on the restorative materials. Samples of the same condition were pooled and centrifuged at 12,000 rpm for 1 min to obtain a cell pellet containing planktonic bacteria. The pellets were re-suspended in 1 mL of Tris-EDTA buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0) and transferred to 2-mL screw-cap vials (Axygen, Union City, CA, USA) with sterile glass beads (4/5 volume of 0.1 mm diameter and 1/5 of 0.5 mm diameter) filled to half of the volume of each vial. The remaining volume in the vials was filled with Tris-EDTA buffer to exclude air, and the vials were pre-chilled in ice. Vial samples were processed in a mini-beadbeater (BioSpec, Bartlesville, OK, USA) at 4,800 oscillations/min for 3 min and then immediately placed on ice. Once cooled, the supernatant containing DNA was immediately used as the template for polymerase chain reaction (PCR).

After removing planktonic bacteria from the supernatant samples, restorative blocks containing bacterial biofilms were washed twice for 30 s with normal saline. Biofilm samples were obtained from the blocks’ surfaces using sterilized wooden toothpicks. The toothpicks were immediately placed in tubes filled with 1 mL of reduced transport fluid. These samples were considered as “loosely adherent biofilm bacteria”, and DNA was extracted from them by bead beating, as described above. For the remaining biofilm bacteria which strongly adhered to the surfaces of the restorative blocks, they were removed by vortexing the blocks with 0.5-g glass beads (0.1 mm diameter) in 1 mL of reduced transport fluid for 1 min. These samples were considered as “strongly adherent biofilm bacteria”, and DNA was extracted from them by bead beating. Biofilm bacteria were also pooled according to the conditions they were grown in before DNA extraction.

**PCR-DGGE**

DNA samples derived from the planktonic, loosely adherent biofilms and strongly adherent biofilms were subjected to PCR-DGGE as previously described, but with a few modifications. 16S rDNA gene (~1500 bp), which is highly conserved, contains variable regions that are targeted for DGGE analysis. The variable region was amplified by PCR and then subjected to DGGE to detect the diversity of the microbial community. PCR products were visualized on a 1.5% agarose gel stained with 1% (v/v) ethidium bromide.

After image analysis, discrepant bands with unstained cores were excised using a sterile scalpel, placed into 1.5-mL tubes containing 50 μL of sterile Milli-Q water, and incubated overnight at 4°C. A volume of 5 μL of the DNA sample was used as a template and re-amplified using the DGGE primers without the GC clamp. The PCR products of V2-V3 region of 16S rDNA gene amplified by the primers HD1A with GC clamp and HD2A were approximately 240 base pairs. The PCR products were subjected to agarose gel electrophoresis and subsequently purified with a PCR product purification kit (Tiangen, Beijing, China). The PCR products were cloned using a pGM-T vector kit (Tiangen, Beijing, China), and sent to Invitrogen Company (Beijing, China) for sequencing using the universal primer T7 (5'-TAA TAC GAC TCA TTA TAG G-3'). The 16S rDNA sequences were aligned with the GenBank sequences using BLAST program in NCBI to find the closest relatives.
RESULTS

Analysis of phylotypes in DGGE profiles
As shown in Fig. 1 and Table 2, quantities of phylotypes in 1-day samples of “loosely adherent biofilm bacteria” and “strongly adherent biofilm bacteria” on the three dental restorative materials were higher than those of HA samples. Among the 1-week samples of the three restorative materials, the glass-ionomer material had the lowest amount of “loosely adherent biofilm bacteria” and “strongly adherent biofilm bacteria”. The number of “loosely adherent biofilm bacteria” decreased from 1 day to 1 week for all restorative material samples. For the “strongly adherent biofilm bacteria”, the number decreased from 1 day to 1 week for amalgam and glass-ionomer samples, but on the contrary increased for the resin composite and HA samples.

WPGMA dendrogram
WPGMA dendrograms (Fig. 2) show the similarities in bacterial compositions obtained from the different materials. There were no clear patterns in the 1-day samples, but some similarities could be observed in the 1-week samples.

Table 2  Quantities of phylotypes detected for different dental restorative materials and hydroxyapatite

<table>
<thead>
<tr>
<th></th>
<th>Amalgam</th>
<th>Glass-ionomer</th>
<th>Resin composite</th>
<th>Hydroxyapatite</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 day</td>
<td>1 week</td>
<td>1 day</td>
<td>1 week</td>
</tr>
<tr>
<td>Planktonic</td>
<td>42.0±1.0</td>
<td>44.0</td>
<td>49.7±1.2</td>
<td>52.7±2.5</td>
</tr>
<tr>
<td>Loosely adhered</td>
<td>50.3±2.1</td>
<td>42.7±1.5</td>
<td>54.7±1.5</td>
<td>47.7±1.2</td>
</tr>
<tr>
<td>Strongly adhered</td>
<td>56.3±0.6</td>
<td>46.0±3.6</td>
<td>47.3±3.1</td>
<td>41.3±2.5*</td>
</tr>
</tbody>
</table>

The data are presented as the mean±standard deviation.
Six blocks of each dental material and six hydroxyapatite discs were used for bacterial inocula for one day and one week, respectively.

* p<0.05 represents significant differences.
In the 1-day samples (Fig. 2 (a)), the planktonic bacterial communities on amalgam (#1) and resin composite (#7) displayed a relatively high similarity (>60% concordance). In the 1-week samples (Fig. 2 (b)), the planktonic bacterial communities on glass-ionomer (#4), resin composite (#7), and HA (#10) displayed moderate similarity (>50% concordance), as did the strongly adherent bacterial communities on glass-ionomer (#6) and resin composite (#9).

Identification of bacterial species by DNA\(/\text{Amann, 1995}\) sequencing

In Fig. 1, the number of bands denotes microbial diversity while the positions of bands indicate different bacterial genera. Discrepant bands might unravel the special bacteria partial to some dental restorative materials.

Table 3 shows the sequencing results. For the 1-day samples, bands that specifically existed in the planktonic groups (bands a, b, c, d and e in Fig. 1(a)) or loosely adherent and strongly adherent groups (bands f, g, h, i,
j and k in Fig. 1(a)) were excised. Four bands (bands a, b, d and e) of planktonic groups and three bands (bands h, j and k) of loosely adherent and strongly adherent groups were confirmed to belong to uncultured bacteria. Different strains of *Lactobacillus fermentum* (bands c, f and g) were found in all groups, and *Streptococcus salivarius* (band i) was found in all loosely adherent and strongly adherent groups.

For the 1-week samples, bands l, m, n, o, p and q in Fig. 1(b) were excised based on their existence in only one of the three restorative materials. *Streptococcus anginosus* (bands l and n) was found in the planktonic groups of amalgam and glass-ionomer cement. *Lactobacillus fermentum*, uncultured *Streptococcus sp.*, and *Bacillus mycoides* (bands m, o and p, respectively) were also found in the planktonic group of glass-ionomer cement. Uncultured *Streptococcaceae* (b and q) was found in the loosely adherent group of glass-ionomer cement.

**DISCUSSION**

Traditional culture-based methods are severely limited in their ability to identify microbial diversity in clinical samples. For example, the majority of viable bacteria do not form visible colony forming units for counting during plate cultivation. Molecular biology techniques have better enabled researchers to discover the existence of new microbial phylotypes in environmental samples. DGGE has been used in many fields to analyze the composition and diversity of microbial communities in different niches. PCR-DGGE is also extensively employed to analyze oral microbial communities. However, there are no studies in published literature that have used DGGE to examine microbial compositions detected on dental materials.

The physical and chemical properties of dental restorative materials influence the bacterial composition on their surfaces. In the present study, DGGE profiles were used to examine the biofilm composition of salivary microbiota on three different restorative materials and compared against those on hydroxyapatite surface.

After 1 day of incubation, the numbers of loosely adherent and strongly adherent bacterial phylotypes in the biofilms of three dental restorative materials were greater than those on hydroxyapatite surface. At 1 week, the numbers of strongly adherent bacterial phylotypes in the biofilms of three restorative materials were greater than that on hydroxyapatite surface. These findings indicated that dental restorative materials acted as exogenous agents which supported the attachment and growth of biofilm bacteria more than hydroxyapatite, which is the main component of tooth enamel.

After 1 week of incubation, the numbers of loosely adherent and strongly adherent bacterial phylotypes on amalgam and glass-ionomer cement decreased. In this study, changes in bacterial diversity were consistent with those observed in previous studies. Amalgam has been reported to have potent and lasting antibacterial properties because soluble ions released from amalgam can kill bacteria in the adherent biofilm. Glass-ionomer cement had the least amount of “strongly adherent biofilm bacteria” among the three restorative materials. Moreover, strongly adherent bacterial phylotypes in the biofilm of glass-ionomer cement decreased from 1 day to 1 week. This finding agreed with those of previous studies in that the antibacterial properties of glass-ionomer cement negatively affected bacterial metabolism.

Fluoride-releasing materials help to prevent secondary caries, which plays a pivotal role in the failure of dental restorations. Future studies on secondary caries prevention, particularly on the influence of glass-ionomer cements on oral microbial composition, can leverage on the results of the present study. However, Montanaro et al. reported that fluoride released from glass-ionomer did not inhibit the adhesion of the cariogenic pathogen, *Streptococcus mutans*. Adding to the debatable role of fluoride in caries prevention, Guida et al. suggested that caries inhibition was due to enamel remineralization, rather than fluoride release.

Resin composite also showed an increase in bacterial diversity from 1 day to 1 week, and this was due to its lack of antibacterial properties. It must also be mentioned that less than 1% of the total number of bacteria could not be detected by DGGE, which thus explained the changes in the observed gel electrophoresis bands of 1-day and 1-week samples.

Analysis of the WPGMA dendrogram of 1-day samples revealed no clear patterns, but some interesting similarities were observed in the 1-week samples. Planktonic bacteria, in general, were closely related in the 1-day and 1-week samples. This result confirmed the fact that there are considerable differences between planktonic and biofilm bacteria in terms of composition and properties. This was further confirmed by the DGGE profiles of 1-week-old strongly adherent biofilm bacteria on the restorative materials, which were closely related. Furthermore, 1-week-old hydroxyapatite bacterial profiles could be clearly differentiated from those of the restorative materials as the profiles were more related to the planktonic bacteria derived from the glass-ionomer cement and resin composite materials. These results provided clear evidence that different restorative materials developed different bacterial compositions on their surfaces, and these microbial consortia were different from planktonic bacteria as well as from those found in biofilms formed on hydroxyapatite (enamel). Therefore, various contributing factors of restorative materials, such as their chemical content, surface roughness, surface free energy and antibacterial properties, affected the formation and composition of dental biofilms.

Each band in the DGGE profile represented a different bacterial phylotype. Of the 17 excised bands in the DGGE profiles, *Lactobacillus fermentum* was a common species detected in the planktonic and biofilm samples. In addition, uncultured *Gemella* and uncultured *Lactobacillales* were found in 1-day-old planktonic samples only. *Streptococcus salivarius*, which is a commensal bacterial species in the oral cavity with
low virulence, was detected in 1-day-old loosely adherent and strongly adherent biofilm samples. *Streptococcus anginosus* was found in 1-week-old amalgam samples.

**CONCLUSION**

For the first time, DGGE profiles and sequencing were used in this study to analyze the biofilm formation of salivary microbiota on dental restorative materials in *vitro*. Results of this study indicated that glass-ionomer cement had an inhibitory effect on biofilm formation of salivary microbiota when compared with amalgam and resin composite. These findings warrant further research to improve the properties of dental restorative materials, which may be helpful for the prevention of secondary caries.

**REFERENCES**


