Bacterial Contamination of Endodontic Materials before and after Clinical Storage

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Abstract

Introduction: The aim of this study was to evaluate the bacterial contamination in endodontic consumables (gutta-percha points, rubber dams, paper mixing pads, caulking agents, and endodontic instrument sponges [EISs]) before and after clinical use and storage. **Methods:** Materials were randomly sampled in triplicates at 3 time points (t_0, at package opening; t_1, at 7 days; and t_2, at 14 days) during their clinical usage. The gutta-percha points and caulking agent (25 mg) were added to 1 mL phosphate-buffered saline (PBS). The rubber dam, paper mixing pad, and EIS were added to 25 mL PBS. After vortexing, centrifuging, and removing the supernatant, the pellet was resuspended in 1 mL PBS, plated on fastidious anaerobic agar, and incubated aerobically and anaerobically. The grown colonies were identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The total bacterial load was calculated in the remaining volume (800 μL) from each sample by quantitative polymerase chain reaction after DNA extraction. **Results:** All tested materials showed a varied number of contaminated samples at the 3 time points (except EIS at t_0) using MALDI-TOF MS. The most isolated genera were *Propionibacterium* (42%) and *Staphylococcus* (32%). By using non–culture-based approaches, all tested materials at the 3 time points (except gutta-percha at t_0 and the caulking agent at t_0, t_1, and t_2) carried bacterial DNA. **Conclusions:** The majority of the tested materials harbored bacteria in their samples before and after clinical storage. Nosocomial infection derived from commonly used consumables could have an impact on the outcome of endodontic treatment. *(J Endod 2017;■:1–5)*

Key Words

Bacterial contamination, clinical storage, endodontic materials, matrix-associated laser desorption/ionization time-of-flight mass spectrometry, quantitative polymerase chain reaction

Significance

Bacterial load may increase with clinical storage of endodontic materials. Nosocomial infections may affect the outcome of root canal treatment, especially in clinical scenarios in which the bacterial load is limited to start with (ie, irreversible pulpitis).

Successful endodontic treatment depends on the eradication of microorganisms present inside the root canal system and the prevention of (re-)infection (1, 2). Therefore, it is essential to create and maintain an aseptic chain throughout the course of endodontic treatment (3). Based on the modern concept of infection control, all materials and instruments used during endodontic procedures should be bacteria free (3, 4). Some endodontic materials (eg, gutta-percha [GP] points and mixing pads) are packaged in a way that renders sterility impossible to maintain throughout clinical storage (5, 6).

Culture techniques have been used for the isolation and identification of bacteria for many years. This method, despite its limitations in the presence of uncultivable pathogens, allows discerning between viable and inactivated pathogens. More recently, the identification of bacteria based on amplification and analysis of the 16S ribosomal RNA (rRNA) gene has overcome some of the limitations of culture-based approaches (7).

In this study, quantitative polymerase chain reaction (qPCR) was used to evaluate the number of bacterial nucleotide sequences present in samples from endodontic material by comparing the number of reaction cycles needed to reach a threshold by the endodontic material samples with the ones needed by their standard control. qPCR was used in this study for its accurate quantification and being regarded as a sensitive and rapid means of sequence enumeration with low probability of nucleic acid contamination (8, 9). Using culture and PCR techniques gives a comprehensive assessment on the nature and level of bacterial contamination.

In addition, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) was used to identify the bacterial colonies isolated from culturing the samples of the tested materials. MALDI-TOF MS is a soft ionization approach and depends on the comparison of specific mass spectra in the bacterial identification through desorption of bacterial proteins in the form of ions (10, 11). The time of flight of the ions to reach the detector is dependent on the mass and charge of the ions. Smaller ions travel faster than larger ions, thus providing a mass spectrum profile based on the difference in composition. Bacteria can be identified by comparing the obtained mass spectrum with the mass spectrum of known strains in the database (10, 12). Its use has increased in microbiology, thanks to its accuracy, rapid identification times, and cost saving (13).

The purpose of this study was to determine the bacterial contamination of endodontic materials such as GP points, rubber dams (RDs), paper mixing pads

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(PMPs), caulking agents, and sponges in their manufacturers' sealed package and after clinical storage using both MALDI-TOF MS and qPCR.

Materials and Methods

Experimental Design

The sampling procedures were performed in a postgraduate endodontic unit at King's College London Dental Institute, London, UK (anonymized for referring purposes). Samples were collected immediately after opening the original packaging (t_0), after 7 days of clinical storage and usage (t_1), and after 14 days (t_2). The clinical staff were not advised of the purpose of the study.

Initial Sampling and Culturing

At t_0, 3 sealed packages of 5 endodontic materials (ie, GP points [ProTaper F2; Dentsply Maillefer, Ballaigues, Switzerland], caulking agent [OraSeal (OS); Ultradent Products, South Jordan, UT], PMP [Kerr, Orange, CA], Endoring Sponge [ERS; Jordco, Beaverton, OR], and RD [Unodent, Essex, UK]) were opened under aseptic laboratory conditions.

Three samples from each sealed package were randomly selected. The GP points and OS (25 mg) were added to tubes containing sterile glass beads (Sigma-Aldrich, St Louis, MO) and 1 mL phosphate-buffered saline (PBS) (Oxoid, Basingstoke, UK). The tubes were vortexed for 2 minutes. Samples of the ERS, PMPs, and RDs were added to Falcon tubes (Corning, Corning, NY) containing sterile glass beads and 25 mL PBS. The tubes were vortexed for 2 minutes and then centrifuged for 30 minutes. The supernatant and materials were removed, and the pellet was resuspended with 1 mL PBS; 100-μL volumes were plated onto nonselective medium (fastidious anaerobic agar supplemented with horse blood [Southern Group Laboratory, Northampton, UK]) (14). The plates were incubated at 37°C aerobically for 3 days and anaerobically for 7 days. Two negative controls with PBS only were cultured with the same conditions. The remainder of each sample (800 μL) was stored at −80°C for qPCR analysis. After 7 days, microbial colonies were counted and subcultured on fastidious anaerobic agar plates. Again, the plates were incubated both aerobically for 3 days and anaerobically at 37°C for 7 days. Sampling was repeated at t_1 and t_2 and cultured aerobically and anaerobically as previously described.

MALDI-TOF Analysis

After culturing, the growth was prepared for MALDI-TOF analysis using the formic acid extraction method according to Schulthess et al's protocol (15). Briefly, the protocol includes suspension of a 1-μL loopful of bacterial growth in 300 μL high-performance liquid chromatography-grade water (Sigma-Aldrich) and 900 μL pure ethanol (Sigma-Aldrich). The supernatant was removed after centrifuging the tube at 13,000*g* for 2 minutes in a centrifuge machine (Thermo Electron Corporation, Waltham, MA). The pellet was left to dry and then resuspended in an equal amount of 70% formic acid (Amresco, Solon, OH) and 100% acetonitrile (Sigma-Aldrich). Then, the mixture was centrifuged at 13,000*g* for 2 minutes. One microliter of the supernatant was added to each spot on a matrix-assisted laser desorption/ionization target plate in duplicate and left to dry. After drying of the extract, 1 μL matrix solution prepared from 475 μL high-performance liquid chromatography-grade water, 25 μL pure trifluoroacetic acid (Alfa Aesar, Haverhill, MA), 500 μL acetonitrile (Sigma-Aldrich), and 10 mg a-cyano-4-hydroxycinnamic acid (Sigma-Aldrich), was added to each spot. After air-drying, the matrix-assisted laser desorption/ionization target plate was inserted into a Microflex LT mass spectrometer (Bruker, Billerica, MA), and the mass spectra generated were analyzed using FlexControl software (version 3.0, Bruker). Identifications with a score of 2.0–3.0, 1.700–1.999, and <1.700 were accepted as reliable identification to the species level, probable genus identification, and no identification, respectively.

qPCR Analysis

DNA Extraction. DNA were extracted from the remainder of each sample previously stored at −80°C using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich). The DNA extraction procedure was performed according to the Sigma-Aldrich's kit gram-positive protocol, with the addition of sterile glass (212–300 μm, Sigma-Aldrich) and tungsten-carbide beads (3 mm; QIAGEN, Hilden, Germany) for the cell disruption by a Fastprep-24 machine (MP Biomedicals, Santa Ana, CA) at 6.5 m/s for 60 seconds (16). Pelleted DNA was resuspended in 200 μL Sigma-Aldrich elution solution and stored at −20°C before use. For all extraction procedures, 3 samples of the negative control were prepared from 1 mL PBS.

Cycling Threshold Enumeration by qPCR. The qPCR assays were prepared with the Retro Gene SYBR Green PCR Kit (QIAGEN) in a final reaction volume of 20 μL consisting of 10 μL Retro Gene SYBR Green Master Mix, 8.8 μL nuclease-free water, 0.1 μL (concentration of 500 nmol/L) of each 16S rRNA gene EubF (5′-TCCTAC GGGAGGCAGCAGT-3′) and EubR (5′-GGACTACCAGGGTATCTAAT CCTGTT-3′) primer, and 1 μL DNA template (17). In the negative control, 1 μL Sigma-Aldrich elution solution was used instead of 1 μL DNA template. Cycling settings were as follows: initial activation at 95°C for 5 minutes followed by 50 cycles at 95°C for 15 seconds (denaturation) and at 58°C for 50 seconds (combined annealing/extension). The numbers of threshold cycles obtained from endodontic material samples and negative control were recorded. Each qPCR reaction was performed in triplicate.

For cycling threshold qPCR assays, colony-forming units (CFUs) in dental material samples were evaluated by comparison with the standard curve generated from *Enterococcus faecalis*. A brain-heart infusion broth culture of *E. faecalis* (OMGS 3202) was incubated at 37°C for 24 hours. Optical density of the culture was adjusted to 1, and CFU/mL was estimated by incubation of dilutions (10^{-5}–10^{-7}) on BHI agar at 37°C for 24 hours followed by colony counts. DNA was extracted from *E. faecalis* culture (optical density at a wavelength of 600 nm = 1) in the same way as for the endodontic material samples. qPCR was performed in triplicate for 10-fold serial dilutions (10^{-1}–10^{-7}) of *E. faecalis* DNA template as mentioned previously for endodontic material sample DNA extracts. The standard curve was generated using *E. faecalis* (qPCR efficiency = 1.08, R^2 = 0.994).

Data Analysis. The *z* test was used to determine whether there was a significant difference between the prevalence of contaminated endodontic material samples at different time points (t_0, t_1, and t_2). The one-sample Wilcoxon test was used to compare the median number of threshold cycles (CTs) for each sample at each time point and the median number of CTs obtained by the standard and negative controls. The Kruskal-Wallis test and the Mann-Whitney test were used to compare the number of CTs obtained by the samples collected at the 3 time points. In all tests, the significance was set at 0.05. Delta CTs (ΔCTs) were calculated to determine the difference in the median number of CTs obtained from the endodontic materials and the standard control as follows: ΔCT = median (CT test material sample) − median (CT standard control).

Fold change was also calculated to assess the difference in the median amounts of 16S rRNA gene present in the endodontic materials and the standard control. It was calculated according to the equation, which was suggested by Schmittgen and Livak (17). The equation is as follows: fold change = median $2^{-\text{CT test material sample}}$/median $2^{-\text{CT standard control}}$.

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