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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, rubber dam, paper mixing pad, caulking agent and endodontic instrument sponge) before and after clinical use and storage.

Methods: Materials were randomly sampled in triplicates at three 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 of phosphate buffered saline (PBS). Rubber dam, paper mixing pad and the endodontic instrument sponge (EIS) were added to 25 ml of PBS. After vortexing, centrifuging, removing the supernatant, the pellet was re-suspended in 1 ml PBS, plated on fastidious anaerobic agar (FAA) and incubated aerobically and anaerobically. The grown colonies were identified by Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF MS). Total bacterial load was calculated in the remaining volume (800 μ l) from each sample by quantitative polymerase chain reaction after DNA extraction.

Results: All tested materials showed varied number of contaminated samples at the three time points (except EIS at t_0) by MALDI-TOF MS. The most isolated genera were *Propionibacterium* (42%) and *Staphylococcus* (32%). By using non-culture based approaches,

all tested materials at the three time points (except gutta-percha at t_0 and caulking agent at t_0, t_1, t_2) carried bacterial DNA.

Conclusions: The majority of the tested materials harboured 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

Introduction

Successful endodontic treatment depends on eradication of microorganisms present inside the root canal system and prevention of (re-)infection (1, 2). Therefore, it is essential to create and maintain an aseptic chain throughout the course of endodontic treatment (Pereira et al., 2011). Based on the modern concept of infection control, all materials and instruments used during endodontic procedure should be bacteria-free (3, 4). Some endodontic materials, for example gutta-percha 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 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 analysing of 16S ribosomal RNA gene has overcome some of the limitations of culture-based approaches (7).

In this study, 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 give a comprehensive assessment on the nature and level of bacterial contamination level.

In this study, 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 of 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 bacterial contamination of endodontic materials such as gutta-percha points, rubber dam, paper mixing pad, caulking agent and sponge in their manufactures sealed package and after the clinical storage by using both MALDI-TOF MS and qPCR.

Materials and Methods

Experimental Design

The sampling procedures were carried out in a Postgraduate Endodontic Unit [REDACTED] [REDACTED] (*anonymised 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). Clinical staff were not advised of the purpose of the study.

Initial Sampling and Culturing

At t_0 , three sealed packages of five endodontic materials; gutta-percha points (GP), ProTaper F2 (Dentsply-Maillefer, Ballaigues, Switzerland), caulking agent; OraSeal (OS) (Ultradent Products, South Jordan, UT USA), paper mixing pad (PMP) (Kerr, Orange, CA, USA) and Endo ring sponge (ERS) (Jordco, Beaverton, OR, USA) and rubber dam (RD) (Unodent, Essex, UK) were opened under aseptic laboratory conditions.

Three samples from each sealed package were randomly selected. The GP and OS (25 mg) were added to tubes containing sterile glass beads (Sigma-Aldrich, Gillingham, UK) and 1 ml phosphate buffered saline (PBS) (Oxoid, Basingstoke, UK). The tubes were vortexed for 2 minutes. Samples of ERS, PMP and RD were added to falcon tubes containing glass beads and 25 ml of PBS. The tubes were vortexed for 2 min and then centrifuged for 30 min. The supernatant and materials were removed and the pellet was re-suspended with 1 ml of PBS. 100 μ l volumes were plated onto non selective medium (fastidious anaerobic agar [FAA] 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 μ l) was stored at -80 °C for qPCR analysis. After 7 days, microbial colonies

were counted and sub-cultured on FAA plates. The plates again incubated both aerobically for 3 days and anaerobically at 37°C for 7 days.

Sampling was repeated at t1 and t2 and cultured aerobically and anaerobically as previously described.

MALDI-TOF analysis

Following culturing the growth was prepared for MALDI-TOF analysis by formic acid extraction method according to Schulthess *et al.* protocol (15). Briefly, the protocol includes suspension of a 1 µl loopful of bacterial growth in 300 µl of HPLC grade water (Sigma-Aldrich, Irvine, UK) and 900 µl of pure Ethanol (Sigma-Aldrich, Irvine, UK). The supernatant was removed after centrifuging the tube at 13,000 g for 2 minute in a centrifuge machine (Thermo Electron Corporation, Waltham, MA, USA). The pellet was left to dry, then re-suspended in an equal amount of 70% formic acid (Amresco, Solon, OH, USA) and 100% acetonitrile (Sigma-Aldrich, UK). Then the mixture was centrifuged at 13,000 g for 2 minute. One microliter of the supernatant was added to each spot on a MALDI target plate in duplicate and left to dry. After drying of the extract, 1 µl of matrix solution: prepared from 475 µl of HPLC grade water, 25 µl of pure trifluoroacetic acid (Alfa Aesar, UK), 500 µl of acetonitrile (Sigma-Aldrich, UK) and 10 mg of a-cyano-4-hydroxycinnamic acid (Sigma-Aldrich, UK) was added to each spot. After air drying the MALDI target plate were inserted into a Microflex LT Bruker mass spectrometer and the mass spectra generated were analysed using FlexControl software (version 3.0) (Bruker, Billerica, MA, USA). 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 was extracted from the remainder of each sample previously stored at -80 °C, by using Sigma GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich, UK). DNA extraction procedure was performed according to Sigma Kit Gram positive protocol with the addition of sterile glass (212- 300 µm, Sigma-Aldrich, UK) and tungsten carbide beads (3mm, QIAGEN, UK) and of a cell disruption by a Fastprep-24 machine (MP Biomedicals, Santa Ana, CA,USA) at 6.5 m/sec for 60 sec (16). Pelleted DNA was re-suspended in 200 µl of Sigma elute solution and stored at -20 °C before use. For all extraction procedures three samples of negative control were prepared from 1 ml PBS.

Cycling threshold enumeration by quantitative polymerase chain reaction

The quantitative polymerase chain reaction assays were prepared with the Retro gene sybr green PCR kit (QIAGEN, Hilden, Germany) in a final reaction volume of 20 µl, consisting of 10 µl of Retro gene sybr green master mix; 8.8 µl of nuclease free water; 0.1 µl (concentration of 500 nM) of each 16S rRNA gene EubF (5'-TCCTACGGGAGGCAGCAGT-3') and EubR (5'-GGACTACCAGGGTATCTAATCCTGTT-3') primer and 1 µl of DNA template (17). In the negative control, 1 µl of sigma elute was used instead of 1 µl of DNA template. Cycling settings were as follows: initial activation at 95°C for 5 min followed by 50 cycles at 95°C for 15 sec (denaturation) and at 58 °C for 50 sec (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 unit in dental material samples were evaluated by comparison with standard curve generated from *Enterococcus faecalis*. A brain heart infusion (BHI) broth culture of *E. faecalis* (OMGS 3202) was incubated at 37 °C for 24

h. Optical density (OD) 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 h, followed by colony counts. DNA was extracted from *E. faecalis* culture (OD₆₀₀ =1) in the same way as for the endodontic material samples. QPCR was carried out in triplicate for tenfold serial dilutions (10^{-1} - 10^{-7}) of *E. faecalis* DNA template as mentioned above for endodontic material samples DNA extracts. The standard curve was generated using *E. faecalis* (qPCR efficiency = 1.08; R² value = 0.994).

Data analysis

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). One-sample Wilcoxon test was used to compare the median number of threshold cycles for each samples at each time point and the median number of threshold cycles obtained by the standard and negative controls. Kruskal-Wallis test and Mann-Whitney test were used to compare the number of threshold cycles obtained by the samples collected at the three time points. In all tests, the significance was set at 0.05. Delta threshold cycles (Δ CT) was calculated to determine the difference in the median number of threshold cycles (CT) obtained from the endodontic materials and the standard control. Δ CT = 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 & Livak, (2008)(17). The equation is: Fold change = median 2^{-CT} test material sample / median 2^{-CT} standard control.

Results

MALDI-TOF MS

By using a culture-based approach, all the endodontic materials (except ERS) when collected from the freshly opened sealed packages (t_0), showed a number of contaminated samples. After t_1 and t_2 the number of contaminated samples increased in all tested materials. A significant difference was present between t_0 and t_2 in ERS and PMP (Fig 1). In total, 107 taxa were identified from the samples of the endodontic materials at the three culture times. They belonged to 26 different bacterial species and 15 *genera*. The most common isolated *genera* were *Propionibacterium* (42.1%), *Staphylococcus* (31.8%), *Micrococcus* (10.3%), *Kocuria* (3.7%) and *Corynebacterium* (2.8%).

The most prevalent identified species were *Propionibacterium acnes* (26.2%), *Propionibacterium acidifaciens* (14%), *Staphylococcus epidermidis* (13.1%), *Micrococcus luteus* (10.3%), *Staphylococcus warneri* (4.7%), *Staphylococcus hominis* (3.7%), *Staphylococcus pasteurii* (3.7%), *Kocuria rhizophila* (2.8%), and *Corynebacterium tuberculostearicum* (1.9%). The number of bacterial species isolated from the contaminated endodontic materials increased at 14 days from clinical storage in all endodontic materials except OS (Fig 4).

qPCR

Results obtained from qPCR at t_0 showed significantly lower mean numbers of threshold cycles for endodontic materials such as RD, PMP, ERS indicating higher bacterial content compared with the standard control ($p < 0.01$). At t_1 and t_2 all tested endodontic materials (except OS) showed a statistically significant difference between the median numbers of threshold cycles obtained from endodontic materials samples and their standard control indicating bacterial contamination ($p < 0.01$).

The increase in the amount of bacterial contamination for each sample was not statistically significant between t_1 and t_2 as per Kruskal-Wallis test and Mann-Whitney test ($p > 0.05$) (Fig 5).

Discussion

The present study demonstrated that endodontic materials such as gutta-percha points, rubber dam, paper mixing pad, caulking agents when taken from freshly sealed packages carried bacteria. An increase in bacterial level was also found in the tested endodontic materials after clinical storage. Culturing revealed the number of contaminated samples increased in all tested endodontic materials. qPCR showed an increase in the median amounts of 16S rRNA gene in the endodontic material samples collected after 7 and 14 days. Longer sampling time was not feasible due to depletion of the stored materials. In case of longer storage, the bacterial contamination may grow linearly over time.

The external validity of the findings may depend on the type of clinical setting where the endodontic procedure is carried out (i.e. teaching hospital vs single specialist surgery).

A selection of commonly used endodontic materials were selected for this study. Although asepsis is regarded as mandatory in the dental settings; sterility is not considered a mandatory requirement for all endodontic consumables (4).

Previous studies assessed bacterial contamination of endodontic materials via culturing methods (5, 18-20). Culture-based approaches have limitations, in particular, the inability to cultivate non-viable microorganisms and to identify large number of bacteria (21). Therefore, in this study culture and non-culture based (qPCR) testing were used to determine the level and type of contamination of the endodontic materials. It has to be acknowledged that qPCR may detects both viable and non-viable bacteria. However, the presence of a 16s machinery may represent cells that were recently and likely metabolically active.

Previous studies determined the presence of bacteria on gutta-percha points (18, 22). The obturation material has been considered as the main source of nosocomial contamination

during root canal procedures. However, other consumables may contribute to environmental cross-contamination within the root canal space (4). An increased awareness is developing regarding the potential for environmental contamination in endodontics (4,14). It has been reported that bacteria can be sampled from over 40% of clinical surface in endodontic settings, out of these contamination only a small percentage (0.9%) pertains to putative endodontic pathogens such as *E. faecalis* (23). Nevertheless, the role of other non-pathogenic species in the event of cross-contamination has not been investigated.

The increase in the level of contamination may be due to airborne bacteria in surgeries that can easily contaminate the endodontic materials especially when they are stored for long time in the clinics. It is well established that dental treatment leads to the formation of aerosol and airborne bacteria that can deposit away from the operation site and the patient(24).

Propionibacterium, *Staphylococcus* and *Micrococcus* were the most commonly identified contaminants of the endodontic materials. This an agreement with Gomes *et al.*, who demonstrated *Staphylococcus*, *Propionibacterium* and *Micrococcus* as the most common genus isolated on gutta-percha points (25). Niazi *et al.* hypothesised that *Propionibacterium* and *Staphylococcus* genera typically isolated from secondary refractory endodontic infection might originate from using contaminated endodontic materials such as gutta-percha points and paper points (26). Although most of the isolated bacteria on the tested endodontic materials are part of the normal skin flora, they may also become opportunistic pathogen and cause life threatening infections (4, 27). In particular there are reports of *Propionibacterium* genus causing endodontic infection (26), sarcoidosis (28), brain abscesses (29) and pulmonary infections (30).

The clinical impact of these results is not clear, however, it could be speculated that endodontic failure may be at least, in part be due to nosocomial infection from endodontic materials. This may explain the failure rate detected even in *de novo* treatment (31). Further studies comparing the microbiota of contaminated endodontic materials and failed root canal treatment may provide further insight.

Conclusions

Commonly used endodontic consumables such as gutta-percha points, rubber dam, paper mixing pad, caulking agents when taken from the freshly opened sealed packages, are contaminated. Opening and storing the endodontic material packages in the endodontic dental surgeries for normal clinical use may increase the contamination level of the endodontic materials. *Propionibacterium*, *Staphylococcus* and *Micrococcus* are the most common genera on the contaminated endodontic materials.

MALDI-TOF represents a valid technique to identify environmental contamination in the endodontic clinical settings. This complements the advantages and disadvantages of qPCR.

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Figures and Tables

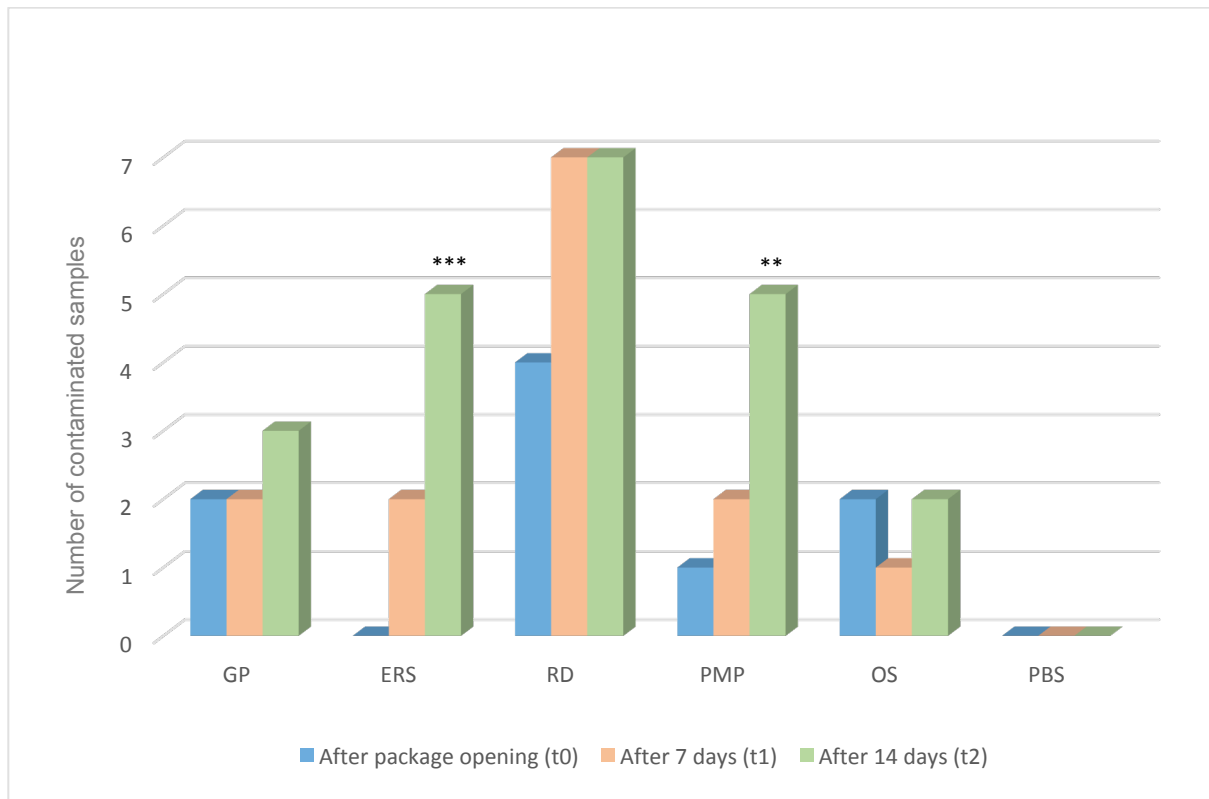


Figure 1: Number of positive samples, as per cultural methods, of the various endodontic materials at three different times from opening the sealed packages (n=9 total per material). PBS (phosphate buffered saline) represents the standard negative control. The tested materials were GP (Gutta-percha points), ERS (Endo ring sponge), RD (Rubber dam), PMP (Paper mixing pad), OS (Caulking Agent, Oraseal), PBS (phosphate buffered saline), ** (Values significantly higher than t0 ($p < 0.05$)) and *** (Values significantly more than t0 ($p < 0.01$)).

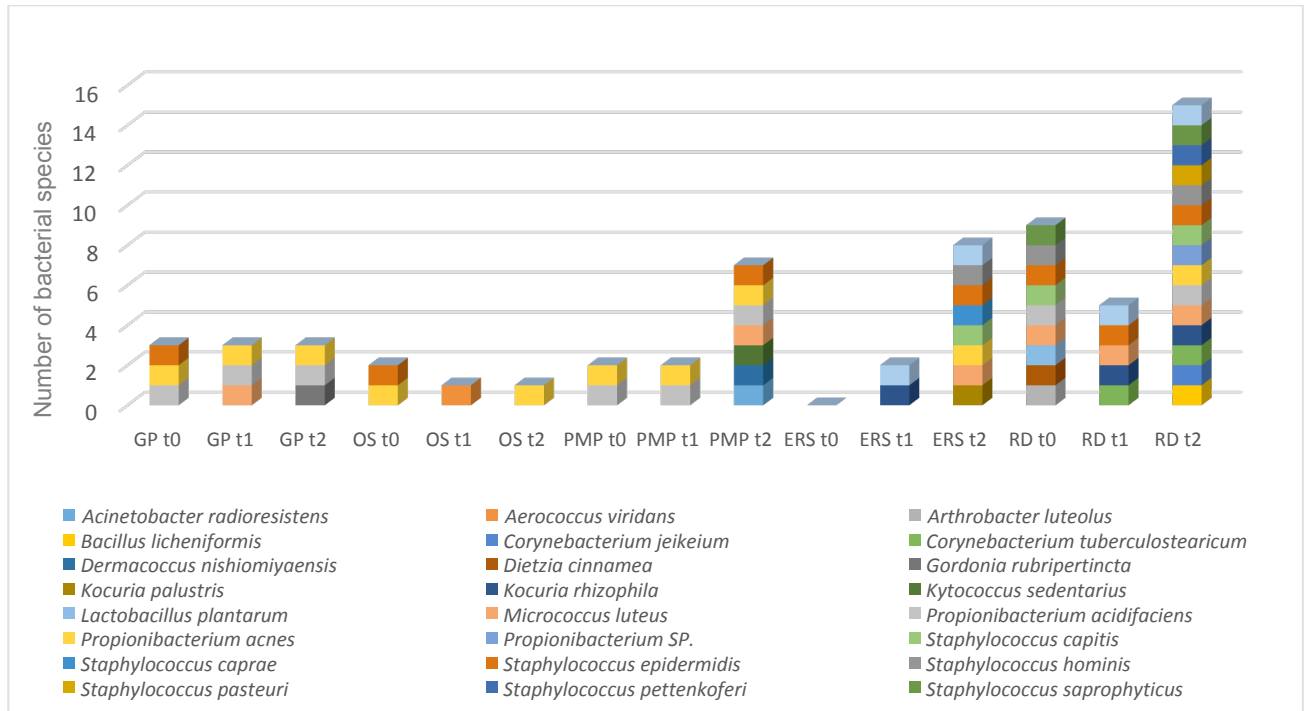


Figure 2: The number and identity of different bacterial species isolated from the endodontic materials at the three culture time points [after package opening (t₀), after seven days (t₁) and after 14 days (t₂)]. The tested materials were GP (Gutta-percha points), ERS (Endo ring sponge), RD (Rubber dam), PMP (Paper mixing pad) and OS (Oraseal).

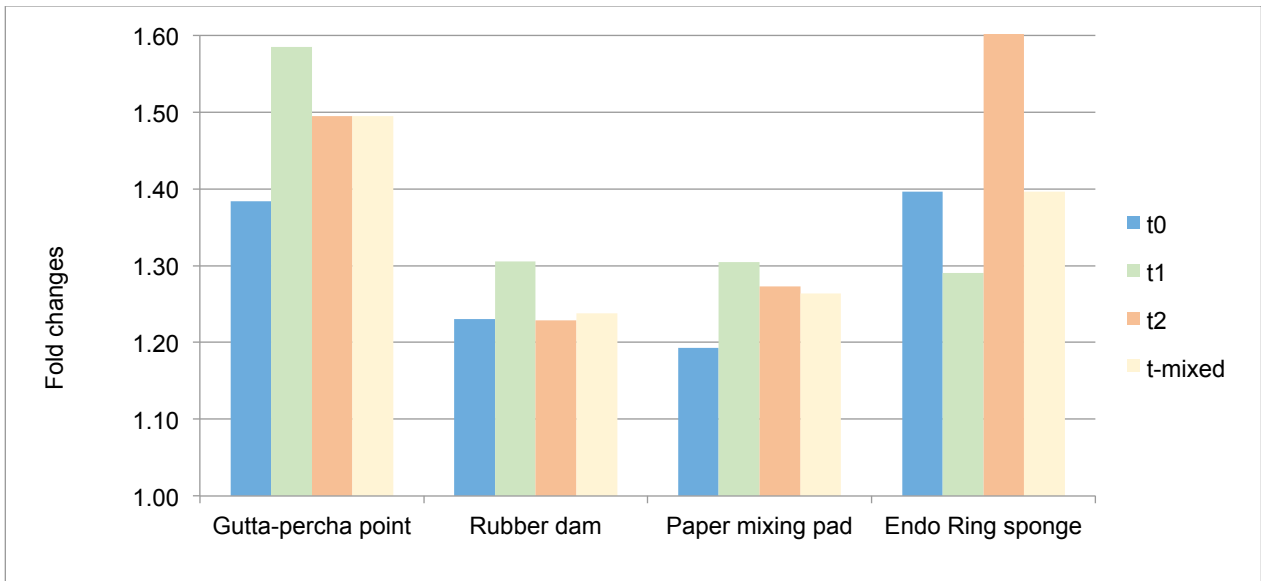


Figure 3: Fold change between some endodontic materials and their standard control as detected by qPCR. X-axis represents time points of sampling. t_0 = after package opening, t_1 = after 7 days and t_2 = after 14 days. t-mixed represents the pooled 27 samples. Y-axis represents the fold change in the median amounts of 16S rRNA gene obtained for each sample compared to standard control. In all time points, the fold change was above one. This means that the mean amounts of 16S rRNA gene obtained from the endodontic materials were higher than the mean amounts of 16S rRNA gene obtained from the standard control.