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Microbiological evaluation of endodontic files after cleaning and steam sterilization procedures

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Abstract

Background: Infection control procedures are essential for modern dental practice and they are continually evolving to meet the dental profession's high standards. The present study evaluated the efficacy of two cleaning procedures to reduce bacterial numbers on endodontic files, and evaluated the effect of biological debris on the subsequent sterilization of files.

Methods: Stainless steel and nickel-titanium (NiTi) files were examined upon removal from the manufacturer's packaging, after instrumentation in root canals of human teeth inoculated with a broth containing two anaerobic species and one facultative anaerobic species of bacteria, and after instrumentation and cleaning with either an ultrasonic bath or a thermal disinfector. For each file, the bacterial numbers were quantified using routine microbiological techniques in an anaerobic chamber.

Results: No bacteria were detected from files direct from their packets. The size, taper and type of file did not affect the ability of either of the cleaning procedures to reduce bacterial numbers. However, an absence of bacteria was more likely when files were cleaned in the thermal disinfector. No bacteria were detected from files that were subjected to steam sterilization irrespective of the type of prior cleaning procedure.

Conclusions: Steam sterilization eliminated all bacteria from the endodontic files irrespective of the presence of biological debris. The majority of bacteria were eliminated from endodontic files after either ultrasonic cleaning or using a thermal disinfector.

Key words: Infection control, cleaning, sterilization, endodontic files.

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INTRODUCTION

Infection control procedures are essential to modern dentistry and have an impact on all clinical practices. There is a lack of evidence linking endodontic treatment with the transmission of disease. However, in the absence of adequate infection control procedures, there is a realistic potential to transmit pathogenic microbes via endodontic instruments. These pathogenic microbes may be sourced from within the root canal system or from the periradicular tissues.

Instruments that contact sterile areas of the body, enter the vascular system or penetrate the oral mucosa are classified as 'Critical Items' and must be sterile before use.[1] This classification includes endodontic files, thus, these instruments should be sterile before use and before re-use. Endodontic files are considered as re-usable instruments. In recent times, the re-use of endodontic files has been scrutinized due to the uncertainty of achieving complete removal of biological debris (the organic and inorganic tissue retained on the surface of the files after being used to instrument a root canal) from these instruments by cleaning procedures. Protocols for the re-use, cleaning and sterilizing of endodontic files are currently being reviewed.[2]

It is thought that the presence of biological debris may prevent the effective penetration of steam. Another possibility is that biological debris with low moisture content may increase the heat-resistance of vegetative bacteria and spores.[3] It has been accepted that the presence of biological debris prevents the antibacterial action of chemical solutions. Organic materials may inactivate germicidal molecules or, if the organic material becomes dry, the proteinaceous layer resists penetration of the chemical solution.[4] Thermal resistance of bacteria and bacterial spores has been shown to increase when the microorganisms were embedded in materials such as soil, oils and fats.[5-7] However, the relevance of these findings to modern infection control practices in dentistry is questionable. In the current literature, there is no evidence to support the premise that biological debris inhibits the steam sterilization of dental instruments.

Few studies have evaluated the efficacy of modern

infection control procedures to reduce microbial numbers on endodontic files. Some studies have investigated the bacterial reduction on files that were submersed in bacterial broths.[8,9] However, the absence of dentine and organic tissue in the biological debris provides less protection for bacteria. Oliet[9] demonstrated the inhibition of sterilization when the files were not cleaned prior to placement in a dry heat sterilizer. However, the cleaning procedures were not representative of modern dental practice and dry heat sterilization procedures may be more susceptible to failure.[10,11]

Johnson *et al.*[12] demonstrated elimination of spores to undetectable levels from ISO size 25 K-Flex files that had been used to instrument canals of teeth and then subjected to steam or chemical sterilization with or without any prior cleaning procedure. These results refute the commonly held belief that sterilization is inhibited if instruments are not cleaned beforehand.[13]

The purpose of the present study was to examine endodontic files when removed from the manufacturer's packaging for the presence of bacteria, to evaluate the efficacy of two modern cleaning procedures, and to evaluate the efficacy of steam sterilization procedures to sterilize endodontic files with retained biological debris.

MATERIALS AND METHODS

A total of 210 Hedström and rotary files were used in the present study. The files included equal numbers of Hedström ISO sizes 15, 25 and 30 files (Antaeos, VDW GmbH, Müncher, Germany), GT 0.08 and 0.06 taper rotary files (Dentsply, Tulsa Dental, Tulsa, Oklahoma, USA) and ProFile 0.04 taper rotary files (Dentsply, Maillefer, Ballaigues, Switzerland). Excluding the files that were examined when removed from the manufacturer's packaging, all files were packaged in sealed sterilization pouches and subjected to steam sterilization (Validator Plus, Siemens, Pelton & Crane, Charlotte, North Carolina, USA), which reached a temperature of 134°C and a pressure of 27psi for at least 12 minutes.

Ethical approval and informed consent was obtained for each tooth used in this study. Fifty premolar teeth, extracted for orthodontic reasons, were stored in phosphate-buffered saline supplemented with 0.001 per cent thymol until required. Standard endodontic access cavities were prepared in the teeth using a high speed Jet #330 bur. Initial instrumentation of the canals was performed using Hedström ISO size 10 files to facilitate future instrumentation. During the initial instrumentation, 0.9 per cent sodium hypochlorite was used as an irrigant to minimize any bacterial contamination within the root canals. Sterile saline was used to flush the sodium hypochlorite from the canals. The teeth were then packaged and sterilized by steam sterilization to eliminate the possibility of foreign bacterial contamination during the experimental procedures.

Bacterial broth preparation

Three species of bacteria were inoculated into the root canals of the teeth to simulate an intra-canal infection. Two obligate anaerobic bacteria, *Fusobacterium nucleatum* (ATCC 10953) and *Porphyromonas gingivalis* (W50), and a facultative anaerobe, *Streptococcus mutans* (Ingbritt), were used to represent the relative proportions of anaerobes and facultative anaerobes found in endodontic infections.[14] The bacterial strains were grown separately in batch cultures under anaerobic conditions. Samples of each organism were transferred from batch cultures and inoculated into a cooked meat broth (Oxoid Pty Ltd, Heidelberg, Victoria, Australia) that was supplemented with 3.7 per cent brain-heart infusion broth (Oxoid Pty Ltd) and incubated at a temperature of 37°C for 24 hours under anaerobic conditions. One millilitre aliquots of each of the three bacterial broths, containing approximately 10^8 cells per millilitre, were combined to make a single bacterial broth to inoculate the root canals.

Inoculation of root canals and contamination of files

For Procedures II-VII (below), the root canals of the extracted teeth were inoculated with 0.3ml of the bacterial broth (containing approximately 10^7 cells). The broth was introduced into the root canals with a sterile syringe and transported along the entire length of the root canals by using sterile finger spreaders in a pumping action. For each of the following procedures, six experimental groups consisting of either five Hedström or rotary files of a single size or taper were examined using standard microbiological techniques.

Procedure I. Unused

Thirty unused files were examined after removal from the manufacturer's packaging. The files were removed from the packaging by the handle or shaft. The fluted sections of the files were removed with sterile wire cutters and each section was placed onto a blood agar plate. The files were handled with tweezers and contact with the fluted sections was avoided.

Procedure II. No cleaning

Thirty files were intentionally contaminated by using them in a root canal that was filled with the bacterial broth. Hedström files were examined after 60 strokes of the file in a coronal direction. Circumferential filing was performed to create biological debris accumulation on all aspects of the fluted sections. Rotary files were examined after use in a canal for 15 seconds at a speed of 250rpm. This group of files also served as positive controls.

Procedure III. Ultrasonication

Thirty files were intentionally contaminated (as in Procedure II) then placed on a gauze square for a maximum of 10 minutes. The files were placed in a perforated metal container (Fig 1) that was placed in a

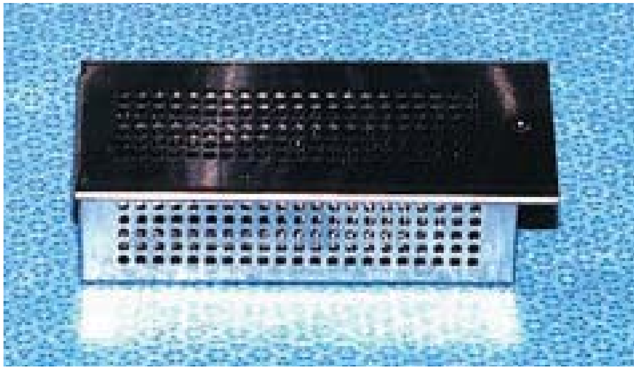

Fig 1. Perforated container (Miele & Cie, Gütersloh, Germany) used to hold files while cleaning in either the ultrasonic bath or the thermal disinfector. Dimensions: L-80mm, W-30mm, H-25mm.

beaker containing the ultrasonic cleaning solution (BioSonic Enzymatic Ultrasonic Cleaner, Whaledent, New Jersey, USA), which was placed in the ultrasonic cleaner (BioSonic Jr, Whaledent, New Jersey, USA) for five minutes. For every cycle, a maximum of 10 files was placed in the container and only one container was placed in the ultrasonic bath.

Procedure IV. Thermal disinfector

Thirty files were instrumented in a contaminated root canal (as in Procedure II) then placed on a gauze square for a maximum of 10 minutes. Files were then placed in a perforated metal container (as in Procedure III), which was then placed in a wire mesh basket in the thermal disinfector (Miele Professional G 7781 TD, Miele & Cie, Gütersloh, Germany). Thermal disinfectors use streams of hot water to physically clean debris from instruments and equipment, and utilize heat as an antibacterial mechanism. The thermal disinfector cycle included a main wash at a temperature of 45-60°C for three minutes and a final rinse at a temperature of 80-93°C for 10 minutes.

Procedure V. No cleaning and steam sterilization

Thirty files were intentionally contaminated (as in Procedure II), placed on gauze squares for a maximum of 10 minutes, packaged in sealed sterilization pouches and sterilized by steam sterilization.

Procedure VI. Ultrasonication and steam sterilization

Thirty files were intentionally contaminated (as in Procedure II), cleaned in an ultrasonic cleaner (as in Procedure III), packaged and sterilized by steam sterilization.

Procedure VII. Thermal disinfector and steam sterilization

Thirty files were intentionally contaminated (as in Procedure II), cleaned in a thermal disinfector (as in Procedure IV), packaged and sterilized by steam sterilization. Negative controls consisted of 15 Eppendorf tubes, containing 1.8ml of Reduced Transport Medium.[15]

Detection of bacteria

For Procedure I, the fluted sections were rolled on the agar with a sterile wire loop to allow contact with the entire surface of each file. The blood agar plates were incubated at 37°C for three days and then examined for any bacterial growth.

For Procedures II-VII and the negative controls, the fluted sections of the files were removed and deposited directly into Eppendorf tubes containing 1.8ml of Reduced Transport Medium. Oxygen levels in the transport medium were minimized by completely filling the Eppendorf tube with the fluid and the inclusion of dithiothreitol (0.2 per cent) as a reducing agent. Within one hour, the samples were transported to an anaerobic chamber (The Mark 3 Anaerobic Work Station, Don Whitley Scientific Pty Ltd, Shipley, West Yorkshire, UK). In the anaerobic chamber, the samples were vortexed for one minute to dislodge the biological debris from the files and disperse the bacteria in the transport medium. For each file that was intentionally contaminated and not cleaned (Procedure II), three-fold serial dilutions were made from the transport medium. Duplicate 25µl aliquots of the transport medium and the dilutions were placed 'drop wise' onto anaerobic blood agar plates. During all procedures, an aseptic technique was employed to prevent contamination from extraneous sources. The agar plates were incubated in the anaerobic chamber for three days under anaerobic conditions (10 per cent hydrogen, 10 per cent carbon dioxide and 80 per cent nitrogen) at a temperature of 36.5°C. Bacterial counts were performed using a colony counter (Gallenkamp, Leicester, England).

Statistical analysis

Mean colony-forming units per millilitre (cfu/ml) were calculated for each file in each sample group. Analysis of variance tests with Tukey post-hoc tests were used to compare bacterial counts between groups of varying size or taper, and the varying cleaning methods. Student's *t* tests were used to determine differences between Hedström and rotary files. Binomial linear regressions were used to detect differences in the efficacy of the cleaning methods to eliminate bacteria to undetectable levels and to examine the effect of file size, taper and type on achieving reduction of bacteria to undetectable levels. The standard level of significance was $p<0.05$.

RESULTS

All negative controls demonstrated no growth of bacteria and all positive controls (Procedure II) demonstrated dense growth of bacterial colonies from the undiluted samples.

Files removed from the manufacturer's packaging (Procedure I)

No bacteria could be detected from any of the rotary or Hedström files that were removed from the manufacturer's packaging.

Table 1. Effect of rotary file taper and method of cleaning on the number of detectable bacteria (cfu/ml)

Cleaning procedure	Rotary file taper 0.04	0.06	0.08	p value
No cleaning	118800	19040	372000*	0.01
Ultrasonic	208†	688†	304†	0.61
Thermal disinfector	240†	0†	0†	0.40
p value	0.01	0.00	0.00	

*ANOVA test demonstrated the GT 0.08 taper files retained significantly greater numbers of bacteria after intentional contamination compared to the other rotary files.
†ANOVA test demonstrated significantly lower numbers of bacteria from files that had been cleaned by either procedure compared to the 'No cleaning' groups. However, for each taper of rotary file there was no significant difference in the numbers of residual bacteria after either cleaning procedure.

Table 2. Effect of Hedström file size and cleaning method on the number of detectable bacteria (cfu/ml)

Cleaning procedure	Hedström file size 15	25	35	p value
No cleaning	19040	49280	660000*	0.01
Ultrasonic	0†	16†	16†	0.30
Thermal disinfector	0†	0†	0†	1.00
p value	0.00	0.02	0.04	

*ANOVA test demonstrated the Hedström ISO size 35 files retained significantly greater numbers of bacteria after intentional contamination compared to the smaller Hedström files.
†ANOVA test demonstrated significantly lower numbers of bacteria from files that had been cleaned by either procedure compared to the 'No cleaning' groups. However, for each size of Hedström file there was no significant difference in the numbers of residual bacteria after either cleaning procedure.

Effect of file size or taper

After intentional contamination of the files, Hedström ISO size 35 files and GT 0.08 taper rotary files retained significantly greater bacterial numbers when compared to smaller files of the same type (Procedure II, Table 1 and 2). After ultrasonication (Procedure III) or a thermal disinfection cycle (Procedure IV), there was no significant difference in the number of colony-forming units retained on the three tapers of rotary files (Table 1) and the three sizes of Hedström files (Table 2). In addition, variation of file size or taper had no significant effect on the ability of the cleaning procedures to reduce the bacterial numbers to undetectable levels.

Effect of type of file

There was no significant difference in the number of bacteria recovered from rotary and Hedström files after intentional contamination with or without a subsequent cleaning procedure (Table 3). However, significantly more rotary files demonstrated bacteria when compared to the Hedström files (14 and 4 respectively, Table 4).

Effect of cleaning method

There were no statistically significant differences in the numbers of bacteria detected when both of the cleaning procedures were compared for all sizes, tapers and types of files (Table 1-3). The average bacterial reductions achieved by the cleaning procedures can be expressed as percent reductions. For Hedström files, ultrasonication reduced the cultivable bacteria by 99.99 per cent and the thermal disinfector achieved 100 per cent reduction. For the rotary files, ultrasonication and thermal disinfection produced 99.76 and 99.96 per cent reductions of bacteria respectively.

An absence of bacteria was more consistently achieved when the files were cleaned by the thermal disinfector (Table 4). This is illustrated by the fact that one of the 30 files cleaned by the thermal disinfector demonstrated bacterial growth compared to 17 of the 30 files cleaned with an ultrasonic bath. The difference in the number of files retaining detectable bacteria after each of the cleaning methods was statistically significant (Table 4).

Table 3. Effect of file type and the method of cleaning for each file type on the number of detectable bacteria (cfu/ml)

Cleaning procedure	Rotary files	Hedström files	p value
No cleaning	169946*†	242773*†	0.53
Ultrasonic	400†	10†	0.07
Thermal disinfector	80†	0†	0.33
p value	0.00	0.08	

*ANOVA test demonstrated that, for both file types, files that were not cleaned after intentional contamination retained significantly larger numbers of bacteria compared to files that were cleaned by either procedure.
†*t* tests demonstrated that there was no significant difference in bacterial counts when the file types were compared for each of the cleaning procedures.

Table 4. Numbers of Hedström and rotary files demonstrating a presence of detectable bacteria after a cleaning procedure

	Cleaning procedure: Ultrasonic	Thermal disinfector	Total
No. Hedström files	4 (15)	0 (15)	4 (30)*
No. rotary files	13 (15)	1 (15)	14 (30)*
Total	17 (30)†	1 (30)†	

( ) indicates the total number of files in each sample group.
*Binomial linear regression test demonstrated a significant difference between the number of Hedström and rotary files that retained detectable bacteria. p value of 0.00.
†Binomial linear regression test demonstrated a significant difference in the number of files that retained detectable bacteria after either of the cleaning procedures. p value of 0.02.

Effect of sterilization

No bacteria were detected from 90 files subjected to a steam sterilization cycle, irrespective of the size, taper or type of file, with or without a cleaning procedure prior to steam sterilization.

DISCUSSION

In the present study, efforts were made to mimic the contamination of files that would occur during clinical endodontic therapy. Instrumentation of extracted teeth

ensured the accumulation of dentine on the files. Injection of the cooked meat broth into the canals provided an organic material, which represented pulp tissue or any other organic material that may be present in infected root canals. The combination of the dentine and the organic tissue in the biological debris provided a suitable challenge for common cleaning and sterilization procedures to eliminate the bacteria from the surface of the files. In addition, the injection of the bacterial broth into the root canals provides relatively consistent bacterial numbers compared to *in vivo* cultivation of bacteria from teeth with varied pathological states and variable numbers of intra-canal bacteria.

This study was designed to minimize the inadvertent elimination of bacteria due to causes other than the cleaning and sterilization procedures. Exposure of the files to air was minimized and would be less than that experienced in clinical practice. The ingredients of the transport medium favoured the maintenance of the viability of the inoculated bacteria. The Eppendorf tubes were filled with the transport medium to minimize the exposure to oxygen. After placement of the files in the Eppendorf tubes, subsequent experimental procedures were performed within an anaerobic chamber to provide an optimal environment for growth of the three bacterial species.

No bacterial growth was detected from the files examined immediately after removal from the manufacturer's packaging. It would be speculative to offer a reason for this result when the exact manufacturing processes are not known. However, it should not be assumed that all unused files are sterile. When the packaging does not completely seal the contents from the external environment, there is a potential for the files to become contaminated. In addition, there is no claim of sterility on the packages of the files examined in this study. The bacterial growth on files removed from the manufacturer's packaging may be expected to be low but Standards Australia[16] recommends that files be sterilized in appropriate packaging prior to use to ensure sterility of the instruments.

At the time of writing, there had been no investigation of the bacterial reduction from endodontic files after instrumentation in teeth and subsequent automated cleaning procedures. The results of the present study indicate no significant difference in the degree of bacterial reduction from the files by either of the cleaning methods. However, there was a greater tendency for the thermal disinfector to reduce the bacterial numbers to undetectable levels. A reason for this may be related to the antibacterial actions of each cleaning method.

The thermal disinfector utilizes two methods of bacterial reduction. Bacterial bioburden is physically removed by streams of hot water originating from various sites within the internal compartment. Heat inactivation of bacteria is also achieved by maintaining a temperature of 93°C within the thermal disinfector for 10 minutes during the cleaning cycle. Reduction of bacteria in an ultrasonic bath relies on the physical removal of biological debris by the propagation of acoustic energy waves through a fluid-filled vessel[17] and the chemical action of the cleaning solution. It has been widely reported that the presence of organic tissue may reduce the effectiveness of a chemical solution due to direct inactivation or by preventing the contact of the chemical solution with bacteria.[4] A similar inhibitory effect on the action of heat is not well documented. Both of the cleaning procedures examined in the present study may not achieve complete removal of biological debris from used files[18] but files cleaned with the thermal disinfector were more likely to have an absence of bacteria. Therefore, the antibacterial action of heat may be less susceptible to inhibition by residual biological debris on the instruments.

The rotary files demonstrated a significantly greater tendency to retain cultivable bacteria and this was more evident when the ultrasonic cleaning was performed. This may be a result of greater retention of biological debris on the rotary files, which protects the bacteria from the antibacterial mechanisms, in particular, the ultrasonic cleaning solution. However, the authors have shown in another study that the rotary files had a lower surface area of biological debris than the Hedström files after cleaning in the ultrasonic bath using a perforated container to hold the files.[18] An explanation for this disparity may be that quantification of the surface area of biological debris is only two-dimensional and does not account for the thickness of the soil layer. The aggressive action of the rotary files induces the packing of biological debris into the flutes of the ProFile and GT rotary files. In addition, the U-shaped flute design of the rotary files may provide more retention of biological debris and greater protection for the bacteria compared to that of the Hedström files.

It is an accepted principle that biological debris on instruments may prevent effective sterilization.[3,13] However, there is no scientific evidence to support this principle in relation to medical or dental instruments that are subjected to steam sterilization. The results of the present study demonstrated no growth of bacteria after the files had been processed by steam sterilization, irrespective of the prior cleaning procedure. Similar results have been demonstrated when ISO size 25 K-Flex files were examined after instrumentation and sterilization, with or without prior ultrasonic cleaning.[12] Johnson *et al.*[12] examined the elimination of *Bacillus stearothermophilus*, a spore-forming bacterium that is extremely resistant to heat. The three bacteria used in the present study are more susceptible to cell death when exposed to air and the steam sterilization process. However, these bacteria accurately represent the bacteria that inhabit root canal systems of teeth with endodontic infections. From the results of the present study and those of Johnson *et al.*,[12] it can be proposed that biological debris on endodontic files

does not reduce the efficacy of the steam sterilization procedures to eliminate microorganisms.

The importance of biological debris removal should not be disregarded. A theoretical risk of CJD transmission *via* oral tissues and maintenance of the cutting efficiency of the files are factors that support achieving effective removal of biological debris after use of the files. When considering that effective elimination of microorganisms is achieved by the sterilization cycle, a major factor in the cleaning procedure should be the removal of biological debris from the instruments. However, if an effective cleaning procedure also has an effective antibacterial action, this may provide an added assurance in the prevention of disease transmission.

CONCLUSION

The presence of biological debris did not affect the efficacy of the steam sterilization procedure for endodontic files. No bacteria were detected from files that were subjected to a steam sterilization cycle after instrumentation in teeth with or without pre-sterilization cleaning. Files removed from the manufacturer's packaging may have minimal or no bacterial contamination but sterility of the files cannot be guaranteed. The results of the present study demonstrated that variation in the size, taper of the files did not affect the efficacy of bacterial reduction of both cleaning procedures but Hedström files were more likely to show the absence of bacteria. There was no significant difference in the efficacy of the cleaning procedures to reduce bacterial numbers from files that had been instrumented in infected human teeth. Overall, a 99.88 per cent reduction in bacterial numbers was achieved after cleaning with the ultrasonic cleaner and a 99.98 per cent reduction was observed after cleaning with a thermal disinfector. However, an absence of detectable bacteria was more consistently demonstrated from files cleaned by the thermal disinfector.

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