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In Vitro Evaluation by Quantitative Real-time PCR and Culturing of the Effectiveness of Disinfection of Multispecies Biofilms in Root Canals by Two Irrigation Systems

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Key words

Biofilm; Disinfection; GentleWave; Quantitative real-time PCR; Ultrasonic

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Abstract

Objectives The purpose of this in vitro study was, by using quantitative real-time PCR and culturing to determine the effectiveness of two irrigation and cleaning systems in removing multispecies oral biofilms from root canals.

Material and Methods Twenty extracted human molars were instrumented to size #15/.02 and then cleaned with the GentleWave system (GW). The teeth were autoclaved to provide the same sterile baseline. The molars were filled with mixed plaque suspended in BHI and centrifuged to inoculate the biofilms. After two weeks of incubation, the teeth were randomly divided into two treatment groups. In GW group (26 canals) , the teeth were further instrumented to size #15/04, and in PiezoFlow (PF) group (30 canals) to #35/.04. The teeth were then cleaned either with GW System or ProUltra PiezoFlow Active Ultrasonic System using 3% sodium hypochlorite NaOCl, 8% EDTA and sterile water as irrigants. Samples (S1, S2 and S3) for bacterial cultures were taken from 13 canals before and after instrumentation and after final cleaning. Quantitative real-time PCR was performed from all 56 canals and universal bacterial, one genus and one species specific primers were used to determine the presence of microorganisms in samples from root canals before and after instrumentation and after final cleaning. Statistical analyses were performed using the Mann-Whitney U test with the significance level set at $P < 0.05$.

Results Bacterial culturing from the canal samples revealed strong reduction of bacteria from S1 to S2 in both groups after instrumentation and irrigation with water only. No growth was detected in any of the S3 samples after cleaning in either group. A highly significant reduction in bacterial DNA was recorded by qPCR for both groups ($P < 0.001$). GW system showed more constant and a significantly higher reduction of total microbial DNA ($P = 0.007$), *Enterococcus faecalis* DNA ($P = 0.011$) and *Streptococcus* spp DNA ($P = 0.029$) than the ultrasonic system. The amount of residual microbial DNA calculated as an average of residual DNA in each individual canal in PF group was 1.99% and in GW group 0.09%.

Conclusions While both systems demonstrated a highly effective reduction of intracanal bacterial DNA, the final total amount and variation in the number of residual bacterial DNA

was significantly smaller in the GW group.

Clinical relevance Elimination of microbes from the infected root canal system is regarded as the key for long term clinical success. While both GentleWave and ultrasonic systems used with NaOCl and EDTA demonstrated a highly effective reduction of intracanal bacterial DNA, GW produced higher reduction and better predictability.

Introduction

Apical periodontitis is a biofilm-related infection. Therefore, one of the primary goals of the treatment is to kill or remove the microbes from the root canal system [1]. In this process, chemomechanical preparation is regarded as the most important step. In recent years, different additional methods have been employed to improve the effectiveness of the irrigation. ProUltra PiezoFlow Active Ultrasonic System (PF) (Dentsply Tulsa, Tulsa, OK, USA), which allows continuous irrigant flow during the use of ultrasound is one of the modern equipment widely applied to clinic [2]. Results from several studies have indicated excellent effectiveness and safety of the ProUltra PiezoFlow system [2,3]. GentleWave (GW) (Sonendo Inc., Laguna Hills, CA, USA) System is a novel type of endodontic device developed for root canal cleaning and disinfection [4]. It uses high speed fluid dynamics to deliver the irrigants into the root canal system without requiring the tip of the instrument to enter the root canals. Instrumentation can therefore be minimized, which contributes to maintaining resistance to root fracture [5]. Some studies have reported that GW used with 3% NaOCl and 8% EDTA as irrigants is superior in soft tissue dissolution and cleaning of the root canals compared to some other irrigation strategies which include the use of ultrasound devices [6-8]. Two clinical studies have reported a high level of success for cases treated by GW System [9,10]. So far, no studies have been published on the effectiveness of the GW System in removing bacteria and biofilm from the root canals in comparison with other cleaning methods.

Culture-based methods have traditionally been used to identify and quantify bacteria in infections [11,12]. However, in the infected root canal, many species are difficult or impossible to grow [13]. Furthermore, low sensitivity and the long time required for culturing are also limitations of the conventional methods [13]. To overcome the limitations of traditional culture-based analyses, molecular analyses have been applied to quantify microbes. Quantitative real-time polymerase chain reaction (qPCR) is increasingly used in microbiological investigations because of its high sensitivity and efficiency. It has been

employed in both *in vitro* and *in vivo* studies to quantify single species or the total bacterial load in polymicrobial infections [14-17]. However, molecular based methods also have some limitations as the viability, physiology and pathogenicity of bacteria cannot be studied by the mere detection of bacterial DNA [13].

The aim of this study was to compare the effectiveness of removing bacteria by GentleWave System and the ultrasonic PF System as measured by qPCR and culturing from contaminated root canals *in vitro*. Total bacterial load, as well as the amount of *Enterococcus faecalis* and *Streptococcus* species, measured as bacterial DNA and colony forming units, were determined before instrumentation, after instrumentation and after the use of the two energy intensive treatments with irrigants.

Materials and Methods

Tooth collection and preparation

Ethics permission was obtained from the University of British Columbia Office of Research Services, Clinical Research Ethics Board (certificate number H15-02793). Twenty extracted human molars were collected and stored in PBS until use. Any teeth with decay or fractures below the cemento-enamel junction, internal or external resorption, open apices, or previous root canal therapy were excluded. Five maxillary and five mandibular first and second molars were included in both groups (PF and GW).

Endodontic access was achieved as per standard practice and patency was confirmed utilizing a #10 K-file. Working length was defined as 1 mm from the radiographic apex [7]. The teeth were instrumented to #15/.02 and then cleaned with the GW System (see details below in paragraph “GentleWave group: second and third sample”) to provide the same baseline for all the teeth. Samples were submerged in 10 mL of PBS and autoclaved for sterility at 121°C for 25 minutes. The apices of the roots of all the teeth were sealed using hot glue.

Inoculation

Supragingival and subgingival plaque was collected from interdental spaces of molar teeth of an adult volunteer using sterile wooden sticks. Plaque was suspended in brain-heart infusion broth (BHI; Becton Dickinson, Sparks, MD, USA) and incubated anaerobically at 37° C for two days. Individual molars were filled with approximately 120 µL of mixed plaque in BHI suspension. The teeth were centrifuged at 3500 g for 5 minutes to introduce bacteria to the fine details of the root canal system. The process was repeated three times with a fresh solution of bacteria during each centrifugation. All the teeth were incubated in BHI broth for two weeks at 37°C for biofilm maturation.

Sampling of the bacteria from the canals, first sample

After two weeks of incubation, samples were divided randomly into two treatment groups. The initial sample (S1) was taken before treatment. Teeth were taken out from the BHI broth by using sterile tweezers, the outer surface of the teeth was first carefully wiped with CaviWipes (Metrex Research, Orange, CA, USA) to clean and disinfect the teeth surface. Under stereo microscope, the pulp chamber was first carefully dried with sterile cotton pellets without affecting the canals. The samples were taken by sterile paper points (#15, Diadent Group International, Seoul, Korea) inserted into each root canal. Several paper points were used until the fluid in the root canal was soaked and the root canal appeared dry. Samples from each canal were collected into separate, sterile 1 mL Eppendorf tubes in PBS and frozen at -20°C until used.

GentleWave group, instrumentation, cleaning, and second and third sample

Ten molar teeth with 26 root canals were randomly allocated into the GentleWave group. A #10 (or smaller) hand K-file was inserted into the canals to measure the working length (WL) (apical foramen minus 1 mm). Pulp chamber was filled with sterile water and the canals were instrumented with #15 hand K-file (Dentsply Tulsa) to WL followed by Vortex Blue™ (Dentsply Tulsa) rotary #15/.04 files at 350 rpm. One mL of sterile water was used to irrigate the canals with a 5mL syringe and a 30G side vented needle after the hand and rotary file. The irrigation needle was placed as deep as possible without binding, but not closer than 1

mm from the WL. A #10 hand K-file was employed to verify that WL could be reached after finished instrumentation [7]. Pulp chamber was dried as described above and a second bacteriological sample (S2) was taken with sterile paper points as above. The teeth were then treated by using the GW System, according to the following protocol as recommended by the manufacturer: 3 min irrigation using 3% NaOCl, sterile water for 30 sec, 8% EDTA for 2 min. Final irrigation was with sterile water for 15 sec. In the GW system, the irrigants circulate simultaneously in all root canals, therefore the number of canals does not affect the overall time of cleaning by irrigation. The pulp chamber was dried as above and the third sample (S3) was taken from each canal. The overall treatment time with GW was 5 min and 45 sec for each tooth.

Ultrasonic system (PF) group, instrumentation, cleaning, and second and third sample

Ten molar teeth with 30 canals were randomly allocated into the Ultrasonic System group. A #10 (or smaller) hand K-file was used to measure WL. Pulp chamber was filled with sterile water and the canals were instrumented with #15 and #20 hand K-file to WL. Vortex Blue Rotary 04 taper files were then used at 350 rpm in the following order: #15, #20, #25, #30 and #35, all to WL. One mL of sterile water was used to irrigate the canals between each file. After instrumentation, a second sample (S2) was taken as described above. Final irrigation was performed with the ProUltra PiezoFlow Active Ultrasonic System as follows: 3% NaOCl 1 min per canal, sterile water 10 sec per canal, 8% EDTA 1 min per canal and sterile water 10 sec per canal. After the ultrasonic treatment, the pulp chamber was dried and the final sample (S3) was taken as described. The overall time for PiezoFlow treatment was 7 min for teeth with three canals and 9 min and 20 sec for teeth with four canals.

DNA extraction and quantitative real-time PCR analysis

The frozen samples were thawed to room temperature, and DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) following the protocol recommended by the manufacturer [17]. The final volume of DNA solution of each sample was 150 μL and was taken into account during calculation.

Total bacterial load was quantified by using 16S ribosomal RNA gene–targeted qPCR. The levels of *Enterococcus faecalis* and *Streptococcus* species (genus specific primer) were evaluated by using specific primers for *E. faecalis* and *Streptococcus* species. Table 1 shows the sequences and annealing temperatures of each primer. The qPCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on a StepOnePlus Real-Time PCR Systems (Applied Biosystems). The total reaction volume was 20 μL. Each reaction included 10 μL of Power SYBR Green PCR Master Mix, 6 μL of sterile distilled water, 1 μL of each 10 μM primer and 2 μL DNA template. The cycling conditions for universal bacteria and *Streptococcus* species contained 10 min at 95° C followed by 40 repeats of 95° C for 1 min, annealing for 1 min (temperatures shown in Table 1), and 72° C for 1 min. The temperature setting for *E. faecalis* was 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. Triplicate measurements were done for all samples. Each measurement included triplicate negative controls with no template DNA. Melting curve analysis was performed after amplification to confirm the specificity of the amplified reaction. Melting curve was detected from 60° C to 95° C. Fluorescence measurements were taken continuously at every 1% increase in temperature. StepOne Software v2.3 (Applied Biosystems) was used to acquire and analyze the data.

Standard curves were constructed by 10-fold diluted DNA extracted from *E. faecalis* ATCC 29212 and *Streptococcus mutans* ATCC 25175. The concentration of the pure extracted DNA was quantified using GeneQuant™ pro RNA/DNA Calculator (GE Healthcare, Little Chalfont, UK). Genome copy levels were calculated using the formula $m = n \times \left(\frac{1\ \text{mole}}{6\times10^{23}\ bp}\right) \times (660\ \text{g/mole}) = n \times 1.096 \times 10^{-21} g/bp$ [15], where m is the genomic mass of a single cell and n is the genome size. *Streptococcus mutans* ATCC 25175 DNA was also used for total bacteria quantification because it contained 5 copies of 16S rRNA gene which is the approximate average number of 16S rRNA genes which most oral bacteria have [14].

Bacterial cultures

Bacteria were cultured from 13 canals (4 in PF group, 9 in GW group) in 5 teeth before and after instrumentation and after final cleaning (samples S1, S2 and S3) with either PF or GW following a previously published protocol [18]. Briefly, samples were collected using sterile paper points for culturing and CFU counting. The paper points were vortexed in 1 mL of freshly sterilized BHI broth. One hundred microliters of the bacterial suspension were mixed with 900 μL BHI broth and serially 10-fold diluted. Droplets of 20 μL from each dilution were cultured on sheep blood agar plates rich in nutrients for anaerobic bacteria and incubated anaerobically for 72 hours and followed for up to 2 weeks [18]. Triplicates of each sample were done.

Statistical analysis

Non-parametric data analysis was performed with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). The % of residual DNA was calculated as the average of the residual DNA% in each of the 30 canals in PF group and 26 canals in GW group. The bacterial reduction from S1 to S2 as well as S1 to S3 were compared between two systems for total bacteria, *E. faecalis* and *Streptococcus* species, respectively by using Mann-Whitney U test. The significance level was set at $P < 0.05$.

Results

Both treatment groups, PF and GW, presented a high percentage of reduction of bacterial DNA from S1 to S3. The reduction by GW was significantly greater than by PF in all three bacterial groups (Table 2). In GW group, a mean number of 3.89×10^7 total bacterial cell equivalents per canal were detected in S1 samples and significantly decreased in S3 to a mean of4.53×10^4 cells per canal ($P < 0.001$); the mean reduction in total bacterial counts was 99.91% with the range from 99.27% to 99.99%. In the PF group, the reduction in the mean number of bacteria from S1 to S3 was from 2.02×10^7 to5.86×10^4 cells per canal, or 98.01%, with individual canals ranging from 85.77% to 99.99%. The difference between GentleWave System and the Ultrasonic System was statistically significant ($P = 0.007$).

E. faecalis was observed at a mean value of 5.91×10^5 (cell number based on qPCR copy numbers) before instrumentation and 1.24×10^3 after treatment in GW System group. The mean reduction (S1 to S3) was 99.75% by GW and 97.28% by the Ultrasound (P = 0.011). Table 2 shows the mean numbers of *E. faecalis* per canal and the mean percentage of reduction in the two treatment groups.

Slightly smaller but still statistically significant difference between the two cleaning methods was measured in the *Streptococcus* group (genus specific primer), 99.67% for GW and 98.61% for the PF (P = 0.029) (Table 2).

There was no significant difference in the decrease of the microbial DNA between the two groups after mechanical instrumentation only (S1 to S2) (P =0.341) (Table 2). Culturing and CFU counting showed strong reduction from S1 to S2 in both groups (Table 3). Canals instrumented to larger size (PF group before the ultrasound) showed a reduction of 99%, while the minimally instrumented canals showed a 96% reduction in CFU (GW group before the multisonic cleaning). Although the difference was relative small, it was statistically significant (p=0.025; Table 3). No growth was detected in any of the S3 samples in either group.

Discussion

Treatment with both the multisonic and the ultrasonic systems greatly reduced the bacterial load in the canals. Based on qPCR, the average reduction of total bacteria, streptococci and *E. faecalis* was 99.67 – 99.91 % in the GW group and 97.28 - 98.61 % in the PF group. Despite the relatively small differences between the two systems, they were statistically significant. The range of reduction of total bacterial counts as counted from the amount of DNA in the individual canals in the GW group was on a higher level and smaller (99.27% - 99.99%) than in the PF group (85.77% - 99.99%). The result indicates that GW more predictably achieved a high level of removal of microbial DNA from the root canal system. The change in

bacterial DNA in the three bacterial groups measured (total bacteria, *E. faecalis*, streptococci*)* from S1 (start) to S2 (after mechanical instrumentation), before cleaning using the two energy driven systems was not significantly different between the two groups (Table 2).

When the reduction of bacteria from S1 to S2 were compared between the three groups (total, *E. faecalis*, streptococci), the % reduction was greater in total bacteria than in enterococcus and streptococci. Although not examined in the present study, this may reflect the role and location of different bacteria in the biofilm layers. Streptococci, which showed smallest reduction from S1 to S2, are typically early biofilm colonizers and may therefore be more protected against mechanical effect on biofilm by the instrumentation.

One of the main benefits of *in vitro* studies is that it is usually possible to standardize the experimental conditions fairly well for different groups and thereby minimize the effect of confounding factors. In the present study, this was not possible to the same extend as in many other *in vitro* studies because of the different type of action of the systems as well as recommendations for use by the manufacturers [6]. The PF system is supposed to be used after conventional size instrumentation (#35/.04 in this study), which allows the tip to be placed freely into the canal [20]. In order to secure maximal efficacy, the tip must avoid contact with canal walls. GW system, on the other hand, is designed to work on minimally or even uninstrumented canals, as instead of being inserted into the canal the tip of the cleaning instrument is placed in the pulp chamber, just above the chamber floor throughout the treatment. For this reason, the canal dimensions between the groups or within the GW group were not standardized. Another key difference is that PF is used in each canal separately, whereas GW actively circulates the irrigant simultaneously in all root canals. In other words, the PF system provides active ultrasonic cleaning in one canal while the other canals at the same time are more passively exposed to the irrigant. With GW, all canals are actively targeted all the time. The experimental design in the present study was made to conform with the recommended use of the systems rather than testing the ultimate cleaning power of the two in identical conditions, i.e. same canal size and same active time of cleaning per canal facilitated by the multisonic and ultrasonic energy. Therefore, conclusions from the results

must be drawn with caution. The NaOCl irrigation per tooth was limited to 3 min with GW and to 3 – 4 min with PF. If the active ultrasonic (PF) irrigation with NaOCl would have been 3 min per canal, the total NaOCl irrigation per a molar in the present study would have been 9 or 12 minutes, depending on the number of canals (3 or 4), instead of the 3 or 4 minutes now used. Nine to twelve-minute of active ultrasonic irrigation is not realistic in a clinical situation, in addition would also come the time for water and EDTA irrigation, multiplied by the number of canals. The recommended time for NaOCl exposure with the GW is five minutes instead of the three minutes used in the present study. The reason for the shorter than normal NaOCl time with GW was the desire to keep overall NaOCl exposure times of the two systems closer to each other. Corresponding studies with GW and other cleaning and treatment methods in the future are likely to set the focus in different ways, resulting in different experimental designs.

Sampling was done with sterile paper points from the root canals. It is generally accepted that paper point sampling is not the most effective way of collecting all microbes from the root canal system [15]. Cutting off the roots and pulverizing them for DNA extraction is likely to yield more bacteria in the samples [21]. However, sequential sampling as in the present study during the process of the treatment cannot be done if the tooth is pulverized. Further, when whole roots are sampled, microbes and even residual DNA from the root surface, contaminated e.g. during extraction, handling and incubating the root canal microbiota would increase the risk for an error. The exterior apical third of the roots in the present study was sealed with hot glue to prevent transfer of microbes between the root surface and the root canal system. Another point worth noting is the different volume of the canals in the two groups. One can argue that from a larger canal more bacteria can be picked than from a narrow canal. It is also possible that, contrary to the previous claim removal of more dentin by instruments in the PF group mechanically removed the bacteria-rich zones around the main canal, which was the sampling area, and improved the effectiveness of the PF system due to better flow of the irrigants. Nevertheless, the canal dimensions in both groups in samples S2 and S3 remained constant throughout the experiment, and the changes (reduction)

in bacterial counts were calculated within each group.

The biofilms in the present study were grown for two weeks before the instrumentation and irrigation. A previous study indicated that 2-week-old biofilms are more sensitive to 1 % NaOCl and 2% chlorhexidine than three-week-old biofilm [22]. However, the effect of biofilm age on the effectiveness of ultrasonic and multisonic energy combined with 3% NaOCl has not been studied. It is possible that older biofilms are more difficult to detach and biofilm microbes more difficult to kill by different methods and compounds used for cleaning and disinfecting the root canal system.

In addition to using qPCR for quantification of bacterial reduction in the canals, bacterial cultures (S1 – S3) were done from 5 teeth (13 canals) to provide additional information about the number of live bacteria in each sample. The CFU results showed strong reduction of culturable bacteria already from S1 to S2 samples. The reduction was higher after instrumentation to 35/04 (99%; PF group) than after minimal instrumentation to 15/04 (96%; GW group), not surprisingly. None of the S3 samples for culture showed growth. While this emphasizes excellent effect by both systems (PF and GW), it also indicates that qPCR is more sensitive than a culture-based method. Negative S3 cultures can be explained by two different ways: sampling and culturing bacteria from complex root canal system is not sensitive enough to detect small amounts of bacteria, many of which are difficult or even impossible to culture, or all bacteria in the area available for sampling were killed by the combination of energy and sodium hypochlorite.

Previous studies have indicated that both PF and GW effectively clean organic matter from hard to reach areas in the root canal system [7,23]. Micro-CT studies of removal of calcium hydroxide (CH) from molar root canals have shown that GW so far is the only system which has completely removed the CH paste from the root canal system [8,24,25]. The present study showed that while no difference between the two systems was detected by culturing, GW removed bacterial DNA at a high level of predictability in minimally (#15/.04) instrumented molar canals. This is an important finding as effective disinfection is one of the

key requirements for minimal instrumentation in endodontics to claim sound scientific basis. Preservation of root dentin can be supposed to contribute to resistance against root fracture [5]. Other studies are needed to address technologies and quality of root fillings in minimally instrumented root canals, another key requirement.

In conclusion, within the limitation of this qPCR study, both the PiezoFlow ultrasonic and GentleWave systems used with NaOCl and EDTA effectively reduced the level of bacterial DNA in the root canals of molar teeth. GentleWave System showed more predictably a constantly high level of bacterial DNA reduction than the Ultrasonic System. Additional *in vivo* studies are also needed for further investigation into the disinfection effectiveness of the PiezoFlow Ultrasonic and the GentleWave Systems.

Compliance with Ethical Standards

Conflict of Interest One of the authors (MH) consults to Sonendo Inc. (manufacturer of GW) and has economic interest in one of the products (GW) used in this study.

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Ethical Approval Ethics permission was obtained from the University of British Columbia Office of Research Services, Clinical Research Ethics Board (certificate number H15-02793).

Informed Consent

Informed consent was obtained from the volunteer providing oral plaque in this study. According to the Clinical Research Ethics Board, no informed consent was required from patients whose teeth, extracted for nonrelated reasons, were used in this study.

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Table 1 Primers used in real-time PCR for bacterial quantification

Target	Primer sequence	Annealing temp (°C)	Reference
Universal 16S rRNA gene	5'-CAD ACT CCT ACG GGA GGC-3' 5'-ATC CTG TTT GMT MCC CVC RC-3'	59	[26]
*Enterococcus faecalis**	5'-CAA ACT GTT GGC ATT CCA CAA-3' 5'-TGG ATT TCC TTT CCA GTC ACT TC-3'	60	
Streptococcus species	5'-AGA GTT TGA TYM TGG CTC AG-3' 5'-TTA GCC GTC CCT TTC TGG T-3'	58	[27]

* A primer for *E. faecalis* previously used in [26] was tried in pilot experiments, but the results showed variation and indicated of less than optimal performance. Therefore, a new primer sequence was chosen based on the analysis of Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and preliminary testing.

Table 2 The DNA level of total bacteria (A), *Enterococcus faecalis* (B) and *Streptococcus* species (C) in root canal samples of teeth taken before instrumentation (S1), after instrumentation (S2) and after GW/PF system (S3)

(A)

Groups	N (Canals)	Mean (copies/canal)			Reduction% S1 to S2	Reduction% S1 to S3		Residual DNA%
		S1	S2	S3	Mean ± S.D.*	Mean ± S.D.*	Range	Mean ± S.D.*
GentleWave	26	$3.89\times10^{7}\pm7.56\times10^{6}$	$8.05\times10^{6}\pm4.98\times10^{6}$	$4.53\times10^{4}\pm1.51\times10^{4}$	83.32%±4.09%	99.91%±0.03%	99.27% - 99.99%	0.09%±0.03%
PF Ultrasonic	30	$2.02\times10^{7}\pm4.64\times10^{6}$	$3.21\times10^{6}\pm2.33\times10^{6}$	$5.86\times10^{4}\pm1.70\times10^{4}$	84.89%±3.50%	98.01%±0.69%	85.77% - 99.99%	1.99%±0.69%

(B)

Groups	N (Canals)	Mean (copies/canal)			Reduction% S1 to S2	Reduction% S1 to S3		Residual DNA%
		S1	S2	S3	Mean ± S.D.*	Mean ± S.D.*	Range	Mean ± S.D.*
GentleWave	26	$5.91\times10^{5}\pm1.61\times10^{5}$	$1.66\times10^{5}\pm3.77\times10^{4}$	$1.24\times10^{3}\pm313\times10^{2}$	72.59%±4.83%	99.75%±0.07%	99.19% - 99.99%	0.25%±0.07%
PF Ultrasonic	30	$3.58\times10^{5}\pm1.09\times10^{5}$	$8.01\times10^{4}\pm1.84\times10^{4}$	$1.44\times10^{3}\pm5.67\times10^{2}$	68.21%±4.98%	97.28%±0.77%	84.21% - 99.99%	2.72%±0.77%

(C)

Groups	N (Canals)	Mean (copies/canal)			Reduction% S1 to S2	Reduction% S1 to S3		Residual DNA%
		S1	S2	S3	Mean ± S.D.*	Mean ± S.D.*	Range	Mean ± S.D.*
GentleWave	26	$2.97\times10^{4}\pm1.32\times10^{4}$	$4.96\times10^{3}\pm9.91\times10^{2}$	$2.92\times10^{1}\pm9.53\times10^{0}$	65.15%±5.24%	99.67%±0.20%	96.31% - 99.99%	0.33%±0.20%
PF Ultrasonic	30	$1.29\times10^{4}\pm3.10\times10^{3}$	$2.55\times10^{3}\pm6.19\times10^{2}$	$3.99\times10^{1}\pm8.07\times10^{0}$	65.33%±5.08%	98.61%±0.60%	86.02% - 99.99%	1.39%±0.60%

*Calculated as the average of DNA reduction% in each individual canal [28].

(C)

Table 3, Colony forming units after 2 days cultivation for root canal samples of teeth taken before instrumentation (S1), after instrumentation (S2) and after final cleaning with the GW/PF system (S3).

Groups	N (Canals)	Mean			Reduction% S1 to S2	Reduction% S1 to S3
		S1	S2	S3	Mean ± S.D.*	Mean ± S.D.
GentleWave	9	$2.18\times10^{6}\pm4.98\times10^{5}$	$6.92\times10^{4}\pm4.45\times10^{4}$	0	96.00%±4.14%	100%
PF Ultrasonic	4	$2.26\times10^{6}\pm3.40\times10^{5}$	$1.34\times10^{3}\pm2.33\times10^{2}$	0	99.94%±0.03%	100%

*Calculated as the average of the DNA reduction% in each individual canal [28].