Title: 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

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Abstract

Aim: Root canal irrigation is an important adjunct to control microbial infection. This study aimed primarily to develop a transparent root canal model to study in *situ* *Enterococcus faecalis* biofilm removal rate and remaining attached biofilm using passive or active irrigation solution for 90 seconds. The change in available chlorine and pH of the outflow irrigant were assessed.

Methodology: A total of forty root canal models (n = 10 per group) were manufactured using 3D printing. Each model consisted of two longitudinal halves of an 18 mm length simulated root canal with size 30 and taper 0.06. *E. faecalis* biofilms were grown on the apical 3 mm of the models for 10 days in Brain Heart Infusion broth. Biofilms were stained using crystal violet for visualisation. The model halves were reassembled, attached to an apparatus and observed under a fluorescence microscope. Following 60 seconds of 9 mL of 2.5% NaOCl irrigation using syringe and needle, the irrigant was either left stagnant in the canal or activated using gutta-percha, sonic and ultrasonic methods for 30 seconds. Images were then captured every second using an external camera. The residual biofilm percentages were measured using image analysis software. The data were analysed using Kruskal-Wallis test and generalised linear mixed model.

Results: The highest level of biofilm removal was with ultrasonic agitation (90.13%) followed by sonic (88.72%), gutta-percha (80.59%), and passive irrigation group (control) (43.67%) respectively. All agitation groups reduced the available chlorine and pH of NaOCl more than that in the passive irrigation group.

Conclusions: The 3D printing method provided a novel model to create a root canal simulation for studying and understanding a real-time biofilm removal under

microscopy. Ultrasonic agitation of NaOCl left the least amount of residual biofilm in comparison to sonic and gutta-percha agitation methods.

1. Introduction

Root canal treatment describes the dental procedure used to either prevent apical periodontitis by the treatment of diseased or infected soft tissue contained in the root canal system, or the procedure used to resolve established apical periodontitis [1], which is caused mainly by bacteria [2]. Bacteria adhere to the root canal surfaces and rapidly form biofilms [3]. A biofilm is defined as a community of microorganisms of one or more species embedded in an extracellular polysaccharide matrix that is attached to a solid substrate [4]. Thus, the essential aim of the root canal treatment involves the microbial control of the root canal system through instrumentation and irrigation. Instrumentation aims to give the canal system a shape that permits the delivery of locally used medications (e.g. irrigant), as well as a root canal filling, which helps to entrap the remaining microbiota [5]. Irrigation also aims to lubricate the instruments, and, remove pathogenic microorganisms (microbiota) in the root canal system through the flushing action [6]. However, as the lubricated instrument is rotated along its long axis to sculpt the inner canal surface which it engages with, the most apical part of the canal remains untouched [7]. Thus, the use of a final irrigation regimen, after the completion of a chemo-mechanical canal preparation, with high volumes of various chemically active solutions may contribute to removing residual biofilm in the non-instrumented part of the root canal system [8].

The debridement action of an irrigant within the root canal system may remain elusive when using a needle and syringe alone [9]. Two phenomena are inherent to irrigant penetration and debridement action in the confined space of a closed root canal system. First, the stagnation of the irrigant flow beyond the irrigation needle tip [10].

Second, the gas bubbles or vapour locks effect ahead of the advancing front of the irrigant [11]. These phenomena may limit the delivery of irrigant to the canal terminus [12]. For the above mentioned reasons, attempts to improve the efficacy of irrigant penetration and irrigant mixing within the root canal system are therefore important [13] since they may improve the removal of residual biofilms. Irrigant agitation may be applied to aid the dispersal of the irrigant into the root canal system, especially into the periapical terminus of the canal [14]. Agitation techniques for root canal irrigants include either manual agitation [13, 15-18] or automated agitation [17, 18].

Manual agitation of the irrigant could be achieved by using a file [19] or a taper gutta-percha cone [16], which is achieved by moving the master file or gutta-percha cone up and down in short strokes within an instrumented canal [20]. Automated devices for agitation of the irrigant in the root canal system include ultrasonic and sonic devices [17].

During ultrasonic agitation, a file oscillates at frequencies of 25 to 30 kHz in a pattern of motion consisting of nodes and anti-nodes along its length [21]. During sonic agitation of the irrigant, the file oscillates at frequencies of 1 to 6 kHz [22], and it produces lower shear stresses compared to ultrasonic agitation [23]. The EndoActivator system is a sonic device with polymer tips with a cordless electrically driven hand-piece [24].

The issue of the efficacy of irrigation protocol to remove bacterial biofilm has received considerable critical attention. It has been investigated by the immersing of samples in a static irrigant that neglect irrigant flow within the confinement of a root canal system [25-27]. Other studies used Computational Fluid dynamics models to measure the physical parameters associated with irrigant flow with in the root canal system, that

lack the ability to estimate the chemical action of irrigant as they provide a virtual view of the root canal irrigation [28, 29].

Although the use of extracted teeth might be clinically relevant, it may not be the optimum method as the root canal components (dentine, cementum) are concealed body compartments [30], making them unavailable for direct visualization. In addition, the use of extracted teeth of a different size introduces many variables to the studies [31].

Attempts to mimic the root canal anatomy using gypsum converted to hydroxyapatite [32, 33] have shown promising anatomical features, but such opaque materials did not allow direct visualisation. The use of 3D printing models to study root canal disinfection has been explored in a preliminary study [34], but the tested steriolithography material, Visijet® EX200 Plastic did not allow bacterial colonization and was not transparent. It seems justifiable to develop an *in vitro* model that provides transparency and generation of multiple samples with the same anatomical features to investigate the real-time interaction between the activated irrigant and biofilm removal during the irrigation process

This study aimed primarily to develop and utilise transparent test models to facilitate an investigation into the influence of NaOCl agitation on the removal rate of *Enterococcus faecalis* biofilm subjected to sodium hypochlorite irrigation. A further aim was to compare the residual biofilm and removal rate of biofilm when subjected to passive (stagnant) and active irrigation (2.5% NaOCl). Finally, the outcomes of chemical interaction between a NaOCl irrigant and bacterial biofilm (*in situ*) represented by the available chlorine and pH of outflow irrigant, as outcome measures were assessed.

2. Materials and Methods

2.1. Construction of transparent root canal models and distribution to experimental groups

A solid computer representation of the root canal model was created using AutoCAD® software (Autodesk, Inc., San Rafael, CA, USA). The design of the model consisted of two equal rectangular moulds (18 mm × 6 mm × 1 mm) (Figure 1).

Each mould contained four holes on either side, as well as a longitudinal half canal. When the two moulds were reassembled, a straight simple canal of 18 mm length, apical size 30, and a 0.06 taper was created.

The AutoCAD format of the model was converted into stereo-lithography format (STL format). Forty root canal models were manufactured using PreForm Software 1.9.1 of Formlabs 3D printer (Formlabs Inc., Somerville, MA, USA). The material used to create the model was a clear liquid photopolymer material (AZoNetwork Ltd., Cheshire, UK). It is composed of a mixture of methacrylates and a photo-initiator. The process of fabrication started by conversion of the digital geometric data of the model into a series of layers that were physically constructed layer-by-layer of 25-μm thickness. Each layer was fabricated by exposing the liquid photopolymer material to a laser light source from the printer causing the liquid to cure into a transparent solid state.

The models (n = 40) divided to four groups (n = 10 per group) (Table 1). In the passive irrigation group, the irrigant was delivered using a 10 mL syringe (Plastipak, Franklin Lakes, New Jersey, USA) with a 27-gauge side-cut open-ended needle (Monoject, Sherwood Medical, St. Louis, MO, USA). In the gutta-percha (GP) irrigation group, the irrigant was delivered as in the previous group and agitated using a cone GP (SybronEndo, Buffalo, New York, USA). In the sonic irrigation group, the irrigant was

delivered as in the first group but agitated using the EndoActivator® device (Dentsply Tulsa Dental Specialities, Tulsa, OK, USA). In the ultrasonic irrigation group, the irrigant was delivered as in the first group but agitated using a Satelec® P5 ultra-sonic device (Satelec, Acteon, Equipment, Merignac, France).

2.2. Generation of single species biofilm (*E. faecalis*) on the surface of the canal models

2.2.1. Preparation of microbial strain and determination of the standard inoculum (CFU/mL)

Biofilms were grown from a single bacterial strain (*Enterococcus faecalis;* ATCC 19433). The strain was supplied in the form of frozen stock in a brain-heart infusion broth (BHI) (Sigma-Aldrich, USA) and 30% glycerol stored at -70 °C. The strain was thawed to a temperature of 37 °C for 10 minutes and swirled for 30 seconds [35]. After thawing, 100 µL of the strain were taken and plated onto a BHI agar plate (Sigma-Aldrich, St. Louis, Montana, USA) with 5% defibrinated horse blood (E&O Laboratories, Scotland, UK) and incubated at 37 °C in the 5% CO_2 incubator (LEEC, Nottingham, UK) for 24 hours. Bacterial morphology and catalase activity were confirmed prior to the generation of the biofilms. For this, two colonies of the strain were separately removed using a sterile inoculating loop (VWR, Leicester, UK), and catalase testing using 3% H_2O_2 (Sigma-Aldrich Ltd, Dorset, UK) and Gram-staining (BD Ltd., Oxford, UK) were performed.

A standard inoculum was used. For this, six colonies were removed from the agar plate, placed into 20 mL of BHI broth, and incubated at 37 °C in a 5% CO_2 incubator for 24 hours. BHI containing *E. faecalis* was adjusted to 0.5 absorbance at a wavelength of 600 nm using a spectrophotometer (NanoDrop™ Spectrophotometer

ND-100, Wilmington, USA) [36]. Inoculum concentration was confirmed using a total of six ten-fold serial dilutions to determine the colony forming units per millilitre (CFUs/mL) corresponding to 1.1×10^8 CFU/mL.

2.2.2. Sterilisation of the canal models

The model halves were packed individually in packaging bags (Sterrad 100S, ASP®, Irvine, CA, USA) and then sterilised using gas plasma with hydrogen peroxide vapour (Sterrad 100S, ASP®, Irvine, CA, USA) for 50 minutes [37].

2.2.3. Generation and staining of E. faecalis biofilm on the canal surface

One mL of standard *E. faecalis* inoculum was delivered into a sterilised 7 mL plastic bijou bottle (Sarstedt Ltd, Nümbrecht, Germany) that contained a single sterilised half model such that the 3 mm apical portion was immersed. This was achieved using a sterile syringe (BD Plastipak™, Franklin Lakes, NJ, USA) and a 21-gauge needle (BD Microlance™, Franklin Lakes, NJ, USA) to insert the inoculum. The samples were then incubated at 37 °C in the 5% CO_2 incubator for 10 days. Every 2 days, half of the inoculum that surrounded the sample was discarded and replaced with fresh BHI broth [38].

After ten days incubation, all samples with biofilms were removed from the plastic bottle and prepared for staining with a crystal violet dye (CV) [39]. The model halves containing the biofilms were placed onto a slide facing up and rinsed with 1 mL sterile distilled water (Roebuck, London, UK) for 1 minute using a sterile 10 mL syringe (Plastipak, Franklin Lakes, New Jersey, USA) to remove loosely attached cells. Using a micropipette (Alpha Laboratories Ltd, Eastleigh, Winchester, UK), 1 µL of CV stain (Merck, Darmstadt, Germany) was applied in the apical 3 mm of the model and left for 1 minute to allow staining. The stained canals were subsequently washed with 3 mL

of sterile distilled water for 1 minute [39]. Subsequently, the models were re-assembled for the irrigation experiments as described below.

2.3. Re-apposition of the model halves

Before reassembling the two model halves, a polyester seal film of 0.05 mm thickness (UnisealTM, Buckingham. UK) was positioned on the half coated with biofilm. Any part of the film that overhung the canal boundary was removed using a surgical blade (Swann-Morton, Sheffield, UK) without disturbing the biofilm. The two halves of the model were then held in position using four brass bolts (size 16 BA) and nuts (Clerkenwell Screws, London, UK).

2.4. Irrigation experiments

The apical end of each canal was blocked using a sticky wax (Associated Dental Product Ltd, Swindon, UK). Each model was fixed to a plastic microscopic slide (75 × 25 × 1.2 mm) (Fisher scientific Ltd, Rochester, NY, USA) using a custom-fabricated clamp. The model half with the biofilm faced the slide. The microscopic slide was placed on a stage of an inverted fluorescence microscope (Leica, UK). The test irrigant used in experiments was NaOCl (Teepol® bleach, UK).

Concentration of available NaOCl was verified before experiments using iodometric titration (British Pharmacopoeia 1973) and adjusted to 2.5%. A total of 9 mL of irrigant (NaOCl) were delivered using a 10 mL syringe (Plastipak, Franklin Lakes, New Jersey, USA) with a 27-gauge side-cut open-ended needle (Monoject, Sherwood Medical, St. Louis, MO, USA). The needle was inserted 3 mm coronal to the canal terminus. The port opening of the needle always faced the model half containing the biofilm. The syringe was attached to a programmable precision syringe pump (NE-1010) to deliver the irrigant in 60 seconds at a flow rate of 0.15 mL s^{-1}, followed by 30 seconds of

irrigant that was either kept stagnant (passive) in the canal or activated using GP, sonic and ultrasonic methods.

For the GP agitation group, a gutta-percha cone with an apical ISO size 30 and .02 taper was placed 2 mm coronal to the canal terminus which was used to agitate the irrigant in the root canal system with a push-pull amplitude of approximately 3-5 mm at a frequency of 50 strokes per 30 seconds. A new GP cone was used with each canal model.

For the sonic agitation group, the agitation was carried out using an EndoActivator® device by placing the polymer tip of an EndoActivator® device with size 25 and .04 taper at 2 mm from the canal terminus, and then the agitation was continued for 30 seconds with high power-setting (Ruddle 2007). Once again, a new tip was used with each canal model.

For the ultrasonic agitation group, the agitation was carried out by placing a stainless steel instrument size and taper 20/02 (IrriSafe; Satelec Acteon, Merignac, France) of Satelec® P5 Newtron piezon unit at 2 mm from the canal terminus, then the agitation was continued for 30 seconds. The file was energized at power setting 7 as recommended by the manufacturer. A new tip was used with each canal model.

Outflow irrigant was collected in a 15 mL plastic tube (TPP, Schaffhausen, Switzerland) using a vacuum pump (Neuberger, London, UK) (Figure 2). The amount of available chlorine (%) and pH of the outflow NaOCl were measured using iodometric titration (British Pharmacopoeia 1973) and a pH calibration meter (HANNA pH 211, Hanna Instrument, UK) respectively.

2.5. Recording of biofilm removal by the irrigation procedure

Removal of biofilm was recorded using a high-resolution CCD camera (QICAM, Canada). The camera was connected to a 2.5 × lens of a fluorescence microscope (Leica, UK). An N2.1 longpass filter was used during the time-lapse recording of interactions between the irrigant and the biofilm.

2.6. Image analysis

One video per irrigation procedure was obtained and images were captured at each second of footage (90 images). The canal surface coverage of biofilm present after every second of irrigation (0.15 mL) was visualised and quantified using Image-pro Plus 4.5 software (MediaCybernetics®, Silver Springs, New York USA) (Figure 3).

2.7. Data analyses

The residual biofilm (%) at each second of 90 seconds irrigation with passive and active NaOCl irrigation was analysed using line plots. An assumption concerning a normal distribution of data for the residual biofilm was checked using a visual inspection of the box and whisker plots. The data representing the percentages of residual biofilm covering the canal surface area were not normally distributed and therefore the non-parametric Kruskal-Wallis test, followed by Bonferroni *post-hoc* comparisons were performed to compare their distributions in the four experimental groups. The effects of irrigant agitation duration on the percentage of residual biofilm covering the canal surface area were analysed by the type of irrigation (passive or GP, sonic, and ultrasonic active irrigation) using a generalised linear mixed model. The differences in median of chlorine and pH values of the outflow NaOCl of the four groups before and after irrigation were compared using the Kruskal-Wallis test. A significance level of 0.05 was used throughout. The data were analysed by SPSS (BM

Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, New York, IBM Corp).

3. Results

The median values of the residual biofilm (%) covering the canal surface-area against duration of irrigation(s), stratified by the type of irrigation are presented in Figure 4.

The data showed that the greatest removal was associated with the ultrasonic group (90.13%) followed by the sonic (88.72%), the GP (80.59%), and the passive irrigation group (control) (43.67%) respectively.

What is important in the box and whisker plots of the data (median values of residual biofilm covering the canal surface) (Figure 5) is that the data do not satisfy the assumptions of normal distribution.

The results of the Kruskal-Wallis test (Table 2) revealed that there was a statistically significant difference between the residual biofilm on the canal surface area in the ultrasonic irrigation group and both GP ($p = 0.002$) and passive irrigation groups ($p = 0.001$). In comparison, the difference was not statistically significant between the residual biofilm in the ultrasonic and the sonic irrigation groups ($p = 0.78$). Interestingly, the difference was not statistically significant between the residual biofilm in the GP group and both sonic ($p = 0.21$) and syringe irrigation groups ($p = 0.34$). Finally, the difference was statistically significant between the residual biofilm of the sonic group and the passive syringe group ($p = 0.001$).

The data of the generalized linear mixed model analysis (Table 3) revealed that the interval of irrigant agitation interestingly had an influence on the removal amount of biofilm. During the 30 seconds of irrigant agitation, the amount of biofilm removal using passive syringe irrigation was significantly less [5.35% s^{-1} (±1.1), 6.66% s^{-1} (±1.1),

7.52% s^{-1} (±1.1)] than the amount of biofilm removal using active GP, sonic, and ultrasonic irrigation respectively (p = 0.001). For the active irrigation groups, the amount of biofilm removal using ultrasonic agitation was significantly more [2.18% s^{-1} (±1.1)], than the amount of biofilm removal using the GP agitation (p = 0.047), whilst it was interestingly not significantly more [0.86% s^{-1} (±1.1)] than the sonic agitation (p = 0.43). On the other hand, the amount of biofilm removal using sonic irrigation was not significantly more [1.32% s^{-1} (±1.1)] than the amount of biofilm removal using GP irrigation (p = 0.23).

The results of the Kruskal-Wallis tests to explore the effect of biofilm NaOCl irrigant interaction on the available chlorine and pH of NaOCl are presented in Table 4. It is noteworthy that the left half of the table revealed that there was a relation between available chlorine reduction and irrigant agitation because there was a statistically significant difference between the available chlorine in the passive group and both the ultrasonic group (p = 0.001) and sonic group (p = 0.016). There was not a statistically significant difference between available chlorine in the passive group and GP group (p = 0.127).

Amongst the active irrigation groups, it was revealed that there was a statistically significant difference between the level of available chlorine in the ultrasonic group and the GP group (p = 0.006). Furthermore, there was not a statistically significant difference between available chlorine in both the sonic and GP groups (p = 1) and ultrasonic agitation group (p = 0.057).

The data from the right half of the table indicated that there was a strong evidence of pH reduction when NaOCl was activated, as a statistically significant difference between the pH in passive irrigation group and active irrigation groups was shown (ultrasonic; p = 0.001, sonic; p = 0.021, and GP; p = 0.029).

Comparing the active irrigation groups, there was a statistically significant difference between the pH in ultrasonic group and both sonic ($p = 0.029$), and GP groups ($p = 0.021$). Furthermore, there was not a statistically marked difference between the pH in the sonic and GP groups ($p = 1$).

4. Discussion

The key attribute of this study was to investigate the rate of *E. faecalis* biofilm removal using passive or activated 2.5% NaOCl irrigant delivered into a simulated root canal model which was made from transparent materials and created using 3D printing. The experiments were successful in testing the aims, which were to compare the efficacy of passive irrigation and three different irrigation protocols (GP, sonic, and ultrasonic) in the removal of biofilm from the root canal system. In addition, the outcomes of biofilm-irrigant interaction were also investigated. A NaOCl irrigant (2.5 %) was selected for the irrigation procedure since it constitutes the most frequently used irrigant in root canal treatment [40, 41] and has been proven to be effective against a broad spectrum of bacteria [42].

For the objective of this study, the model proposed herein was made from transparent resin materials (acrylic), and created using 3D printing. The selection of this material was due to its excellent optical transparency, which enabled direct and real-time imaging of biofilm removal by antibacterial agents (e.g. NaOCl), as well as the 3D printing technique which provided an accurate representation of the simple root canal anatomy and allowed numerous variables to be tested [43]. Moreover, the use of resin canal models is recommended from other studies [21, 44]. However, the surface and composition of such a synthetic material (resin) differs from that of the natural surface found in the root canal dentine. The porous nature of dentine (due to dentinal tubules) may act differently from a solid resin material. An *in vitro* study that uses *ex vivo*

(extracted teeth) to test the antimicrobial action of irrigants would be more relevant in terms of reflecting the clinical situation. Yet tooth structures are opaque, which makes them unsuitable for the direct visualisation needed to assess the antibacterial action of an irrigant during the process of irrigation within the root canal. Researchers have thus had to resort to using indirect methods of analysis in order to gain an insight into the efficacy of irrigation (for example, splitting the tooth). Accordingly, it is not possible to assess the rate of biofilm removal by an irrigant during the irrigation regimen in *ex vivo* models.

The model proposed herein relied upon an adequate seal between the two model halves in order to minimize leakage of the irrigant during the irrigation procedure. This was achieved by using a seal film between the two halves as recommended in another study that assesses the efficacy of the antimicrobial agent in flow chambers [45]. Indeed, a pilot experiment to compare between models (n = 3) with the seal film and other models (n = 3) without the film showed that the leakage was minimal in models with film. For this, the placement of the seal film between the model halves, and the holding of this construction in position using nuts and bolts, is important in that it provides a seal and thus facilitates the irrigation and minimizes irrigant leakage from the canal model. However, the model used in this study does not account for root canal complexities such as the lateral canal, isthmus area and accessory canals.

In the present study, each model was designed with an apical size 30 and taper 0.06 because this is the apical size most commonly used in clinical practice [46]. The selection of a side cut 27-gauge endodontic needle was made for this study, again due to common use in clinical practice but also to avoid the greater pressure required to deliver the irrigant at a rate of 9 mL per minute, as is the case when using a flat

ended 30-gauge needle. A total of 9 mL per minute (0.15 mL s^{-1}) irrigant was used since this falls within the range of 0.01–1.01 mL s^{-1} reported in other studies [47].

The Gram-positive facultative *E. faecalis* type strain was selected to generate the biofilms as these species have been associated with secondary root canal infections [48]. It has been reported that *E. faecalis* exhibits an inherent resistance to antimicrobial agents, as well as possessing the capacity to adapt to changes in environmental conditions [49]. Furthermore, *E. faecalis* is able to develop a biofilm under different growth conditions, including aerobic, anaerobic, nutrient-rich and nutrient-deprived environments [50]. Moreover, this species has been used to evaluate the efficacy of irrigation solutions [48, 51]. However, single species biofilm may be considered a limitation of the present study and future investigations using multi-species biofilms, including Gram-negative species may be valuable to be explored in the future.

The initial inoculum concentration was in accordance with other studies [51, 52], which was around 10^8 CFU/mL. In addition, this concentration represents cell concentrations (of total bacteria) found in infected root canal systems determined by culture [53].

A total of ten days of *E. faecalis* biofilm growth was chosen for this study as it has been shown to produce standardised biofilm models for testing the efficacy of antimicrobial agents [51], which has been confirmed in preliminary studies using microscopy (data not shown) demonstrating the evidence of microbial colonisation.

In this study, a fluorescence microscope was used to record biofilm removal by NaOCl. Fluorescence microscopy has been used as a technique by which to assess biofilms [54-56] when stained with a fluorescent marker. One major advantage of this technique is that it allows a direct visualisation of the biofilm removal without fixation, dehydration or the disturbing of biofilm structures during the irrigation regimen.

Nevertheless, there were some limitations associated with the microscopy for the test models. High-resolution imaging proved difficult because of the steeply curved sides of the canal walls, causing poor light transmission/reflection from these areas. Also, it was not possible to observe the degradation of single bacterial cells in the biofilm since a low magnification 2.5-x objective lens was used in this study to capture the apical 3mm of the canal. Finally, crystal violet stain was added to the biofilms as a way of making them visible and slightly fluorescent. To examine the effect that crystal violet may have on the oxidative capacity of NaOCl, preliminary experiments (based on iodometric titration and the pH calibration of NaOCl) were performed. Crystal violet, which exhibits slight fluorescence properties [57], proved neutral towards NaOCl and did not exhibit any effect on the oxidative capacity of NaOCl, as represented by the available chlorine and pH. This may be due to the fact that, firstly, the stain was alkaline and secondly, the concentration was not high enough to cause an effect on NaOCl.

Image analysis software (Image-Pro Plus) has been used to analyse the images from fluorescence microscopy. This software has also been adopted in other studies in order to analyse images [16, 58]. One criticism that can be made in relation to all image-analysis techniques is that the areas measured are, to some extent, subjectively chosen by the examiner. In order to reduce this limitation, inter- and intra-examiner assessments were carried out. A semi-automatic approach to measuring the biofilms was applied and imaging software was used to manually draw the template of the root canal outline and quantify the biofilm. The same template was used to obtain and calculate the biofilm area after washing, without further interference of the operator.

Although the method of quantifying the biofilm from the root canal wall showed marked results, a single assessor performed the measurements and therefore there was a possibility of bias. In order to reduce this, a methodology was agreed using a standard protocol for outlining the root canal and for setting the threshold of the stain to be measured. The principal assessor and another observer who was experienced in using image analysis software measured 10% of the images and this was repeated until sufficient inter-observer agreement was achieved [59]. Another attempt to reduce bias was attained by assessment of the intra-observer reliability. This was performed by recording ten replicate measurements of the residual biofilm in each group at specific intervals (every 10 seconds of the 90 second irrigation) and comparing the values taken. This comparison showed good agreement between the measurements [60]. This semi-automatic method provided operator-independent quantitative results. The amount of residual bacteria in the canal models in active irrigation groups (GP, sonic, and ultrasonic) decreased from the passive irrigation group (control). This could be explained by the fact that the NaOCl agitation may refresh the consumed irrigant within the canal [61], which increased the biofilm degradation by the chemical action of new NaOCl [19]. Furthermore, irrigant agitation may have intensified the fluid dynamics and increased wall shear stresses. Nevertheless, the difference in efficacy of the agitation techniques to agitate NaOCl inside the root canal may be related to space restrictions of the root canal that interfere with the agitation method [43]. The same above mentioned reasons may once again be responsible for the important finding that the reduction in the total remaining amount of available chlorine and pH of NaOCl was obvious in agitation groups in comparison to the passive syringe group.

The difference between GP, sonic, and ultrasonic agitation may be attributed to the fact that the manual push–pull motion of gutta-percha point generated frequency is

less efficient than the automated methods [14]. The difference between EndoActivator sonic and ultrasonic agitation can be due to the driving frequency of ultrasonic device being higher than that of the sonic device. A higher frequency results in a higher flow velocity of NaOCl irrigant [62]. This may be the result of more biofilm removal by ultrasonic than EndoActivator irrigation.

The results of this study are broadly consistent with the earlier study of Halford et al. (2012) [63], who showed that the ultrasonic agitation of NaOCl effectively reduces viable *E. faecalis* bacteria in root canal models when compared to syringe and sonic agitation. In contrast, the reduced efficacy of manual agitation (e.g. GP) compared to sonic and ultrasonic agitation, presented in this study, is not consistent with the results of the Townsend and Maki study (2009) [64], who suggested that manual agitation, sonic, and ultrasonic were similar in their ability to remove bacteria from the canal walls. These differences can be explained in part by the differences in canal preparation as Townsend and Maki used a size and taper 40/0.10 and 35/0.08; size 30 and taper 0.06 was used herein. For that, the larger apical sizes and taper may enhance irrigant exchange and the hydrodynamic forces generated by manual agitation.

Based on the findings, the efficacy of passive irrigation using 2.5% NaOCl was less than that achieved by active irrigation protocols using 2.5% NaOCl. Manual agitation (GP agitation) was associated with greater residual biofilm than the automated agitation (sonic & ultrasonic). Hence, it could conceivably be hypothesised that the automated agitation provides optimum efficacy of 2.5% NaOCl within the root canal system, as the difference between the automated agitations was not statistically significant ($p > 0.05$).

Despite these promising results, there are still many unanswered questions about the efficacy of activated NaOCl on multispecies biofilms in simple and complex root canal system. Further studies, which take these variables into account, will need to be undertaken.

5. Conclusion

Within the limitations of the current study, the bacterial biofilm models used herein provide a simple method by which to visualise and examine the efficacy of root canal irrigants during irrigation within root canal systems. This study shows that the agitation of NaOCl irrigant is essential for increasing the efficacy of 2.5% NaOCl to remove biofilm. In addition, the use of automated agitation (sonic & ultrasonic) is recommended when compared to manual GP agitation in the removal of biofilm in the main root canal as a final irrigation protocol.

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7. References

[1]. European Society of E. Quality guidelines for endodontic treatment: consensus report of the European Society of Endodontology. International Endodontic Journal. 2006;39(12):921-30.

[2]. Kakehashi S, Stanley H, Fitzgerald R. The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. Oral Surgery, Oral Medicine, Oral Pathology. 1965;20(3):340-9.

[3]. Costerton JW, Stewart PS, Greenberg E. Bacterial biofilms: a common cause of persistent infections. Science. 1999;284(5418):1318-22.

[4]. Wilson M. Susceptibility of oral bacterial biofilms to antimicrobial agents. Journal of Medical Microbiology. 1996;44(2):79-87.

[5]. Nair P, Henry S, Cano V, Vera J. Microbial status of apical root canal system of human mandibular first molars with primary apical periodontitis after “one-visit” endodontic treatment. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2005;99(2):231-52.

[6]. Baker NA, Eleazer PD, Averbach RE, Seltzer S. Scanning electron microscopic study of the efficacy of various irrigating solutions. Journal of Endodontics. 1975;1(4):127-35.

[7]. Peters O, Peters C, Schonenberger K, Barbakow F. ProTaper rotary root canal preparation: effects of canal anatomy on final shape analysed by micro CT. International Endodontic Journal. 2003;36(2).

[8]. Ballal NV, Kandian S, Mala K, Bhat KS, Acharya S. Comparison of the efficacy of maleic acid and ethylenediaminetetraacetic acid in smear layer removal from instrumented human root canal: a scanning electron microscopic study. Journal of Endodontics. 2009;35(11):1573-6.

[9]. Jiang L-M, Lak B, Eijsvogels LM, Wesselink P, van der Sluis LW. Comparison of the cleaning efficacy of different final irrigation techniques. Journal of Endodontics. 2012;38(6):838-41.

[10]. Ram Z. Effectiveness of root canal irrigation. Oral Surgery, Oral Medicine, Oral Pathology. 1977;44(2):306-12.

[11]. Tay FR, Gu L-s, Schoeffel GJ, Wimmer C, Susin L, Zhang K, et al. Effect of vapor lock on root canal debridement by using a side-vented needle for positive-pressure irrigant delivery. Journal of Endodontics. 2010;36(4):745-50.

[12]. Gulabivala K, Ng Y, Gilbertson M, Eames I. The fluid mechanics of root canal irrigation. Physiological Measurement. 2010;31(12):R49.

[13]. Druttman A, Stock C. An in vitro comparison of ultrasonic and conventional methods of irrigant replacement. International Endodontic Journal. 1989;22(4):174-8.

[14]. Macedo R, Robinson J, Verhaagen B, Walmsley A, Versluis M, Cooper P, et al. A novel methodology providing insights into removal of biofilm-mimicking hydrogel from lateral morphological

features of the root canal during irrigation procedures. International Endodontic Journal. 2014;47(11):1040-51.

[15]. Cecic PA, Peters DD, Grower MF. The comparative efficiency of final endodontic cleansing procedures in removing a radioactive albumin from root canal systems. Oral Surgery, Oral Medicine, Oral Pathology. 1984;58(3):336-42.

[16]. Huang TY, Gulabivala K, Ng YL. A bio-molecular film ex-vivo model to evaluate the influence of canal dimensions and irrigation variables on the efficacy of irrigation. International Endodontic Journal. 2008;41(1):60-71.

[17]. Sabins RA, Johnson JD, Hellstein JW. A comparison of the cleaning efficacy of short-term sonic and ultrasonic passive irrigation after hand instrumentation in molar root canals. Journal of Endodontics. 2003;29(10):674-8.

[18]. Cunningham WT, Martin H, Forrest WR. Evaluation of root canal debridement by the endosonic ultrasonic synergistic system. Oral Surgery, Oral Medicine, Oral Pathology. 1982;53(4):401-4.

[19]. Bronnec F, Bouillaguet S, Machtou P. Ex vivo assessment of irrigant penetration and renewal during the final irrigation regimen. International Endodontic Journal. 2010;43(8):663-72.

[20]. Gu L-s, Kim JR, Ling J, Choi KK, Pashley DH, Tay FR. Review of contemporary irrigant agitation techniques and devices. Journal of Endodontics. 2009;35(6):791-804.

[21]. Weller RN, Brady JM, Bernier WE. Efficacy of ultrasonic cleaning. Journal of Endodontics. 1980;6(9):740-3.

[22]. Tronstad L, Barnett F, Schwartzben L, Frasca P. Effectiveness and safety of a sonic vibratory endodontic instrument. Dental Traumatology. 1985;1(2):69-76.

[23]. Ahmad M, Pitt Ford TR, Crum LA. Ultrasonic debridement of root canals: acoustic streaming and its possible role. Journal of Endodontics. 1987;13(10):490-9.

[24]. Ruddle CJ. Hydrodynamic disinfection. International Dentistry Sa. 2007;11(4):6-18.

[25]. Spratt D, Pratten J, Wilson M, Gulabivala K. An in vitro evaluation of the antimicrobial efficacy of irrigants on biofilms of root canal isolates. International Endodontic Journal. 2001;34(4):300-7.

[26]. Clegg M, Vertucci F, Walker C, Belanger M, Britto L. The effect of exposure to irrigant solutions on apical dentin biofilms in vitro. Journal of Endodontics. 2006;32(5):434-7.

[27]. Bryce G, O'Donnell D, Ready D, Ng Y-l, Pratten J, Gulabivala K. Contemporary root canal irrigants are able to disrupt and eradicate single-and dual-species biofilms. Journal of Endodontics. 2009;35(9):1243-8.

[28]. Boutsioukis C, Gogos C, Verhaagen B, Versluis M, Kastrinakis E, Van der Sluis LW. The effect of apical preparation size on irrigant flow in root canals evaluated using an unsteady Computational Fluid Dynamics model. International Endodontic Journal. 2010;43(10):874-81.

[29]. Shen Y, Gao Y, Qian W, Ruse ND, Zhou X, Wu H, et al. Three-dimensional numeric simulation of root canal irrigant flow with different irrigation needles. Journal of Endodontics. 2010;36(5):884-9.

[30]. Cate-Ten A. Oral histology: development, structure, and function. Saint Louis, Mosby; 1998.

[31]. Khalilak Z, Fallahdoost A, Dadresanfar B, Rezvani G. Comparison of extracted teeth and simulated resin blocks on apical canal transportation. Iranian endodontic journal. 2008;3(4):109.

[32]. Turner D, NM, JvdM, KG, Y-LN. Investigation of artificial tooth models for assessing the efficacy of root canal irrigation protocols using sodium hypochlorite. Abstract: International Endodontic Journal; 2011. p. 1189.

[33]. Papageorgopoulou L. Pilot studies to develop an artificial tooth model with standardized apical anatomies for the evaluation of the efficacy of root canal irrigation London, UK: Eastman Dental Institute, UCL; 2013.

[34]. Thakrar M. Development of an in vitro artificial infection model with standardised apical root canal anatomy. London, UK: Eastman Dental Institute, UCL; 2014.

[35]. Siqueira Jr JF, Rôças IN, Lopes HP. Patterns of microbial colonization in primary root canal infections. Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology. 2002;93(2):174-8.

[36]. Al Shahrani M, DiVito E, Hughes CV, Nathanson D, Huang GT-J. Enhanced Removal of Enterococcus faecalis Biofilms in the Root Canal Using Sodium Hypochlorite Plus Photon-Induced Photoacoustic Streaming: An In Vitro Study. Photomedicine and Laser Surgery. 2014;32(5):260-6.

[37]. Precautions MSB, Flush AC. Guideline for Disinfection and Sterilization in Healthcare Facilities, 2008. Online at: http://www.cdc.gov/hicpac/pdf/guidelines/Disinfection_Nov_2008.pdf Accessed July 8, 2015.

[38]. De-Deus G, Brandão M, Fidel R, Fidel S. The sealing ability of GuttaFlow™ in oval-shaped canals: an ex vivo study using a polymicrobial leakage model. International Endodontic Journal. 2007;40(10):794-9.

[39]. Izano EA, Wang H, Ragunath C, Ramasubbu N, Kaplan JB. Detachment and killing of Aggregatibacter actinomycetemcomitans biofilms by dispersin B and SDS. Journal of Dental Research. 2007;86(7):618-22.

[40]. Byström A, Sunvqvist G. The antibacterial action of sodium hypochlorite and EDTA in 60 cases of endodontic therapy. International Endodontic Journal. 1985;18(1):35-40.

[41]. Baumgartner JC, Cuenin PR. Efficacy of several concentrations of sodium hypochlorite for root canal irrigation. Journal of Endodontics. 1992;18(12):605-12.

[42]. Siqueira J, Machado A, Silveira R, Lopes H, Uzeda Md. Evaluation of the effectiveness of sodium hypochlorite used with three irrigation methods in the elimination of Enterococcus faecalis from the root canal, in vitro. International Endodontic Journal. 1997;30(4):279-82.

[43]. Layton G, Wu W-I, Selvaganapathy PR, Friedman S, Kishen A. Fluid Dynamics and Biofilm Removal Generated by Syringe-delivered and 2 Ultrasonic-assisted Irrigation Methods: A Novel Experimental Approach. Journal of Endodontics. 2015.

[44]. Lee SJ, Wu MK, Wesselink P. The efficacy of ultrasonic irrigation to remove artificially placed dentine debris from different-sized simulated plastic root canals. International Endodontic Journal. 2004;37(9):607-12.

[45]. Chin MY, Busscher HJ, Evans R, Noar J, Pratten J. Early biofilm formation and the effects of antimicrobial agents on orthodontic bonding materials in a parallel plate flow chamber. The European Journal of Orthodontics. 2006;28(1):1-7.

[46]. Ng YL, Mann V, Gulabivala K. A prospective study of the factors affecting outcomes of nonsurgical root canal treatment: part 1: periapical health. International Endodontic Journal. 2011;44(7):583-609.

[47]. Boutsioukis C, Lambrianidis T, Kastrinakis E, Bekiaroglou P. Measurement of pressure and flow rates during irrigation of a root canal ex vivo with three endodontic needles. International Endodontic Journal. 2007;40(7):504-13.

[48]. Endo MS, Signoretti FGC, Marinho ACS, Martinho FC, de Almeida Gomes BPF. PCR identification of endodontic pathogens and DNA quantification in samples from teeth with posttreatment apical periodontitis. Clinical and Laboratorial Research in Dentistry. 2014;20(4):197-208.

[49]. Laplace J-M, Thuault M, Hartke A, Boutibonnes P, Auffray Y. Sodium hypochlorite stress in Enterococcus faecalis: influence of antecedent growth conditions and induced proteins. Current Microbiology. 1997;34(5):284-9.

[50]. George S, Kishen A, Song P. The Role of Environmental Changes on Monospecies Biofilm Formation on Root Canal Wall by Enterococcus faecalis. Journal of Endodontics. 2005;31(12):867-72.

[51]. Sena N, Gomes B, Vianna M, Berber V, Zaia A, Ferraz C, et al. In vitro antimicrobial activity of sodium hypochlorite and chlorhexidine against selected single-species biofilms. International Endodontic Journal. 2006;39(11):878-85.

[52]. Vianna ME, Horz HP, Gomes BPFA, Conrads G. In vivo evaluation of microbial reduction after chemo-mechanical preparation of human root canals containing necrotic pulp tissue. International Endodontic Journal. 2006;39(6):484-92.

[53]. Zavistoski J, Dzink J, Onderdonk A, Bartlett J. Quantitative bacteriology of endodontic infections. Oral Surgery, Oral Medicine, Oral Pathology. 1980;49(2):171-4.

[54]. Briones AM, Okabe S, Umemiya Y, Ramsing N-B, Reichardt W, Okuyama H. Influence of different cultivars on populations of ammonia-oxidizing bacteria in the root environment of rice. Applied and Environmental Microbiology. 2002;68(6):3067-75.

[55]. Roth KA, Friedman S, Levesque CM, Basrani BR, Finer Y. Microbial Biofilm Proliferation within Sealer-Root Dentin Interfaces Is Affected by Sealer Type and Aging Period. Journal of Endodontics. 2012;38(9):1253-6.

[56]. Tawakoli PN, Al-Ahmad A, Hoth-Hannig W, Hannig M, Hannig C. Comparison of different live/dead stainings for detection and quantification of adherent microorganisms in the initial oral biofilm. Clinical Oral Investigations. 2013;17(3):841-50.

[57]. Kleinman SL, Ringe E, Valley N, Wustholz KL, Phillips E, Scheidt KA, et al. Single-molecule surface-enhanced Raman spectroscopy of crystal violet isotopologues: theory and experiment. Journal of the American Chemical Society. 2011;133(11):4115-22.

[58]. McGill S, Gulabivala K, Mordan N, Ng YL. The efficacy of dynamic irrigation using a commercially available system (RinsEndo®) determined by removal of a collagen 'bio-molecular film'from an ex vivo model. International Endodontic Journal. 2008;41(7):602-8.

[59]. Hartmann DP, Wood DD. Observational methods. International handbook of behavior modification and therapy: Springer; 1990. p. 107-38.

[60]. Koppe T, Meyer G, Alt K. Comparative dental morphology. Preface. Frontiers of Oral Biology. 2009;13:XI.

[61]. Moorer W, Wesselink P. Factors promoting the tissue dissolving capability of sodium hypochlorite. International Endodontic Journal. 1982;15(4):187-96.

[62]. Ahmad M, Pitt Ford T, Crum L, Walton A. Ultrasonic debridement of root canals: acoustic cavitation and its relevance. Journal of Endodontics. 1988;14(10):486-93.

[63]. Halford A, Ohl C-D, Azarpazhooh A, Basrani B, Friedman S, Kishen A. Synergistic Effect of Microbubble Emulsion and Sonic or Ultrasonic Agitation on Endodontic Biofilm in Vitro. Journal of Endodontics. 2012;38(11):1530-4.

[64]. Townsend C, Maki J. An In Vitro Comparison of New Irrigation and Agitation Techniques to Ultrasonic Agitation in Removing Bacteria From a Simulated Root Canal. Journal of Endodontics. 2009;35(7):1040-3.

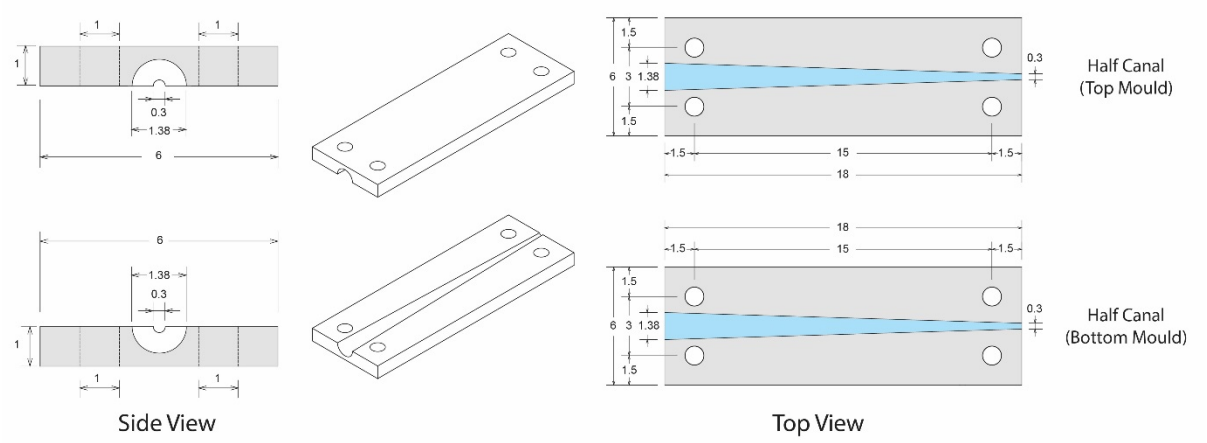

Figure 1: Image illustrates the design of the root canal model. The top view shows half of a simulated canal of 18 mm; the left side is the coronal portion of the simulated canal with 1.38 mm diameter and the right side represents the apical portion with 0.3 mm diameter. The lower view shows the other half and when the two halves are reassembled, a straight simple canal of 18 mm length, apical size 30, and a 0.06 taper is created.

Table 1: Allocation of the model samples

Group	Type of irrigation
(passive irrigation) (n = 10)	Syringe and needle + passive irrigant stagnation
(GP irrigation) (n = 10)	Syringe and needle + GP irrigation
(sonic irrigation) (n = 10)	Syringe and needle + Sonic irrigation
(ultrasonic irrigation) (n = 10)	Syringe and needle + Ultrasonic irrigation

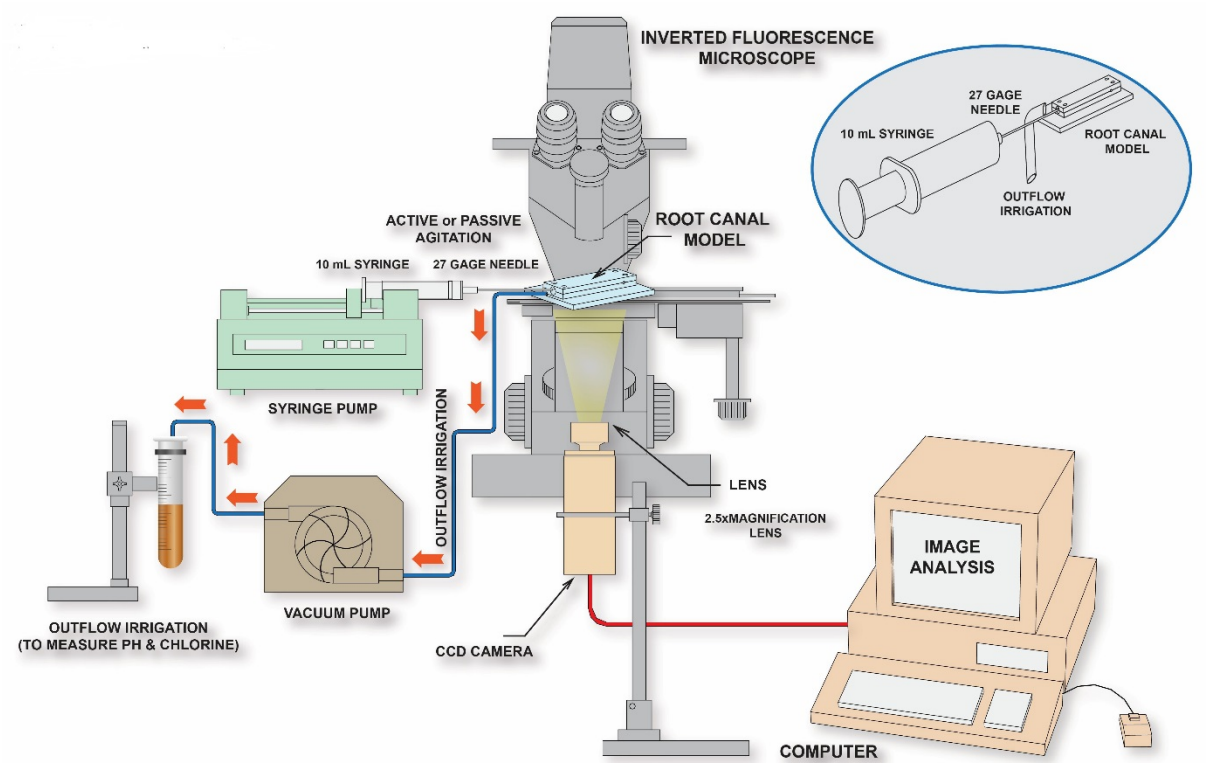

Figure 2: Sketch illustrating the set-up of equipment for recording of the biofilm (biofilm was generated on the apical portion (3 mm) of the canal model) removal by active or passive NaOCl irrigation protocol using a camera connected to a 2.5 × lens of a fluorescence microscope an inverted fluorescence microscope. The irrigant were delivered using a syringe with a 27-gauge side-cut open-ended needle, which was attached to a programmable precision syringe pump. The residual biofilm was quantified using computer software (Image-pro Plus 4.5). Outflow irrigant was collected in a plastic tube using a vacuum pump. The amount of available chlorine (%) and pH were measured using iodometric titration and a pH calibration meter respectively.

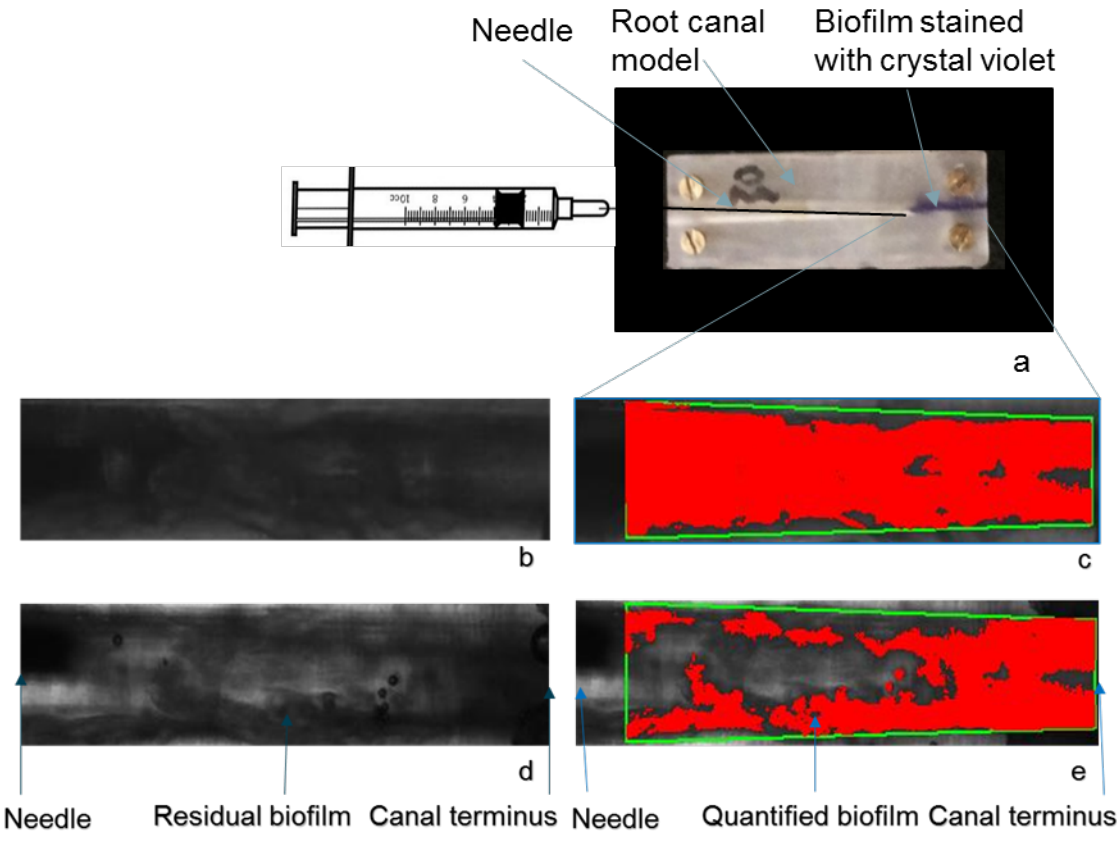

Figure 3: Images of stained *E. faecalis* biofilm on the canal surface of the root canal model (a); before (b) and after (c) 90 seconds of irrigation protocol using 2.5% NaOCl. Image-pro plus 4.5 software depicts the respective stained biofilm in red before (d) and after (d) irrigation.

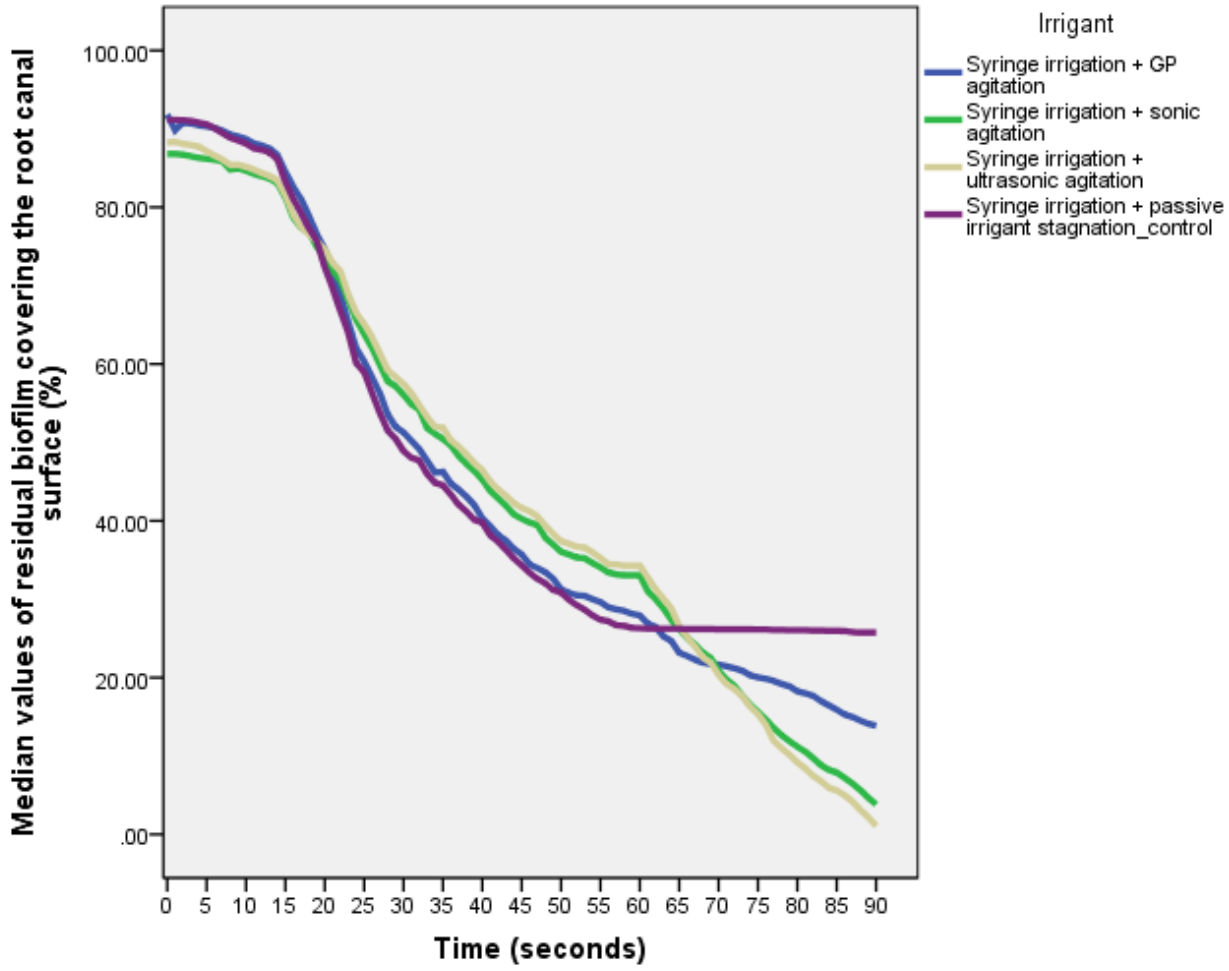

Figure 4: Median values of the residual biofilm (%) covering the root canal surface-area over duration (s) of irrigation for each group, stratified by the type of irrigant agitation (n = 10 per group).

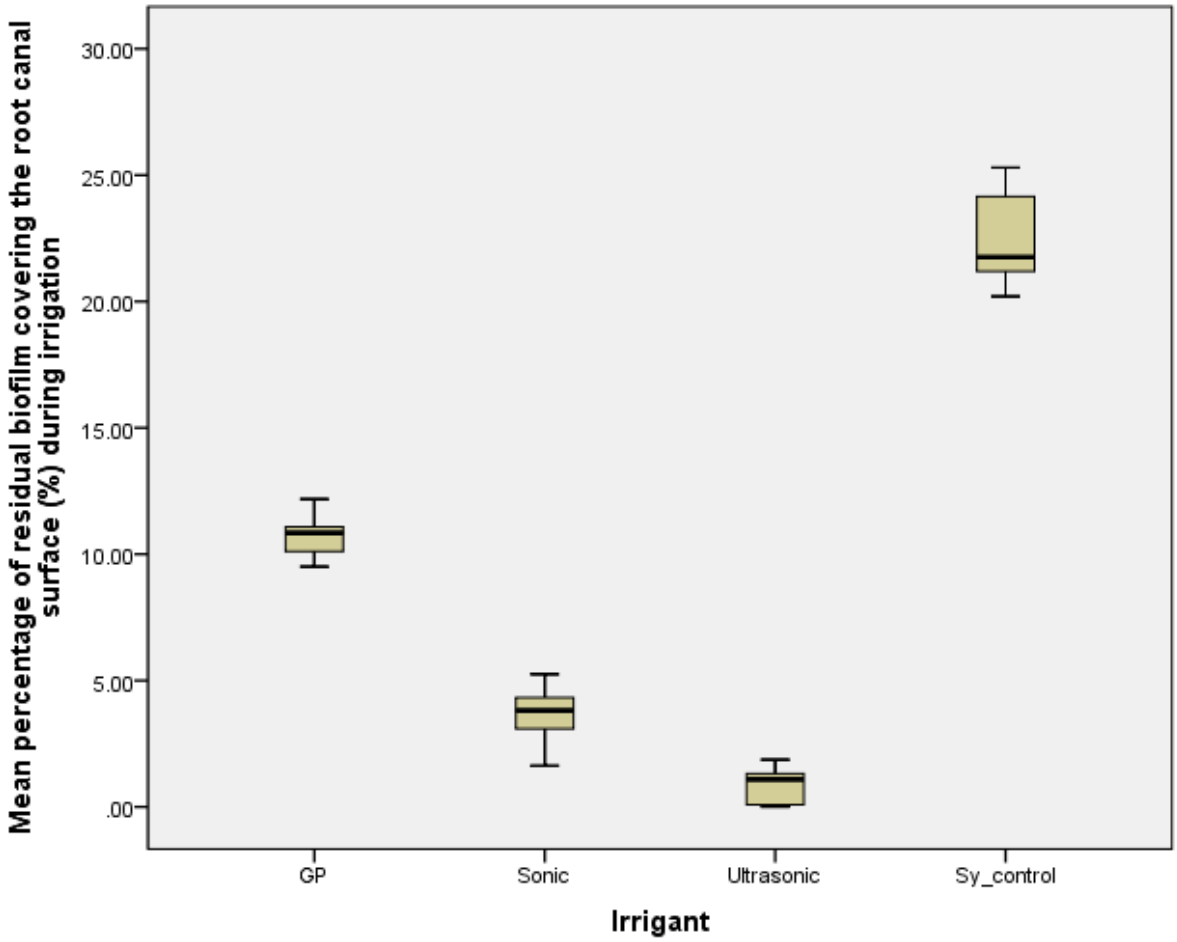

Figure 5: Box and whisker plots of the median values (%) of the residual biofilm covering the root canal surface area (n = 10 per group).

Table 2: Kruskal-Wallis analysis to compare the difference in the amount of residual biofilms covering the canal surface following passive or active irrigation time (30 seconds) with 2.5 % NaOCl irrigant (n = 10 per group).

Comparable groups		***Median (minimum, maximum) (%)**		
Group 1	**Group 2**	**Group 1**	**Group 2**	**p value**
ultrasonic	GP	1.09 (0, 5.25)	13.85 (12.51, 15.18)	**0.002**
ultrasonic	passive syringe	1.09 (0, 5.25)	25.76 (20.23, 29.30)	**0.001**
ultrasonic	sonic	1.09 (0, 5.25)	3.82 (1.63, 5.25)	0.78
sonic	GP	3.82 (1.63, 5.25)	13.85 (12.51, 15.18)	0.21
sonic	passive syringe	3.82 (1.63, 5.25)	25.76 (20.23, 29.30)	**0.001**
GP	passive syringe	13.85 (12.51, 15.18)	25.76 (20.23, 29.30)	0.34

* The median difference is significant at the 0.05 level.

Table 3: Generalized linear mixed model analysing the effect of time (second) on the amount of biofilm removed from the canal surface of each experimental group (n = 10 per group).

Experimental groups	*Coefficient for time effect (±SE)	95% CI	p value
GP agitation vs passive syringe irrigation	-5.35 (±1.1)	-7.49, -3.19	**0.001**
sonic agitation vs passive syringe irrigation	-6.66 (±1.1)	-8.81, -4.51	**0.001**
ultrasonic agitation vs passive syringe irrigation	-7.52 (±1.1)	-9.67, -5.37	**0.001**
GP agitation vs ultrasonic agitation	2.18 (±1.1)	0.03, 4.323	**0.047**
sonic agitation vs ultrasonic agitation	0.86 (±1.1)	-1.29, 3.01	0.43
sonic agitation vs GP agitation	-1.32 (±1.1)	-3.47, 0.83	0.23

*Coefficient for time effect represents the rate of biofilm removal, SE= standard error.

Table 4: Kruskal-Wallis analysis analysing the effect of biofilm NaOCl irrigant interaction on the available chlorine (left) and pH (right) of NaOCl as dependent variables (n = 10 per group).

Comparable groups		***Median available chlorine (minimum, maximum) (%)**		**P value**	***Median pH (minimum, maximum)**		**p value**
Group 1	**Group 2**	**Group 1**	**Group 2**		**Group 1**	**Group 2**	
syringe	ultrasonic	0.43 (0.29, 0.61)	1.35 (1.26, 1.52)	**0.001**	0.56 (0.41, 0.68)	3 (2.15, 4.39)	**0.001**
syringe	sonic	0.43 (0.29, 0.61)	0.89 (0.52, 1.12)	**0.016**	0.56 (0.41, 0.68)	1.71 (1.56, 1.88)	**0.021**
syringe	GP	0.43 (0.29, 0.61)	0.69 (0.53, 0.81)	0.127	0.56 (0.41, 0.68)	0.69 (0.53, 0.81)	**0.029**
ultrasonic	sonic	1.35 (1.26, 1.52)	0.89 (0.52, 1.12)	0.057	3 (2.15, 4.39)	1.71 (1.56, 1.88)	**0.029**
ultrasonic	GP	1.35 (1.26, 1.52)	0.69 (0.53, 0.81)	**0.006**	3 (2.15, 4.39)	0.69 (0.53, 0.81)	**0.021**
sonic	GP	0.89 (0.52, 1.12)	0.69 (0.53, 0.81)	1	1.71 (1.56, 1.88)	0.69 (0.53, 0.81)	1

* The median difference is significant at the 0.05 level.