

Microbial Leakage of Gutta-percha and Resilon™ Root Canal Filling Material: A Comparative Study Using a new Homogeneous Assay for Sequence Detection

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ABSTRACT: The sealing ability of gutta-percha/sealer root canal filling was compared to a new thermoplastic synthetic polymer-based obturation material (Resilon™), using a microleakage model and a new sequence detection assay One Cut Event Amplification (OCEAN™). Eighty-eight extracted human teeth, shaped with K-Files and the ProTaper Technique, were randomly assigned to four groups ($n = 22$) and obturated in the apical 5 mm. Group R were obturated with the Resilon/Epiphany technique; group GP were obturated with gutta-percha and Zinc oxide eugenol sealer; group RCH and GPCH received calcium hydroxide intracanal medication before being obturated. Sterilized specimens were inoculated with *Enterococcus faecalis* and incubated in sterile medium for 47 days. DNA extracted from the specimens was amplified by PCR and then identified by the OCEAN technique. Samples obturated with Resilon root canal filling material showed a greater number of microleakage events than the other

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Figure 1 appears in color online: <http://jba.sagepub.com>

JOURNAL OF BIOMATERIALS APPLICATIONS Volume 00 — 2007

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0885-3282/07/00 0001-16 \$10.00/0 DOI: 10.1177/0885328207077411
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groups ($p=0.036$). Calcium hydroxide medication did not have a relevant impact on the quality of the apical seal ($p=0.044$).

KEY WORDS: root canal filling, resilon, gutta-percha, microleakage, PCR, *Enterococcus faecalis*, materials science.

INTRODUCTION

Successful endodontic treatment depends upon three-dimensional obturation of the root canal system to the end of the canal [1]. Since it is virtually impossible to completely eliminate bacteria from the canal system, the obturation must provide a hermetic seal at the apex in order to prevent reinfection of periapical tissue [2]. Thus, the main objective of root canal filling is the entombment of most surviving bacteria and the creation of a barrier to stop periapical tissue fluids from reaching them [3].

Gutta-percha associated with a sealer has, from its introduction into clinical practice in 1848 until the present time, been the most widely used root canal obturation material; still today it is considered the 'standard of care' in endodontic therapy. A recent review of clinical studies has shown the favorable long-term prognosis of root canal therapy performed with gutta-percha and a sealer [4]. Teeth without preoperative apical periodontitis can remain free from disease after initial or orthograde treatment in 92–98% of cases; teeth with apical periodontitis can completely heal after the root canal treatment or retreatment in 74–86% of cases, being however functional in 91–97%. However, the seal of root canal filling in gutta-percha is not always fully hermetic, revealing microleakage, in different study conditions, both apically and coronally [5–8]. The long-term clinical implication may be an increased risk of failure of the endodontic therapy.

With the aim of rectifying this possible unfavorable aspect of the gutta-percha seal in some clinical situations, a new thermoplastic synthetic polyester polymers-based root canal filling material (Resilon™) has recently been proposed [9–12]. The system comprises master cones or pellets for backfill with thermoplastic characteristics, similar to those of gutta-percha, used in combination with a dual curable dental resin composite sealer. Initial studies showed significantly less microleakage in Resilon sealed groups than in gutta-percha sealed groups [9] and higher mean fracture loads in the former ones [10]. An *in vivo* model in dogs has also determined greater resistance to coronal bacterial infiltration in subjects obturated with Resilon, with consequently weaker periapical inflammatory response [12]. All these

advantages appear to derive from the so-called 'Resilon Monoblock System.' The dual curable composite sealer forms a bond with the dentin structure on one side and the synthetic polymer of the Resilon on the other side, creating an intimate contact between the material and the canal walls. Thus a more hermetic seal and improved mechanical properties of the endodontically treated tooth should be established. However, some other studies demonstrate that both gutta-percha and Resilon seem to be unable to reinforce root-filled teeth [13,14]. Calcium hydroxide is widely used in endodontics as intermediate dressing between two sessions of root canal therapy, with the aim of improving the disinfection of the canal system before obturation [15,16]. The hypothesis has been advanced that calcium hydroxide may have a negative influence on the quality of apical seal of the gutta-percha root canal filling [17–19], due to residues present on the canal walls [19,20], especially when a zinc oxide eugenol (ZOE) sealer is used [20,21].

The primary objective of this *in vitro* study was to evaluate the occurrence of *Enterococcus faecalis* microleakage events through gutta-percha and sealer root canal filling (active controls), compared to Resilon obturation (test groups) using a new homogeneous method for sequence identification known as One Cut Event AmplificatioN (OCEAN™). (Figure 1). Its reaction exploits the action of a restriction enzyme and is based on interaction of the target sequence with two probes: an *anchor* probe, complementary to the target for most of its extension, and a probe marked with a fluorophore (F) known as the *amplifier*. The duplex target-anchor is stable at the reaction temperature of 65°C and favors formation of a ternary complex with the amplifier. This complex contains the recognition site of a restriction enzyme present in the solution. Reaction of this enzyme causes fragmentation of the amplifier into two segments that dissociate from the complex, while the anchor-target again becomes available for bonding with a new amplifier, promoting a linear amplification reaction.

The secondary objective was to analyze the influence of calcium hydroxide root canal dressing on the apical seal with both tested materials.

MATERIALS AND METHODS

Specimen Preparation

Extracted human single-root teeth with fully formed apex (upper central incisors and canines with substantially equal canal curvature

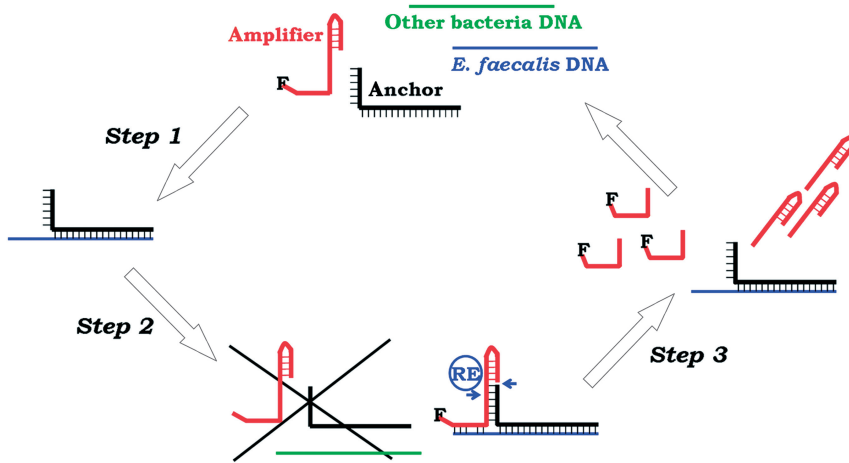


Figure 1. The OCEAN reaction. Step 1: a stabilizing probe (*anchor*) hybridizes to the target DNA, complementary of most of its extension. Step 2: the duplex target–anchor is stable at the reaction temperature of 65°C and a fluorescently labeled (F) probe (*amplifier*), specific for the *E. faecalis* primer pair, hybridizes to the target–anchor complex. The ternary structure forms the recognition site for a restriction endonuclease (RE) present in the solution. Step 3: fragmentation by the enzyme produces a cut amplifier which dissociates into two segments, while the anchor–target again becomes available for bonding with a new amplifier, promoting a linear amplification reaction. Production of the specific signal for *E. faecalis* was visualized by resolving the fragment of amplifier marked with the fluorescence generated by the reaction on polyacrylamide gel.

and morphology) that had not undergone prior endodontic treatment were utilized. After debriding the root surface, specimens were immersed in a 5% solution of sodium hypochlorite (Niclör 5, OGNA, Muggiò, Italy) for 1 h and then stored in 0.9% sodium chloride solution until preparation. Each specimen was then sectioned so as to obtain a residual root of length 15 mm. Each root canal was preflared using K-Flexofiles (Dentsply Maillefer, Ballaigues, Switzerland) up to #20 and then shaped using ProTaper S1-S2-F1-F2-F3 (Dentsply Maillefer, Ballaigues, Switzerland) at the working length. The working length was established under microscopic vision (Pro Magis, Carl Zeiss, Oberkochen, Germany) at 10× magnification, when the tip of the instrument was visible at the apical foramen. Irrigation was performed with a 22 gauge needle syringe: 33 mL of 5% sodium hypochlorite at 50°C (Niclör 5, OGNA, Muggiò, Italy), alternating with 2 mL of 10% EDTA (Tubuliclean, OGNA, Muggiò, Italy), total irrigation time 10 min per specimen. After drying with paper points, the roots were inspected

under the microscope at 10× magnification to check the integrity and shape of the apical foramen, the absence of cracks, and the canal cleanliness. The diameter of the apical foramen was checked with a K-File Nitiflex (Dentsply Maillefer, Ballaigues, Switzerland) fitting at the apex at the working length. Only specimens with an apical diameter of #30 and #35 entered the study.

One hundred and fifty-two specimens were selected. Fifty-six specimens were used for training on the use of the new EpiphanyTM Soft Resin Endodontic Obturation System (Pentron Clinical Technologies, Wallingford, CT, USA) and were not included in the study. Four specimens were used as positive controls and four as negative controls. The remaining 88 specimens were randomly subdivided by a blind examiner into four groups (GP, GPCH, R, RCH) of 22 each using a random numbers table.

Before canal obturation specimens in groups RCH and GPCH, were medicated with calcium hydroxide (DT Temporary Dressing, Dental Therapeutics AB, Sweden), delivered with a lentulo, sealed coronally with a temporary filling (CavitTM W, 3M ESPE, Seefeld, Germany) and then stored in 100% relative humidity. After 7 days, the root canal dressing was removed as follows: flushing with 5 mL 5% NaOCl at 40–50°C; K-Flexofile 20 ISO (Dentsply Maillefer, Ballaigues, Switzerland) 1 mm short of the working length (WL); flushing with 5 mL 10% EDTA; flushing with 5 mL 5% NaOCl at 40–50°C; K-Flexofile 20 ISO 1 mm short of the WL; flushing with 5 mL 10% EDTA; K-Flexofile 20 ISO 1 mm short of the WL; final flush with 5 mL 10% EDTA for 2 min.

Specimens in groups GP and GPCH were obturated in the apical 5 mm with nonstandard medium gutta-percha cones and the continuous wave of condensation technique with System B and medium plugger (Analytic Technologies, Redmond, WA, USA) at 220°C and Pulp Canal Sealer EWT (Kerr, Orange, CA, USA). Before obturation, a final flush with 96° ethyl alcohol was applied followed by drying with paper points.

Specimens in groups R and RCH were obturated in the apical 5 mm with nonstandardized ResilonTM cones and the dual curable dental resin composite sealer (Pentron Clinical Technologies, Wallingford, CT, USA), following the manufacturer's instructions. Before obturation, a final flush with 17% EDTA was applied followed by drying with paper points. A paper point soaked in primer was used to transfer the liquid into the canal; excess was removed with a dry paper point. The resin-based cement was then placed in the apical third of the root canal with a lentulo. A nonstandardized medium ResilonTM cone (Pentron Clinical

Technologies, Wallingford, CT, USA) adapted to the apical diameter was inserted into the root canal and the specimen obturated with the continuous wave of condensation technique with System B and medium plugger (Analytic Technologies, Redmond, WA, USA) at 150°C. Lastly the material was polymerized for 40 s using a photopolymerizing lamp XL 3000 (3M ESPE Dental Products, St Paul, MN, USA). Negative controls ($n = 4$) were obturated (two with gutta-percha/ZOE sealer and two with ResilonTM/composite sealer) and completely sealed on their entire surface with a layer of varnish and then covered with sticky wax, also covering the apex; positive controls ($n = 4$) were not obturated and were sealed externally with varnish and sticky wax to 3 mm from the apex.

All obturated specimens in all groups were then checked radiographically to verify the quality of the canal obturation, sealed with temporary filling (CavitTM W, 3M ESPE, Seefeld, Germany) and stored in 100% relative humidity. After 15 days, all specimens were prepared for microleakage testing by a double chamber model as has been previously described [22]. A pipette tip was placed in each canal and cemented to the dentine with cyanoacrylic cement. Surfaces were sealed with varnish and sticky wax to cover 5 mm of the pipette tip adjacent to the root, and the root walls from the pipette tip to 3 mm from the apical foramen. Each specimen was fixed with cyanoacrylic cement onto a steel wire support (diameter 0.80 mm) which was placed in a stable, vertical position in a 50 mL Falcon tube with screw stopper, such that the canal apex corresponded to the 25 mL notch on the tube. The tubes were placed in envelopes and sterilized in ethylene oxide, which does not alter the structure of the materials when it comes into contact with and does not produce a temperature increase. It is a volatile gas that, at the end of the sterilization cycle, does not leave any residue, even inside the dentin tubules, not influencing the growth of vitality of bacterial inoculated subsequently [23]. The procedure was as follows: 6 h at 40°C, 3 h humidifying at 70–75% humidity, 6 h application of 10% ethylene oxide, and total removal of the gas from the envelope by repeated replacement of the air content.

Inoculation

Under a laminar flow hood (CLANLAF - VFR 1206, USA) each tube was filled with 30 mL of sterile culture medium (yeast extract 1%, glucose 1%), completely immersing the root apex in medium

(lower chamber). The upper chamber was inoculated with 150 μ L of overnight culture of *E. faecalis* ATCC 29212 and incubated at 37°C. The culture medium of each specimen was inspected for contamination by the same examiner for 47 days of incubation via optical examination. Specimens showing turbidity were stored in a refrigerator at 4°C and sent for analysis of the target DNA with PCR followed by One Cut Event Amplification (OCEAN, Diasorin, Crescentino, Italy) assay [24], performed by another examiner. Both examiners were blind to experimental groups. At the end of the experiment session all the remaining specimens were checked for sterility by PCR and OCEAN.

DNA Extraction and PCR Amplification

DNA was extracted from 1 mL culture with NucleoSpin[®] (Macherey-Nagel, Germany) spin columns and was eluted in a 100 μ L volume. Ten microliter DNA were amplified by PCR with PCR primers, specific for *E. faecalis* 16S/23S rDNA: EF2fwd 5'-CAA-GGC-ATC-CAC-CGT -3' (forward primer) and EF2rv 5'-GAA-GTC-GTA-ACA-AGG -3' (reverse primer). Fifty microliter PCR reaction medium contained: DMSO 10%, EF2fwd and EF2rv 1 μ M, 1 \times Taq polymerase reaction buffer (Sigma-Aldrich, USA), dNTPs 0.8 nM, Taq polymerase 0.05 U/ μ L (Taq polymerase SuperPak (D5938) Sigma-Aldrich, USA). The PCR cycling conditions applied were: an initial denaturation step at 95°C for 2 min, 40 cycles of 95°C for 1 min (denaturation), 55°C for 1 min (primer annealing), 72°C for 1 min (extension), and a final extension at 72°C for 5 min. PCR cycling was carried out in a DNA thermocycler (PCR Sprint, Hybaid, USA). The reaction produced two amplicons of 314 and 414 bp, which were resolved on 1% agarose gel containing ethidium bromide and visualized under UV.

One Cut Event Amplification (OCEAN) Assay

The identity of the PCR product was then confirmed through the OCEAN reaction (Figure 1). Production of the specific signal for *E. faecalis* was visualized by resolving the fragment of amplifier marked with the fluorescence generated by the reaction on polyacrylamide gel.

A total reaction volume of 19 μ L containing 2 μ L di 10 \times BSA, 2 μ L 10 \times NEB2 buffer (New England Biolabs, Ipswich, MA), 11 μ L di-sterile deionized water, 2 μ L EF9F amplifier (2 μ M) marked with FAM fluorophore, 1 μ L EF11 anchor (10 nM), and 1 μ L of amplified DNA

was incubated for 5 min at 95°C to denature the amplified DNA and the probes, then incubated for 10 min at 65°C to enable formation of the OCEAN reaction complex (amplifier + anchor + target DNA). One microliter of the enzyme BsoBI (New England Biolabs, Ipswich, MA USA) was then added to the reaction mixture and maintained for 1 h at 65°C. The reaction was blocked by adding a loading buffer 2× (0.0892 M Tris, 0.0889 M boric acid, 0.052 EDTA, 7 M urea, 120 g/L Ficoll (R) 400, 0.1 g/L bromophenol blue), 8 μL of this mixture were loaded onto a 15% denatured polyacrilamide gel [15% acrylamide (acryl/bis 19:1)], 7 M urea, 0.0892 N Tris(hydroxymethyl)amminomethane, 0.0889 M boric acid, 0.002 M EDTA, 3.6 mM ammonium persulphate, 6.67 mM TEMED) and resolved by vertical electrophoresis. The gel was then visualized by fluorimetry with wavelengths specific for the FAM fluorophore (excitation wavelength 492 nm, emission wavelength 517 nm). Two negative controls were inserted to check that no contamination had occurred: 1 μL of 1 × BSA was placed in one, and in the other 1 μL of sterile deionized water instead of the amplified DNA.

Statistical Analysis

The number of events (microleakage) for each group was described with observed and expected values by cross-tabulation analysis. The analysis was performed first for the four groups separately and then calcium hydroxide medicated (GPCH and RCH) Versus nonmedicated (GP and R). The differences among groups were investigated with χ^2 test. The *null hypothesis* tested was that there would be no significant difference ($\alpha=0.05$) between the groups considered in terms of microleakage events under standard experimental conditions. All statistical analyses were performed using the SPSS for Windows 12.0 package (SPSS, Inc., Chicago, IL USA).

RESULTS

Positive controls showed turbidity after 24 h and the PCR-OCEAN analysis confirmed the presence of the target DNA, whereas negative controls did not exhibit any infection of the culture medium until the end of the experiment, thus validating the experimental model.

Table 1 shows the infiltration number separately for the four groups. All the other remaining samples, checked for sterility by PCR and OCEAN at the end of the experiment, resulted noncontaminated. Analysis of the four groups separately (Table 2) showed a greater number of events in group R ($\chi^2=8.57$; $df=3$; $p=0.036$). When the

Table 1. Specimens affected by microleakage in the four groups as detected by OCEAN analysis.

Days	Group GP (gutta-percha/ZOE sealer)	Group GPCH (gutta-percha/ZOE sealer)*	Group R (Resilon TM /resin sealer)	Group RCH (Resilon TM /resin sealer)*
10			3	1
15				1
20	1		1	
30	1		1	
40			1	
47	2	0	6	2

*Calcium hydroxide medication performed before obturation.

Table 2. Cross-tabulation analysis of the number of events (microleakage) for each group.

			Event		Total
			Yes	No	
Groups	GP	<i>N</i>	2	20	22
		Expected value	2.5	19.5	22
	GPCH	<i>N</i>	0	22	22
		Expected value	2.5	19.5	22
	R	<i>N</i>	6	16	22
		Expected value	2.5	19.5	22
	RCH	<i>N</i>	2	20	22
		Expected value	2.5	19.5	22
Total		<i>N</i>	10	78	88
		Expected value	10	78	88

groups were reclassified as (GPCH + RCH) Versus (GP + R) the differences found were at the margin of the statistical significance ($\chi^2 = 4.06$; $df = 1$; $p = 0.044$).

DISCUSSION

In light of the results of this microleakage study, the *null hypothesis* that there is no difference between ResilonTM and gutta-percha has to be rejected. The ResilonTM groups showed a greater number of microleakage events than the gutta-percha/sealer groups. This seems to be in contrast with the results of another study evaluating apical microleakage with the same two materials [9], where the gutta-percha groups, associated with AH26 and Epiphany sealers, leaked significantly more

than Resilon/Epiphany groups. These results were confirmed in another comparative study based on a fluid filtration model [25]. A greater tendency to microleakage has been reported with AH26 cement than with ZOE sealer [26,27] or glass-ionomer cement [28], when used with thermoplasticized gutta-percha techniques. These studies hypothesized that the thermophysical properties of an epoxy resin based sealer could be negatively influenced by the increased temperature due to the thermoplasticization of gutta-percha during warm vertical condensation. Wiener & Schilder [29] found that AH26 expanded at 1 week, followed by a shrinkage from 1 week to 30 days. In our study the gutta-percha groups were associated with a ZOE sealer (Pulp Canal Sealer EWT, Kerr, USA) and this could explain their better performance. Furthermore, the lower rate of microleakage in gutta-percha/sealer groups may be supposed to be due to the antimicrobial effect of eugenol and antiseptics in the sealer. Another study, based on a dye penetration model, found that ResilonTM resulted in less microleakage than gutta-percha [30]. However, dye penetration studies suffer from several limitations and their relevance is actually considered questionable [31–33]. Bacterial leakage studies, although they do not determine the ability of filling materials to prevent leakage of bacterial metabolites and their by-products, are recommended to test the sealing properties of materials in endodontics [34].

The present bacterial leakage model used *E. faecalis* to test the sealing properties of the obturation materials under comparison. *Enterococcus faecalis* is not particularly demanding from the nutritional standpoint. This resistant microorganism can survive extreme challenges and is frequently isolated in cases of endodontic failure [35,36], since it can penetrate the dentine tubules and escape chemomechanical treatment of the root canal system [37].

In this study, the use of calcium hydroxide as intracanal dressing appears not to have drastically influenced the degree of microleakage of Resilon and gutta-percha, compared to specimens in which an intermediate medication was not used. These findings are in agreement with another study on ResilonTM [38]. On the contrary, Porkaew et al. [39] and Holland et al. [40] reported a reduced tendency to apical leakage in specimens treated with calcium hydroxide *versus* untreated specimens. It has been hypothesized that calcium hydroxide may influence apical seal, due to residues present on the canal walls in the apical zone that are not removed by irrigant solutions [17–20]. Actually, there is agreement that the best protocol for the removal of calcium hydroxide consists in alternating irrigation with 5% sodium hypochlorite and EDTA in concentrations

between 10% and 17% with the use of manual files, as was done in this study.

Bacterial leakage was evaluated through PCR followed by a new homogenous assay for sequence detection denominated OCEAN (OCEAN™ – Diasorin SpA, Crescentino, Italy) [24]. It has been demonstrated that molecular methods are superior in terms of sensitivity and specificity if compared to cultivation procedures [41], which can be limited by the difficulty of cultivation and identification [42]. Molecular genetic methods offer several advantages in microbial identification and, in consequence, in microleakage studies. They enable cultivable and uncultivable microbial species or strains to be detected, directly from samples, with no need for cultivation, and do not require carefully controlled anaerobic conditions during sampling and transportation of anaerobic bacteria or other fragile microorganisms; they are faster and less time-consuming and they offer a rapid diagnosis [42]. In particular, a protocol for PCR detection of *E. faecalis* and *E. faecium* from the root canal has shown high sensitivity and specificity in detecting microbiota from root canal specimens [43]. These techniques are not quantitative and do not require a viable organism in order to generate a PCR product. Nevertheless, the objective of the present study was not to assess the viability of *E. faecalis*, but to determine the effectiveness of a barrier (the tested material) against bacterial infiltration (the event considered). However, PCR may be inherently prone to generate false positives due to the extreme performance of the reaction, which may result in nonspecific amplification of even traces of contaminants. Thus, the identity of a PCR product needs to be further assessed by some post-PCR technique. Commonly used techniques such as reverse dot blot rely on specific capture of the PCR product by hybridization to a complementary DNA strand immobilized on a solid surface. The hybrid double strand is then labeled by different techniques. This approach results quite labor intensive and time consuming. The OCEAN reaction provides an innovative way to verify the identity of a PCR product. The architecture of this assay, relying on the formation of a triplex (target–amplifier–anchor) gives to the system a very high specificity. Furthermore, the assay involves a recycling of the labeled amplifier on the target DNA resulting in a sequence-specific signal amplification. The OCEAN assay produces an easily detectable amount of cleaved labeled amplifier in <1 h. Being a fast, homogenous assay, OCEAN can be used for rapid detection and typing of bacteria, without the need for running gels. As a future development, a quick multiplex OCEAN assay could be employed for rapid diagnosis to identify different bacteria in a point of care format.

In our study, all the specimen were obturated in the apical 5 mm, with the aim of analyzing microleakage in the most critical portion of the root canal seal. In this situation, which is very frequent in clinical practice, when a post space is prepared for optimal preprosthetic reconstruction, the gutta-percha/sealer groups showed better apical seal than those obturated with ResilonTM. Moreover, adhesion to root canal walls is still considered an unfavorable clinical situation, compared to indirect intracoronary restorations [44]. A weak link may exist in ResilonTM-filled root canals along the sealer-dentin interface [45–47]: the gaps were hypothesized to have been created by the inability of the material to counteract the rapid polymerization contraction of the methacrylate-based resin sealer. This may be due to high concavity configuration factors that might have contributed to polymerization stresses along the root canal walls [48]. Furthermore, manipulation of partially polymerized sealer during condensation could also negatively affect the bonds developing between the self-etching primer and dentin surface. Another question has been considered about the biodegradability of ResilonTM if a hermetic seal is not established: whereas gutta-percha is a relatively inert material, polycaprolactone, which gives thermoplasticity to the polymer of which ResilonTM is constituted, seems to be susceptible to alkaline hydrolysis [49] and biodegradation under microbial attack [50], due to enzymatic hydrolysis [51] by bacterial lipases that can cleave the ester bonds [52,53].

CONCLUSIONS

Within the limitations of the study, it may be concluded that the traditional root canal filling technique using thermoplasticized gutta-percha and a ZOE sealer appears to provide a better apical seal than the ResilonTM filling technique, which has been introduced as an alternative method to obturate root canals, challenging the use of gutta-percha. There is a need for further investigation and long-term clinical studies to increase our knowledge of an item of current and future great interest, i.e., the use of obturation systems that exploit adhesive techniques within the canal system. These will enable immediately postendodontic reconstruction to be applied, reducing the risk of bacterial infiltration by providing an immediate coronal seal [54] and thus improving the long-term prognosis of prosthetic restorations [55].

ACKNOWLEDGEMENTS

The authors gratefully thank Prof. Alessandra Angeretti and Dr Narcisa Mandras (Department of Public Health and Microbiology, University of Turin) for valuable support and Dr Annalisa Castella (Department of Public Health and Microbiology, University of Turin) for the cooperation in statistical analysis.

A special thank to Dr. Alessandra Bosia who participated in the design of the OCEAN assay used in this study.

Dr. Daniel Adlerstein is leading the Molecular Diagnostics team at DiaSorin SpA.

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