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Antimicrobial efficacy of different irrigant solutions using a novel biofilm model: an *in vitro* confocal laser scanning microscopy experiment

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Running Title

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Declaration

The authors explicitly declare no conflicts of interests in relation to this article

Key Words

Biofilm; CLSM; EDTA; Enterococcus faecalis; Irrigants; Sodium Hypochlorite

Abstract

Aim: Determine the ability of different irrigation solutions to biomechanically remove Enterococcus faecalis biofilm from a novel artificial root canal model during chemomechanical preparation.

Methods: High resolution micro-computer-tomography scans of a mandibular molar's mesial root were used to produce 50 identical 3D-printed resin root canal models. These were cultured with *E.faecalis* over seven days to generate biofilm and subjected to chemomechanical preparation using: saline; 17% ethylenediaminetetraacetic acid (EDTA) or 2% sodium hypochlorite (NaOCl) alongside positive/negative controls (n=10). Canals were prepared to 40/.06 taper, with 1mL irrigation between instruments, followed by 5mL penultimate rinse, 30s ultrasonic activation and 5mL final rinse. Residual biofilm volume (pixels) was determined following immunofluorescent staining and confocal-laser-scanning-microscopy imaging. Statistical comparisons were made using Kruskal-Wallis with *post-hoc* Dunn's tests ($\alpha<0.05$).

Results: In all canal thirds, the greatest biofilm removal was observed with NaOCl, followed by EDTA and saline. The latter had significantly higher *E.faecalis* counts than NaOCl and EDTA ($P<0.01$). However, no statistical differences were found between EDTA and NaOCl or saline and positive controls ($P>0.05$).

Conclusions: Within limitations of this model, 17% EDTA was found to be as effective as 2% NaOCl at eradicating *E.faecalis* biofilm following chemomechanical preparation. Further investigations with multi-species biofilms are encouraged.

Introduction

Putative endodontic microbial communities, and their by-products, are the causative agents of pulp and periapical diseases (1, 2). Current therapeutic strategies therefore aim to disrupt these biofilms and disinfect root canals so that the bacterial load is below the critical threshold required for periradicular healing (3). This is typically achieved by using a combination of hand or rotary instruments alongside constant irrigation with antibacterial solutions. Greater emphasis is placed on cleaning the canal as opposed to shaping it due to the inherently complex nature of the endodontic anatomy (4). For these reasons, irrigant selection plays a critical role in determining the success of root canal treatment.

Currently, the most commonly used endodontic irrigants are sodium hypochlorite (NaOCl) and ethylenediaminetetraacetic acid (EDTA; 5, 6). Whilst the former possesses potent antimicrobial and tissue dissolving properties (7), it is also a caustic agent that when used incorrectly has the capability to inflict diffuse soft tissue swelling, bruising, ulceration and in severe cases, necrosis and neurological damage (8). This is compounded by its cytotoxic potential (9), inability to remove infected inorganic debris from within the surgical smear layer (7) and detrimental effects to the dentine's flexural strength (10, 11). For these reasons, NaOCl is often administered at concentrations less than 3%, with strengths of 2% still being considered an effective dose for disinfection (5). Conversely, EDTA, which is widely available at 17% concentration and is conventionally used for its chelating action on the smear layer (12), overcomes many of these limitations but at present has questionable antimicrobial properties. For instance, there are numerous studies that have reported this solution as being effective against a broad spectrum of endodontic bacteria (13 – 18), including *Enterococcus faecalis* which is known for its invasive, adherent and pH

resistant properties (19). However, almost an equal number of studies have observed limited or no antimicrobial effect despite using similar analytical methodologies (20 – 27). The results of these studies must also be interpreted with caution as they were conducted using relatively simple *in vitro* experimental model systems in addition to planktonic forms of bacteria rather than biofilms (28). Such investigations do not reflect *in vivo* conditions well.

In recent years, technological advancements have allowed for the development of resin-based materials that facilitate growth of microbial biofilms. These have been found to possess similar properties to dentine, with respect to bacterial attachment (29), and through rapid processing techniques can be manufactured into three-dimensional (3D) models that accurately mimic the intricate anatomy and unique environment of the root canal system (30). These experimental models show promise in overcoming many of the limitations of those that have been previously used. They also allow for the antimicrobial activity of irrigant solutions to be evaluated throughout the course of both chemical and mechanical preparation of root canals, a feature which is difficult replicate with extracted teeth. At the same time, highly sensitive methods of microbial analysis are now available to quantify viable micro-organisms within biofilms by way of fluorescent staining and confocal laser scanning microscopy ([CLSM] 23, 31, 32). These offer more careful morphological observation through higher resolution imaging, the possibility of 3D reconstructions and overcoming many of the limitations associated with dentine demineralisation and microtome sectioning. As very few studies have employed such techniques in relation to EDTA, further and more sophisticated investigations into the antimicrobial capabilities of this solution are warranted (23). The resulting information could contribute to the development of more

biocompatible irrigant regimes and an improved understanding of the potential mechanisms in which these solutions interact with microbes.

The aim of this *in vitro* experiment was therefore to determine the ability of saline, 17% EDTA and 2% NaOCl to biomechanically remove an *E. faecalis* biofilm from a more clinically relevant and novel root canal model during chemomechanical preparation. The tested null hypothesis was that there were no significant differences between the different irrigant solutions.

Materials and Methods

Root Canal Model

Following ethical approval (REC Ref: 14/SW/1148), 20 extracted human mandibular molars were randomly selected from the University of Birmingham's Dentistry Research Tissue Bank. Teeth with extensive caries and restorations, root fractures, open apices, resorptive defects, previous root fillings and fused roots were excluded. A high-resolution micro–computed tomographic scan (μCT; 13.6 μm/pixel; Skyscan 1172; Chelmsford, UK) was subsequently obtained for each tooth. The resulting image slices were uploaded onto ImageJ software (National Institutes of Health, Bethesda, USA) and reconstructed into 3D.

The μCT series of a mandibular molar's mesial root, which contained two distinct unprepared canals and complete isthmus, was selected to generate the model as demonstrated in Figure 1 due to its complexity. Initially, the mesial root was segmented at the point of furcation and the curvature straightened so the endodontic system could be centrally bounded in rectangular geometry and subsequently divided into two equal halves. Three-dimensional replicas were then printed from a transparent auto-

fluorescent resin material (Accura; 3D Alchemy, Shropshire, UK), via stereolithographic rapid processing technology at 50 μm/layer resolution and ± 0.2 mm surface accuracy (3D Alchemy). A precisely fitting white polypropylene cubic frame was also fabricated so the rectangular halves of each model could be firmly approximated during chemomechanical preparation to mimic a closed root canal system with standardised dimensions. More specifically the canal length, inter-canal distance and range of isthmus width in each assembled model were measured as being 9.5 mm, 3.6 mm, and 0.08 to 0.31 mm respectively using the calibrated line tool on ImageJ software.

Biofilm Cultivation

Under aseptic condition in a laminar flow hood, the NCTC 12697 strain of *E. faecalis* (Public health England, Wiltshire, UK) was cultivated on Brain Heart Infusion (BHI) agar (Sigma-Aldrich, Gillingham, UK) for 24 h at 37°C in a 5% CO_2 incubator. Thereafter, a single colony was transferred into 10 mL BHI broth and incubated under the same conditions alongside a sterile control. The resulting bacterial suspension was diluted 100-fold in fresh BHI broth and adjusted to an optical density of 1 using a flow cytometer (BD accuri, California, USA). This standardised the bacterial concentration to 1.6 x 10^6 CFU/mL.

Prior to inoculation, all blocks and frames were autoclaved for 30 minutes at 121°C. The models were then positioned into a 24-well tissue culture plate, so the internal canal surface faced towards the plate cover. Two millilitres of bacterial suspension were subsequently added into each well, after which plates were cultured for 7 days in a 5% CO_2 incubator. The BHI growth medium was replenished every 48 h and following this period, biofilms were washed with Phosphate Buffered Saline (PBS) and fixed for 10 minutes with 2.5% glutaraldehyde (Sigma-Aldrich).

Control & Test Groups

Infected root canal blocks were inserted into the polypropylene cubic frame and then randomly distributed into 3 groups (n = 10) according to irrigant solution. These included i) Saline (CD Medical, Bolton, UK), ii) 17% EDTA (Cerkamed, Stalowa-Wola, Poland) and iii) 2% NaOCl (Cerkamed). To confirm adequate biofilm growth (positive control), 10 blocks were contaminated but not chemomechanically prepared and a further 10 samples were cultured in sterile BHI broth to determine background staining (negative controls). As a power calculation could not be conducted, due to the lack of prior data, the sample size was determined using previous studies investigating similar hypotheses (20, 21, 23)

Root Canal Preparation

Root canal preparation was performed by a single blinded operator (SSV) to a pre-determined working length (WL) of 9 mm. After assembled blocks were firmly clamped to the bench top, a glide path was established using a stainless-steel size 10 K-file (Dentsply Sirona, Ballaigues, Switzerland) in a watch winding motion. Root canals were then prepared up to a ProTaper Gold F4 (size 40/.06 taper) rotary file at speeds and torques recommended by the manufacturer (Dentsply Sirona). Between instruments, 1 mL of irrigant was expressed into each canal with the tip of a 27 gauge side vented needle positioned 2 mm short of the WL (Monoject, Covidien, Mansfield, USA). A 5 mL penultimate rinse was then administered followed by 30 seconds passive ultrasonic irrigation, with an ISO size 20 Irrisafe tip (Acteon, Norwich, UK) activated half power 1 mm from WL (MiniEndo II; SybronEndo, California, USA). To terminate the irrigation sequence, a final 5 mL rinse was performed as above followed 5 mL sodium thiosulphate or 5 mL saline to arrest NaOCl and EDTA activity

respectively. Root canal blocks were then disassembled and washed with PBS prior to immunofluorescent staining.

Biofilm Staining

To label the residual *E. faecalis* biofilm, samples were incubated for 24 h at 4°C with 50 µl of a primary non-conjugated polyclonal antibody (Rabbit anti-*Enterococcus* species*;* MyBioSource, San Diego, USA). Blocks were then washed in PBS, incubated in a dark environment for 24 h at 4°C with 50 µl of a secondary tetramethyl rhodamine-isothiocyanate (TRITC) conjugated polyclonal antibody (Donkey Anti-Rabbit IgG H&L; Abcam, Cambridge, UK) and then washed again in PBS. Both antibodies were diluted 300-fold with 3% w/v bovine serum albumin.

Confocal Laser Scanning Microscopy

Labelled models were mounted onto a customised glass slide and viewed under a CLSM at 5 x magnification (Carl Zeiss, Oberkochen, Germany). A plastic seating jig standardised the position of each block and a copper grid (TedPella, California, USA) with unique patterns allowed images to be captured between samples at reproducible positions (Figure 2). Multi-track lasers, set at 488 nm and 555 nm, were used to reduce cross talk between the green auto-fluorescence inherently emitted by the Accura resin material and the red fluorescence emitted by the TRITC labelled biofilm. Sixteen images (seven coronal, six middle and three apical) were captured per model at optimal focus and fixed resolution (512 x 512 pixels) with the same objective and laser settings (Table 1). All images were saved in .tiff format, coded and then analysed in ImageJ software by a blinded assessor (SSV).

A semi-automated method was used to quantify the remaining biofilm (29). For each image, the four central squares were isolated (“clear outside”) to standardise the area

of analysis and split into individual colour channels ("split channel"). The resulting green and red grey-scale images represented the Accura material and residual biofilm respectively, the latter of which was used in subsequent analyses. Background fluorescence was removed ("subtract background") and an "auto-threshold" applied to allow the residual biofilm to be quantified via a calibrated "voxel counter" tool.

Statistical Analysis

Statistical tests were performed using SPSS software (V.25; IBM, New York, USA). The Shapiro-Wilk test revealed data to be non-normally distributed and therefore, comparisons between groups were made using Kruskal-Wallis and *post-hoc* Dunn's tests with the initial alpha values set at 0.05. Additionally, 10 randomly selected images from each group were analysed one month apart to determine intra-rater reliability via the intra-class correlation coefficient (ICC). Data was presented as medians and means alongside the interquartile range and standard deviation respectively.

Results

The volume of residual biofilm for each group is summarised in Table 2 and Figure 3 with representative CLSM images displayed in Figure 4. The ICC demonstrated intra-rater agreement at greater 0.95.

In all canal thirds, the greatest biomechanical removal of biofilm following chemomechanical preparation was found in the 2% NaOCl group, followed by 17% EDTA and then saline. However, no solutions were able to eradicate the entirety of the biofilm. Nevertheless, when compared to the positive control group, the greatest percentage reductions for any given regime were observed in a coronal to apical

direction. The positive controls presented the largest *E. faecalis* counts whereas negative controls showed zero *E. faecalis* presence.

Highly significant differences were identified between irrigant solutions in all canal thirds ($P < 0.001$). Root canals prepared with 17% EDTA and 2% NaOCl resulted in significantly less residual *E. faecalis* biofilm than saline ($P < 0.01$). However, no statistical differences were found between 17% EDTA and 2% NaOCl or between saline and positive control groups ($P > 0.05$).

Discussion

The current study used a novel model for testing the antimicrobial efficacy of several commonly used irrigants throughout chemomechanical preparation of artificial root canals. Under these parameters, 17% EDTA was found to be comparable to 2% NaOCl at biomechanically removing *E. faecalis* biofilms in all canal thirds. However, saline was significantly less effective and so the null hypothesis was rejected.

Endodontic disinfection involves both chemical and mechanical debridement within a closed root canal system. However, previous investigations into the antimicrobial efficacy of EDTA have seldom been conducted under such conditions. More than often, test solutions have been administered onto infected agar plates (14, 17, 22), cover slips (24), cell suspensions (15, 20) and dentine disks (23). Whilst extracted teeth were used in more recent experiments (25, 26), the root canals in these samples were inoculated only after instrumentation, where they then underwent a distinct chemical disinfection protocol. The resin model employed in the present study however overcame these limitations by allowing solutions to be delivered into an infected and closed endodontic system prior to and throughout the entire

chemomechanical debridement process. This is more akin to how root canal treatment is performed clinically and at present is difficult to test with an *ex vivo* or intratubular infection approach. The precise manufacturing process employed in this study also offered a degree of anatomical replication that far surpassed prior synthetic models (33 – 35), giving way for more representative irrigant flow dynamics and methodological standardisation that cannot be achieved with extracted teeth (36). Additionally, the model could be longitudinally split on demand without disturbing the residual biofilm, which would allow continued analysis following chemomechanical debridement. This feature would be particularly useful for investigating bacteria that have *in vitro* demonstrated a potential for regrowth, such as E.*faecalis,* after NaOCl administration (37). Furthermore, the use of the copper ring depicted in Figure 2 to select specific regions of the canal to image and the semi-automated quantitative method of analysis offers a more reproducible and accurate approach to investigating endodontic biofilm removal than many preceding studies.

The most apparent limitations of this study however arise from the model's material composition and structure, in that there is no peri-, intra- and inter-tubular dentine or tubules for micro-organisms to penetrate into. This non-biological substrate could alter the inherent mechanisms of bacterial surface adherence thus, affecting subsequent biofilm formation. There would also be more freely available chlorine ions for NaOCl disinfection due to the absence of collagen, fluid and necrotic debris (38). Additionally, single-species biofilms exhibit less biomass and resistance to endodontic irrigants than their more representative multi-species counterparts and those made of E.*faecalis* in particular have demonstrated the potential to re-establish themselves following exposure to common endodontic irrigants (37, 39, 40). Collectively, these limitations could overestimate the antimicrobial efficacy of the tested irrigant solutions;

however, attempts were made to reduce the impact of these confounding variables. For instance, Accura resin was selected as *E. faecalis* attached to its surface at a force comparable to that of dentine, which precluded the need for any prior collagen coating (29). Similarly, this species was used as the test micro-organism due to its ability to rapidly form biofilms on resin materials and remain within root canals even after thorough chemomechanical disinfection protocols (18). Furthermore, lower NaOCl concentrations were administered to compensate for the lack of organic matter and biofilms were cultured until they reached peak biomass at seven days, which is significantly longer than other simulated root canal studies (33 – 35). The disinfection challenge these measures created was further potentiated by the complicated endodontic anatomy of the model, as evidenced by high treatment failure rates associated with the tooth it was based on (41). However, despite these endeavours, some caution must still be taken when extrapolating the present results into the clinical setting and further investigations using multi-species biofilm models are encouraged to reinforce these findings.

In the present study, 17% EDTA and 2% NaOCl were found to be equally as effective at eradicating *E. faecalis* biofilm from within artificial root canals. These findings contrast previous studies that reported the antimicrobial efficacy of the former to be absent, limited or vastly inferior to NaOCl even after prolonged periods of exposure (20 – 27). This discrepancy could be attributed to methodological heterogeneity, varying irrigation protocols and the differing mechanisms in which these solutions interact with micro-organisms. For instance, NaOCl disassociates into its bactericidal hydroxyl and chlorine ion derivatives and then rapidly eliminates microbes by disrupting enzymatic processes essential to their physiology (42). Conversely, EDTA only destabilises gram-negative bacteria by chelating cations from within their outer

cell membranes (43). Whilst this effect alone may not always induce cell death, it could potentially be sufficiently enhanced enough to do so when combined with mechanical instrumentation, a feature which has only been tested in the current study. This chelating action has also shown to promote cellular detachment and weaken the macrostructures of established biofilms, which can then be more easily flushed from root canals via the mechanical shearing forces created by conventional irrigant flow dynamics and agitation techniques (24). Furthermore, higher EDTA concentrations and exposure times, as used in this experiment, have demonstrated greater disinfection capabilities (15, 16). Collectively, these mechanisms could potentially equate to the antimicrobial activity of 2% NaOCl and would explain why 17% EDTA was found to be comparable to this solution post root canal preparation but significantly more effective than saline, the latter of which possesses no antibacterial properties and was also used to provide additional validation of the experimental model.

Whilst 2% chlorhexidine has previously been considered an alternative endodontic irrigant to NaOCl, evidence highlighting its negative association with periradicular healing and increased incidents of anaphylaxis has recently emerged (44, 45). Consequently, this has deterred its use as demonstrated by a recent national survey which found 15 of the 18 undergraduate dental schools across the UK & Ireland abstaining from teaching its use during root canal treatment (5). For these reasons, it was not included as an additional test group.

Conclusion

Based on the results of this study, which were derived from a novel biofilm model, it can be proposed that 17% EDTA is as effective as 2% NaOCl at biomechanically removing *E. faecalis* biofilm following chemomechanical preparation of a complex root canal system. Further investigations however with a multi-species biofilm model are encouraged to reinforce the promising results observed within the present study.

References

1. Kakehashi, S., Stanley, H.R., Fitzgerald, R.J. The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. Oral Surg Oral Med Oral Pathol 1965; 20:340–9.

2. Siqueira, Jr J.F., Rocas, I.N., Ricucci, D. Bioflims in endodontic infection. Endod Topics 2012; 22:33–49.

3. Siqueira, Jr J.F., Rocas, I.N. Clinical implications and microbiology of bacterial persistence after treatment procedures. J Endod 2008; 34:1291–301.

4. Peters, O.A., Schönenberger, K., Laib, A. Effects of four Ni-Ti preparation techniques on root canal geometry assessed by micro computed tomography. Int Endod J 2001; 34:221-30.

5. Virdee, S.S., Ravaghi, V., Camilleri, J., *et al*. Current trends in endodontic irrigation amongst general dental practitioners and dental schools within the United Kingdom and Ireland: a cross-sectional survey. Br Dent J 2020; [Online ahead of print]

6. Dutner, J., Mines, P., Anderson A. Irrigation trends among American association of endodontist members: A web-based survey. J Endod 2012; 38:37-40.

7. Zehnder, M. Root canal irrigants. J Endod 2006; 32:389-98.

8. Guivarc'h, M., Ordioni, U., Ahmed, H.M., *et al*. Sodium hypochlorite accident: a system-atic review. J Endod 2017; 43:16–24.

9. Martin, D.E., De Almeida, J.F., Henry, M.A., *et al*. Concentration-dependent effect of sodium hypochlorite on stem cells of apical papilla survival and differentiation. J Endod 2014;40: 51-5.

10. Sim, T.P., Knowles, J.C., Ng, Y.L., *et al.* Effect of sodium hypochlorite on mechanical properties of dentine and tooth surface strain. Int Endod J 2001; 34:120–32.

11. Marending, M., Luder, H.U., Brunner, T.J., *et al*. Effect of sodium hypochlorite on human root dentine–mechanical, chemical and structural evaluation. Int Endod J 2007; 40:786–93.

12. Mohammadi, Z., Shalavi, S., Jafarzadeh, H. Ethylenediaminetetraacetic acid in endodontics. Eur J Dent 2013;7: S135-42.

13. Yoshida, T., Shibata, T., Shinohara, T., *et al*. Clinical evaluation of the efficacy of EDTA solution as an endodontic irrigant. J Endod 1995; 21:592-3.

14. Siqueira, J.F. Jr., Batista, M.M.D., Fraga, R.C., *et al*. Antibacterial effects of endodontic irrigants on black-pigmented Gram-negative anaerobes and facultative bacteria. J Endod 1998; 24:414– 6.

15. Zehnder, M., Schmidlin, P., Sener, B., *et al*. Chelation in root canal therapy reconsidered. J Endod 2005; 31:817– 820.

16. Ballal, N.V., Yegneswaran, P.P., Mala, K., *et al*. In vitro antimicrobial activity of maleic acid and ethylenediaminetetraacetic acid on endodontic pathogens. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2011;112;696–700.

17. Prado, M., Silva, E.J., Duque, T.M., *et al.* Antimicrobial and cytotoxic effects of phosphoric acid solution compared to other root canal irrigants. J Appl Oral Sci 2015; 23:158-63.

18. Giardino, L., Savadori, P., Generali, L., *et al.* Antimicrobial effectiveness of etidronate powder (Dual Rinse® HEDP) and two EDTA preparations against Enterococcus faecalis: a preliminary laboratory study. Odontology 2020; [Epub ahead of print]

19. Nakajo, K., Komori, R., Ishikawa, S., *et al.* Resistance to acidic and alkaline environments in the endodontic pathogen Enterococcus faecalis. Oral Microbiol Immunol 2006; 21:283-8.

20. Arias-Moliz, M.T., Ferrer-Luque, C.M., Espigares-Rodríguez, E., *et al.* Bactericidal activity of phosphoric acid, citric acid, and EDTA solutions against Enterococcus faecalis. Oral Surg Oral Med Oral Pathol Oral Radiol Endod 2008; 106:84– 9.

21. Arias-Moliz, M.T., Ferrer-Luque, C.M., Espigares-Garcia M., *et al*. Enterococcus faecalisbiofilms eradication by root canal irrigants. J Endod 2009; 35:711–4.

22. Fidalgo, T.K., Barcelos, R., Portela, M.B., *et al.* Inhibitory activity of root canal irrigants against Candida albicans, Enterococcus faecalis and Staphylococcus aureus. Braz Oral Res 2010; 24:406– 12.

23. Ordinola-Zapata, R., Bramante, C.M., Cavenago, B., *et al.* Antimicrobial effect of endodontic solutions used as final irrigants on a dentine biofilm model. Int Endod J 2012; 45:162– 8.

24. de Almeida, J., Hoogenkamp, M.H., Felippe, W.T., *et al*. Effectiveness of EDTA and modified salt solution to detach and kill cells from Enterococcus faecalis biofilms. J Endod 2016; 42:320– 3.

25. Hartmann, R.C., Neuvald, L., Barth, V., *et al.* Antimicrobial efficacy of 0.5% peracetic acid and EDTA with passive ultrasonic or manual agitation in an Enterococcus faecalis biofilm model. Aust Endod J 2019; 45:57– 63.

26. Matos, F.S., Khoury, R.D., Carvalho, C.A.T., *et al*. Effect of EDTA and QMIX Ultrasonic Activation on the Reduction of Microorganisms and Endotoxins in Ex Vivo Human Root Canals. Braz Dent J (2019); 30:220-6.

27. Souza, M.A., Hoffmann, I.P., Menchik, V.H.S., *et al.* Influence of ultrasonic activation using different final irrigants on antimicrobial activity, smear layer removal and bond strength of filling material. Aust Endod J 2019; 45:209-15.

28. Camilleri, J., Arias-Moliz, T., Bettencourt, A., *et al.* Standardization of antimicrobial testing of dental devices. Dent Mater. 2020;36: e59-73.

29. Albaaj, F.S. (2018) Development of a model to assess cleaning and disinfection of complex root canal systems (PhD Thesis). Birmingham, UK: University of Birmingham.

30. Khalil, W., Ezeldeen, M., Van De Casteele., *et al.* Validation of cone beam computed tomography–based tooth printing using different three-dimensional printing technologies. Oral Surg Oral Med Oral Pathol Oral Radiol 2016;121:307-15.

31. Zapata, R.O., Moraes, I.G., Bernardineli, N., *et al.* Confocal laser scanning microscopy is appropriate to detect viability of Enterococcus faecalis in infected dentin. J Endod 2008; 3:1198–201.

32. Parmar, D., Hauman, C.H., Leichter, J.W., *et al.* Bacterial localisation and viability assessment in human ex vivo dentinal tubules by fluorescence confocal laser scanning microscopy. Int Endod J 2011; 44:644–51.

33. De Meyer, S., Meire, M.A., Coenye, T., *et al*. Effect of laser-activated irrigation on biofilms in artificial root canals. Int Endod J 2017; 50:472–9.

34. Mohmmed, S.A., Vianna, M.E., Penny, M.R., *et al.* A novel experimental approach to investigate the effect of different agitation methods using sodium hypochlorite as an irrigant on the rate of bacterial biofilm removal from the wall of a simulated root canal model. Dent Mater 2016; 32:1289–300.

35. Layton, G., Wu, W.I., Selvaganapathy, P.R., *et al*. Fluid dynamics and biofilm removal generated by syringe-delivered and 2 ultrasonic-assisted irrigation methods: a novel experimental approach. J Endod 2015; 41:884–9.

36. Ozdemir, H.O., Buzoglu, H.D., Calt, S., *et al.* Effect of ethylenediaminetetraacetic acid and sodium hypochlorite irrigation on Enterococcus faecalis biofilm colonization in young and old human root canal dentin: in vitro study. J Endod 2010; 36:842–6.

37. Guerreiro-Tanomaru JM, Chávez-Andrade GM, de Faria-Júnior NB, *et al*. Effect of Passive Ultrasonic Irrigation on Enterococcus faecalis from Root Canals: An Ex Vivo Study. Braz Dent J. 2015; 26:342-6.

38. Macedo, R.G., Wesselink, P.R., Zaccheo, F., *et al.* Reaction rate of NaOCl in contact with bovine dentine: effect of activation, exposure time, concentration and pH. Int Endod J 2010; 43:1108–15

39. Ozok, A.R., Wu, M.K., Luppens, S.B., *et al.* Comparison of growth and susceptibility tosodium hypochlorite of mono- and dual-species biofilms of Fusobacterium nucle-atum and Peptostreptococcus (micromonas) micros. J Endod 2007; 33:819–22

40. Madsen, J.S., Røder, H.L., Russel, J., *et al.* Coexistence facilitates interspecific biofilm formation in complex microbial communities. Environ Microbiol 2016; 18:2565–74.

41. Laukkanen, E., Vehkalahti, M.M., Kotiranta, A.K. Impact of type of tooth on outcome of non-surgical root canal treatment. Clin Oral Invest 2019; 23:4011–8.

42. Guida, A. Mechanism of action of sodium hypochlorite and its effects on dentin. Minerva Stomatol 2006; 55:471–82.

43. Finnegan, S, Percival, S.L. EDTA: An antimicrobial and antibiofilm agent for use in wound care. Adv Wound Care (New Rochelle) 2015; 4:415-21.

44. Ng, Y., Gulabivala, K., Mann, V. A prospective study of the factors affecting outcomes of non-surgical root canal treatment: part 1 periapical health. Int Endod J 2011; 44:583−609.

45. Nakonechna, A., Dore, P., Dixon, T. et al. Immediate hypersensitivity to chlorhexidine is increasingly recognised in the United Kingdom. Allergol Immunopathol (Madr) 2014; 42:44–9.

Figure Legend

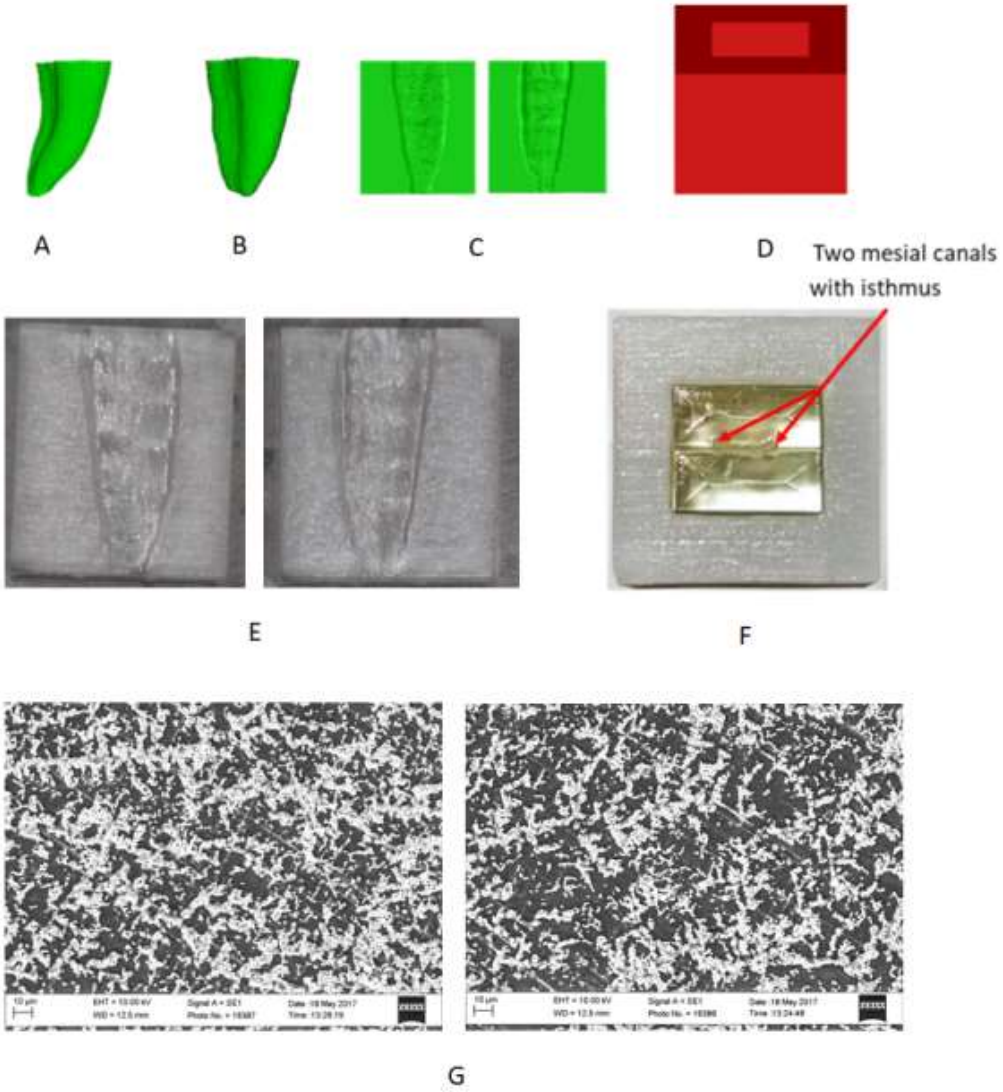
Figure 1 - The stages involved in developing the 3D printed root canal model: (A) 3D image of a segmented mesial root of a mandibular molar, (B) root curvature straightened, (C) the root canal divided into two equal rectangular halves, (D) 3D image of the cubic frame, (E) model parts following 3D printing, (F) an assembled model before root canal preparation, (G) Field emission scanning electron micrographs of *E. faecalis* biofilms grown on Accura resin after seven days incubation (magnification x 1200, scale bars represent 10 μm).

Figure 2: A schematic diagram illustrating the positions of images acquired for the first (red) and second half (green) of the root canal model when disassembled and positioned in the jig. The number of images per canal segment were distributed in relation to the percentage volume of each canal third. The unique patterns of the superimposed copper grid facilitated reproducible positioning of images between samples.

Figure 3 – Volume of residual *E. faecalis* biofilm following root canal preparation with different irrigant solutions. Data presented as medians alongside upper and lower interquartile ranges, minimum and maximum values, and outliers. Statistically significant comparisons ($P < 0.01$; Dunn's test) between groups are presented as superscripts (* vs. control and saline groups).

Figure 4 – Representative confocal laser scanning microscopy images of residual *E. faecalis* biofilm following root canal preparation with different irrigant solutions. The green fluorescence represents the transparent auto-fluorescent resin Accura material (i.e. eradicated biofilm) and the red fluorescence represents the residual TRITC labelled biofilm (scale bars represents 50 μm).

Figures



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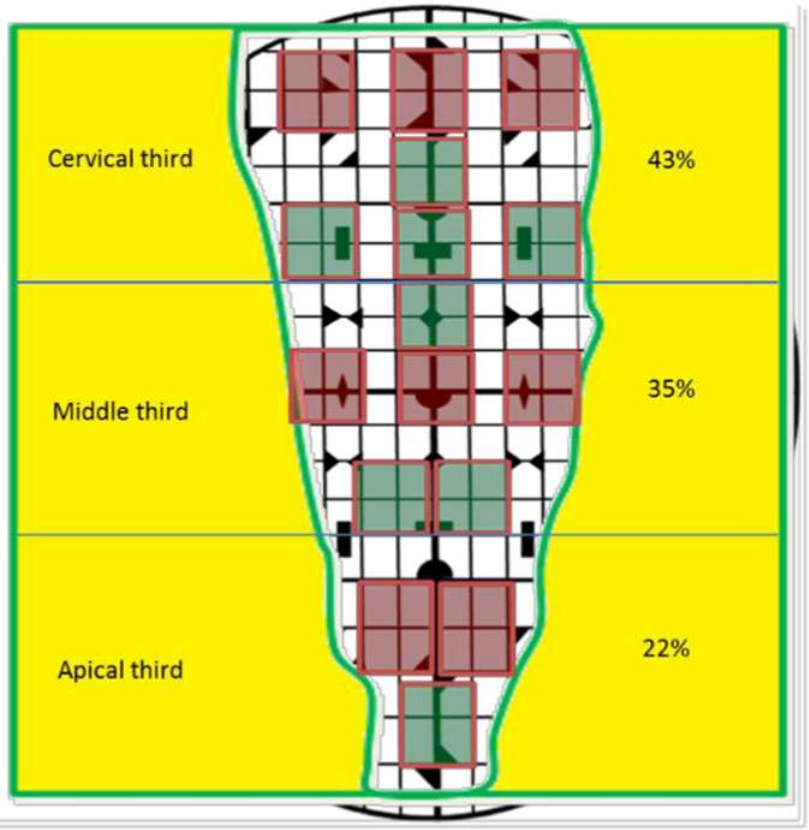

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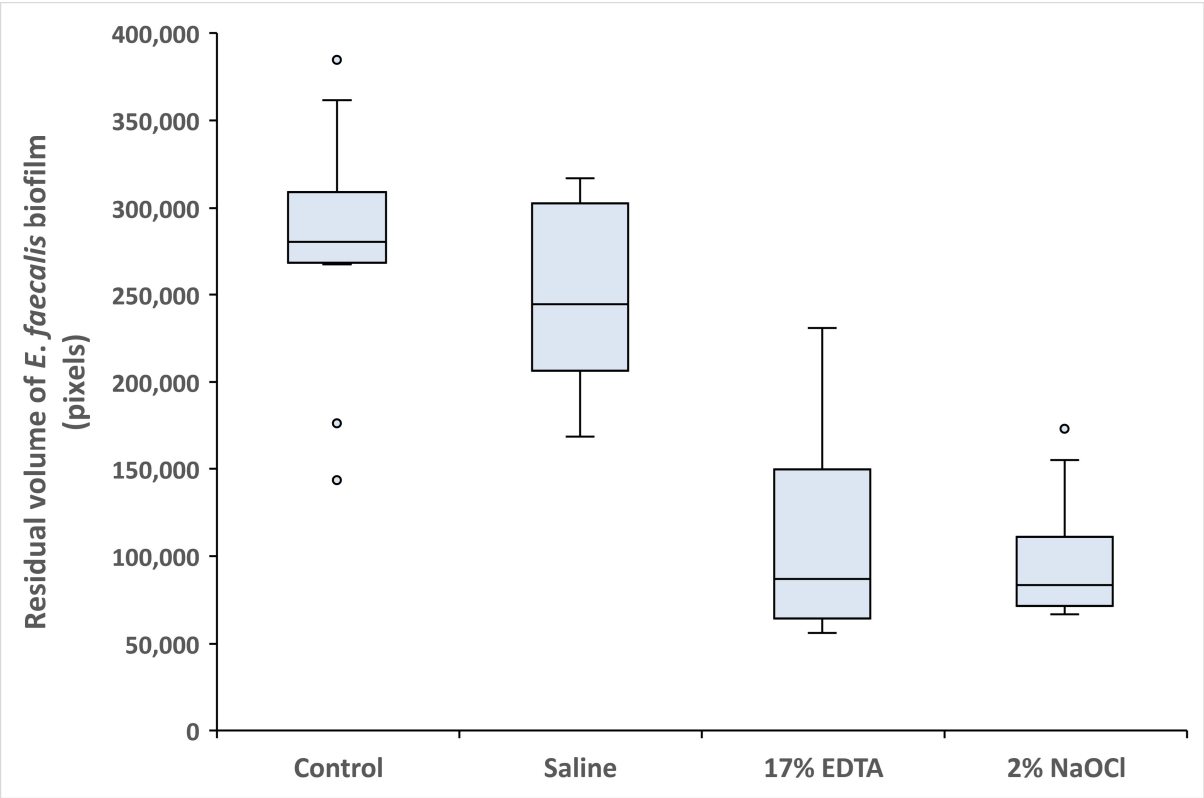
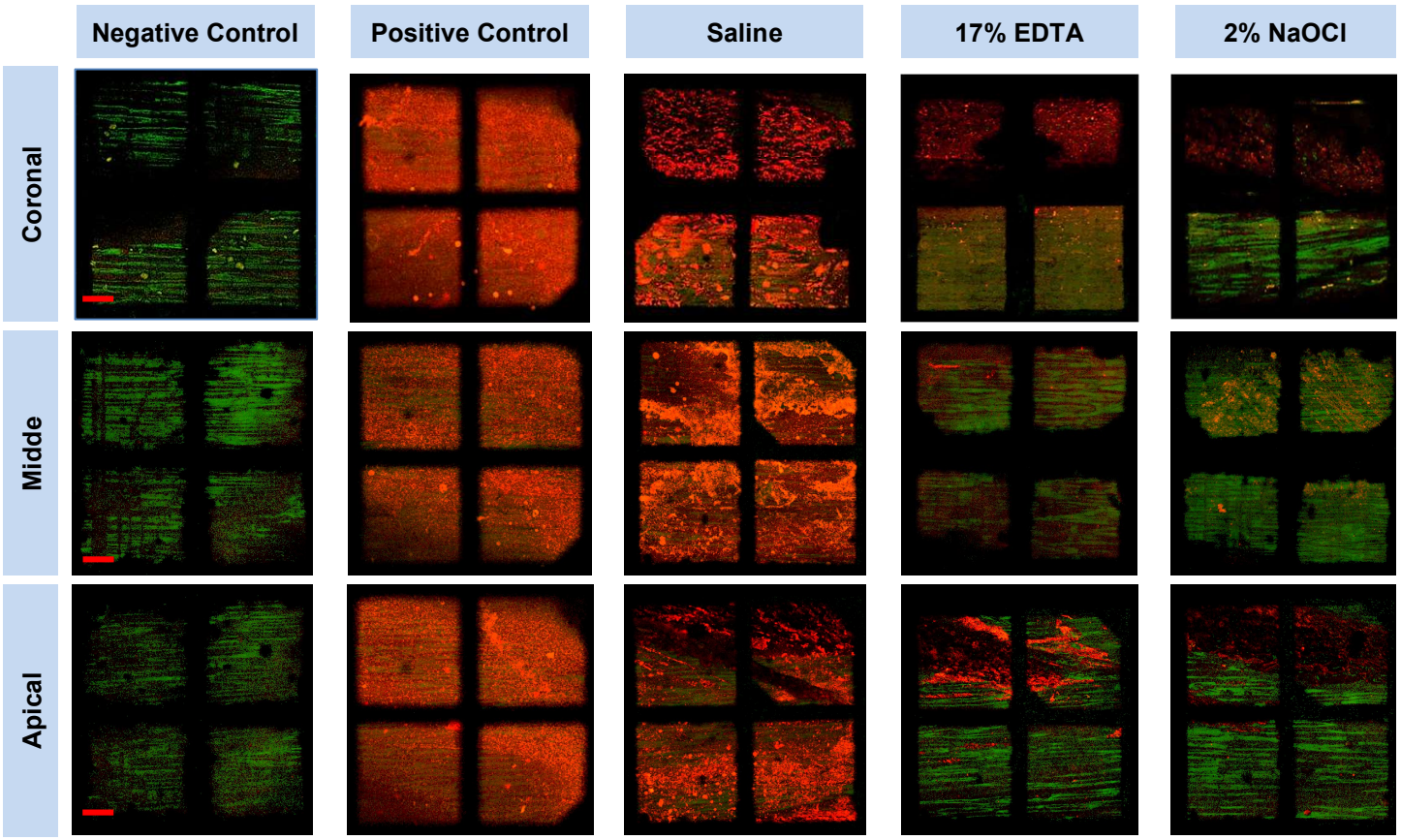


Figure 3 – Volume of residual *E. faecalis* biofilm following root canal preparation with different irrigant solutions. Data presented as box and whisker plots where the central bar represents the median alongside upper and lower interquartile ranges at the edge of boxes, minimum and maximum values for the whiskers, and outliers shown as open circles. Statistically significant comparisons ($P < 0.01$; Dunn's test) between groups are presented as superscripts (* vs. control and saline groups).



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Tables

Table 1 – Laser settings used during confocal laser scanning microscopy imaging.

	Scanning speed	Range (Frame)	Pin hole size	Gain master	Digital offset	Colour	Wavelength (nm)
Track 1	3	2	54.2	732	0	Green (AF)	488
Track 2	3	2	54.2	732	0	Red (TRITC)	555

AF: Alexa fluor 488; TRTIC: Tetramethyl rhodamine-isothiocyanate

Table 2 – Residual volume of *E. faecalis* biofilm following chemomechanical preparation of root canals with different irrigant solutions.

		Volume of residual *E. faecalis* biofilm (pixels)			
Group		**Total**	**Coronal**	**Middle**	**Apical**
Control	**Median ± IQR**	280 109 ± 40 972	111 364 ± 45 868	97 956 ± 48 872	78 273 ± 40 101
	Mean ± SD	277 626 ± 73 590	110 323 ± 23 575	102 076 ± 37 211	65 227 ± 27 220
Saline	**Median ± IQR**	244 572 ± 96 201 [13%]	89 124 ± 43 171 [20%]	84 519 ± 31 410 [14%]	57 625 ± 28 682 [26%]
	Mean ± SD	250 670 ± 53 865 [10%]	93 827 ± 25 333 [15%]	94 203 ± 24 443 [8%]	62 640 ± 17 922 [4%]
17% EDTA*	**Median ± IQR**	87 063 ± 85 255 [69%]	28 856 ± 20 159 [74%]	37 170 ± 36 316 [62%]	26 212 ± 22 165 [67%]
	Mean ± SD	114 412 ± 65 344 [59%]	40 285 ± 28 606 [63%]	47 813 ± 26 382 [53%]	26 315 ± 13 348 [60%]
2% NaOCl*	**Median ± IQR**	83 590 ± 39 465 [70%]	23 686 ± 16 606 [79%]	35 056 ± 17 593 [64%]	29 185 ± 14 131 [63%]
	Mean ± SD	99 194 ± 37 669 [64%]	24 785 ± 9 189 [78%]	42 336 ± 17 207 [59%]	32 073 ± 14 766 [52%]

EDTA: ethylenediaminetetraacetic acid; IQR; interquartile range; NaOCl: sodium hypochlorite; SD: standard deviation; [%]: percentage change in point median or mean value with respect to control group.

* vs. control and saline group ($P < 0.01$) [Kruskal-Wallis & *post-hoc* Dunn's test]