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ORIGINAL ARTICLE

Molecular microbiological analysis of ProTaper Next, XP-Endo Shaper and Reciproc Blue systems in severely curved canals

ABSTRACT

Aim: *This study evaluated the effectiveness of the mechanical reduction of intracanal bacteria produced by the endodontic systems Reciproc Blue (VDW GmbH), XP-Endo Shaper (FKG Dentaire) and ProTaper Next (Dentsply Sirona Endodontics) in severely curved canals by means of a molecular microbiological analysis.*
Methodology: *A total of 42 severely curved mesiobuccal canals of human permanent mandibular molars were selected and prepared. Then, canals were contaminated with* Enterococcus faecalis *strains (ATCC 29212) by incubation during 21 days at 37 °C for formation of a mature biofilm. After that, contaminated specimens were randomly divided in 3 groups (*n=*14): ProTaper Next (*G1*), XP-Endo Shaper (*G2*) and Reciproc Blue (*G3*). Microbial samples were obtained before (S1) and after root canal preparation (S2). Analysis of intracanal* E. faecalis *reduction was performed using quantitative polymerase chain reaction (qPCR), and the difference between groups was analyzed by Kruskal-Wallis test. Significance level was set at p<0.05.*
Results: *All systems presented effective bacterial reduction (p<0.05), but still had bacterial growth. No significant difference between the evaluated file systems was demonstrated (p>0.05).*
Conclusions: *ProTaper Next, Reciproc Blue and XP-Endo Shaper presented similar mechanical reduction of intracanal bacteria. No file system was capable of rendering severely curved canals completely free from bacteria.*

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Introduction

Success in root canal therapy relies on the reduction of microorganisms and their by-products from the root canal system (1). The mechanical action of endodontic files underscores high levels of bacterial decrease (2, 3). However, endodontic instruments are frequently unable of reaching all root canal walls; thus the complete elimination of microorganisms is not achieved and the untouched areas may remain colonized (1, 4). Aiming to increase the treatment prognosis, endodontic instruments undergone a considerable evolution with progressive generations of NiTi files presenting modifications mainly as regards files design, manufacturing process, alloy processing and heat treatment (3, 5).

Culture analysis by means of counting colony-forming units is the most common method used in literature to evaluate the bacterial reduction produced by endodontic instruments (2, 4). However, this method presents limitations such as not considering low bacterial amounts and detecting exclusively viable and cultured microorganisms (1). Molecular microbiological methods, such as quantitative polymerase chain reaction (qPCR), are capable of overcoming these issues; but, to date, very few studies used molecular assays to analyze the effectiveness of mechanical reduction of intracanal bacteria produced by different files systems (6-9). Multi-files systems, such as ProTaper Next (Dentsply Sirona Endodontics, York, PA, USA), are broadly used in root canal therapy since wide evidence support these instruments (2, 3). Nevertheless, large interest towards single-file systems has emerged resulting from treatment optimization and a growing body of studies that also indicate their use (4, 5, 10). XP-Endo Shaper (FKG Dentaire, La Chaux-de-Fonds, Switzerland) is a newly developed snake-shaped single-file rotary system with high flexibility, which is expected to produce minimal stress on dentinal walls (4, 5). Moreover, Reciproc Blue (VDW GmbH, Munich, Germany), a single-file reciprocating system with a S-shaped cross-section, was also introduced (11). This instrument presents a new heating process that increase flexibility and resistance to cyclic fatigue (5, 10). Previous reports have shown some advantageous properties of Reciproc Blue as regards dentinal microcracks formation (5), cyclic fatigue resistance (12), removal of root canal filling materials and regaining apical patency (10). However, the capacity of these newly developed files in mechanical bacterial reduction, which is a relevant aspect for the treatment success, still scarce in literature. Therefore, the purpose of this study was to evaluate the mechanical reduction of intracanal bacteria produced by ProTaper Next, XP-Endo Shaper and Reciproc Blue systems in severely curved canals by means of the molecular microbiological analysis.

Materials and Methods

Specimens' selection and preparation

This study was approved by the University Ethics Committee (2.705.981). The mesiobuccal canal of 48 permanent human mandibular molars with complete root formation, extracted for periodontal reasons not related to this study, were selected. Initially, specimens were maintained in 4% sodium hypochlorite solution (NaOCl) during 2 hours and cleaned by periodontal curettes. Periapical radiographs were taken from each tooth in a buccolingual and a mesiodistal direction. This stage aimed to select only similar radiographic morphology specimens with one isolated mesiobuccal canal and severely curved (20-35°) mesial roots, according to Schneider's method (5, 13). Then, the crown of each tooth was sectioned near the cementoenamel junction, and the mesial roots were standardized to a length of 13 mm from the anatomic apex, using a diamond disc. The following features excluded teeth from this study: dental caries, previous root canal treatment, root resorption, root canal calcification, initial apical diameter larger than a size 15 K-file, dentinal crack or root fracture. The exclusion criteria were detected using periapical

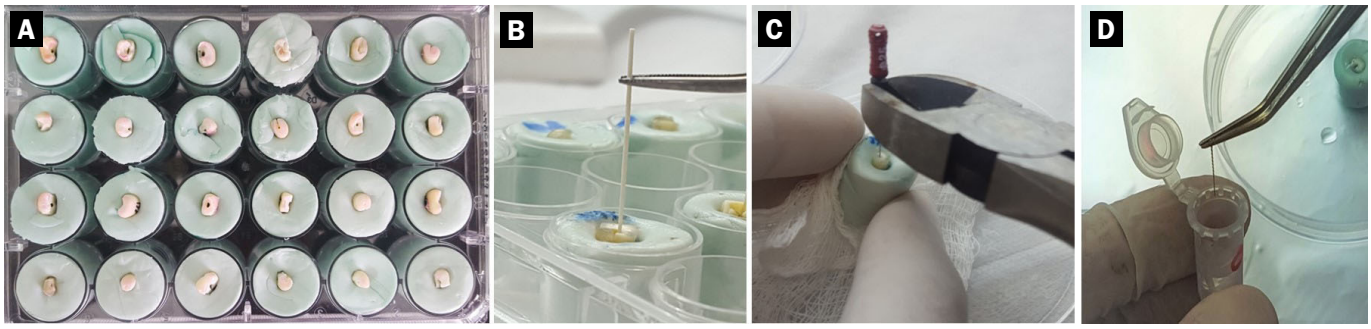

Figure 1
Representative images of bacterial samples collected for quantitative polymerase chain reaction (qPCR).
A) Individual models of each specimen.
B) Collection of root canal sample by a sterile paper point.
C) Sectioning of Hedstroem file with the aid of sterile tweezers for post root canal preparation quantification of bacterial contamination (S2).
D) Insertion of sectioned Hedstroem file into a sterile Eppendorf containing sterile saline solution.

radiographs, a clinical microscope (10x magnification), and a size 15 K-file (Dentsply Sirona).
Firstly, 5 mL of 2.5% NaOCl was used and root canals were explored with a size 10 K-file (Dentsply Sirona Endodontics, York, PA, USA). The working length was established in the total root length, 13 mm. Next, the intracanal contents were removed with a size 15 K-file (Dentsply Sirona), and specimens were newly irrigated with 5 ml of 2.5% NaOCl. The smear layer was removed using 3 mL of 17% EDTA during 3 minutes and a final irrigation with 5 mL of sterile saline solution was obtained. Root canals were dried with the aid of capillary tips (Ultradent products, South Jordan, UT, USA) and sterile paper points in the working length. The apical foramen of each specimen was sealed with Z 100 composite resin (3M, Saint Paul, MN, EUA) in order to prevent apical bacterial leakage and to create a closed-end channel, producing the vapor lock effect (6). Afterwards, the external apical surface was sealed with nail varnish.
In laminar flow chamber, individual models of each specimen were prepared using silicone impression material (Zetaplus, Zhermack, RO, Italy) with the aim to simplify root handling and shaping (Figure 1A). Teeth were prepared vertically up to the cervical third with the produced models, inserted into individual wells in 24-well cell culture plates (Costar, Washington DC, USA) and sterilized in an autoclave at 134 °C for 15 min.
Following this, aseptic conditions were confirmed after retaining teeth at 37° during 24 h, in which no bacterial growth was detected.

Root contamination with Enterococcus faecalis
Pure culture suspension of *Enterococcus faecalis* strains (ATCC 29212) was obtained by cultivation in brain heart infusion agar (BHI; Difco, Detroit, MD, USA) and then standardized on the McFarland nephelometric scale $30x10^8$ bacteria/mL. From the total sample, 6 specimens were selected as control, being filled solely with sterile BHI. At the same time, 42 roots were contaminated with 10 µl of *E. faecalis* suspension using sterile micropipette tips. Samples were incubated at 37 °C for 21 days in CO_2, during which the BHI was removed and replenished in 20 µL every 24 h (2, 14). This procedure was performed under laminar flow by means of sterile micropipettes.
Bacterial viability and intracanal sampling purity was checked every week by a selection of 2 random samples. For this, a sterile paper point #15 was maintained into the root space during 1 min, and immediately spread in BHI and incubated at 37 °C and 5% CO_2 for 24 hours. After growing, Gram staining and colony morphology on Columbia Agar with 5% Sheep Blood (CA-SB) (Becton Dickinson GmbH, Heidelberg, BW, Germany) was performed.

Initial quantification of bacterial contamination (S1)
After incubation period, root canals were rinsed with 1 mL sterile saline solution. Then, the initial sample (S1) was obtained by the sequential use of 2 sterile paper points #15 placed inside the root canal during 1 min each (Figure 1B). Following this, paper points were transferred with sterile tweezers to sterile polypropylene flasks containing 500 µL of sterile saline solution, and vortexed for 30 seconds.

Root canal preparation

The process of endodontic treatment and bacterial samples collection are represented in Figure 2. The 42 contaminated specimens were randomly assigned to the following groups, according to the file system used (n=14): ProTaper Next (G1), XP-Endo Shaper (G2) and Reciproc Blue (G3). The working length (WL) was established in the total root length.

- **G1 ProTaper Next (Dentsply Sirona).** Files were driven in continuous rotation in a crown-down technique using a gentle up-and-down motion. Firstly, the X1 file (17.04) was used for cervical and middle thirds shaping. This file was reused until reaching the WL. Following this, the X2 file (25.06) was used in the same manner as previously described.
- **G2 XP-Endo Shaper (FKG Dentaire).** XP-Endo Shaper file (30.01 as initial taper; however, during use, this instrument expands to a minimum taper of 0.04) was activated in continuous rotation at 800 rpm and 1.0 Ncm. Long and light up-and-down movements were applied inside root canals until reaching the cervical and middle thirds. After that, the file was reused in the same manner until reaching the WL.
- **G3 Reciproc Blue (VDW GmbH).** Reciproc Blue R 25 (25.08) was activated in reciprocating motion. The file was gently inserted with an up-and-down pecking motion that presented a maximum amplitude of 3 mm until the shaping of cervical and middle thirds was completed. After 3 up-and-down movements, when superior pressure was necessary to advance the instrument along the canal, the file was removed for cleaning of the flutes. Then, the file was reused in the same manner until reaching the WL.

Root canal preparation was performed by a single operator, an endodontist, who had been previously trained for each system. All files had single use. The protocol of each system followed manufacturer's instructions as previously explained. All systems were powered by a torque-controlled motor (X-Smart Plus; Dentsply Sirona Endodontics, York, PA, USA), set at the designated function according to the used system.

Root canals were irrigated during and after finishing instrumentation with a total volume of 10 mL sterile saline solution. Solution was delivered using a 24-G needle (Ultradent products, South Jordan, UT, USA) by means of a peristaltic pump (LAP-101-3; MS Tecnopon, Piracicaba, SP, Brazil) and a flow rate of 5 mL/min. For this, irrigation method was standardized. Initially, root canals were irrigated with 2.5 mL of saline solution. Next, after cervical and middle third shaping, 2.5 mL was used for root canal irrigation while instruments were cleaned with sterile gauzes. After completing preparation of specimens in the WL, a final irrigation with 5 mL was performed. During the use of all instruments, when a resistance requiring more apical pressure was detected, the file was removed and the flutes were cleaned.

Six non-contaminated root canals were instrumented for each file system (*n*=2) and used as negative controls.

Post root canal preparation quantification of bacterial contamination (S2)

Finishing endodontic instrumentation and irrigation, a post root canal preparation sample (S2) was obtained. For this, a size 25 sterile Hedstroem file (Dentsply Sirona) was introduced in the WL with circumferential strokes on all root canal surfaces (2). The file was then sectioned below the handle and, with the aid of sterile tweezers, dropped into a sterile Eppendorf containing 500 µL of sterile saline solution (Figure 1C, 1D). Following this, a sequential use of 2 sterile paper points #25 placed inside the root canal during 1 min each was performed. Collected paper points were stored in the same tube as the file.

Quantitative analysis by qPCR

Collected samples were vortexed for 30 s and subjected to DNA extraction for further qPCR analysis. DNA was extracted by the use of the QIAamp DNA Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer's directions. Aiming to maximize DNA extraction, a pre-incuba-

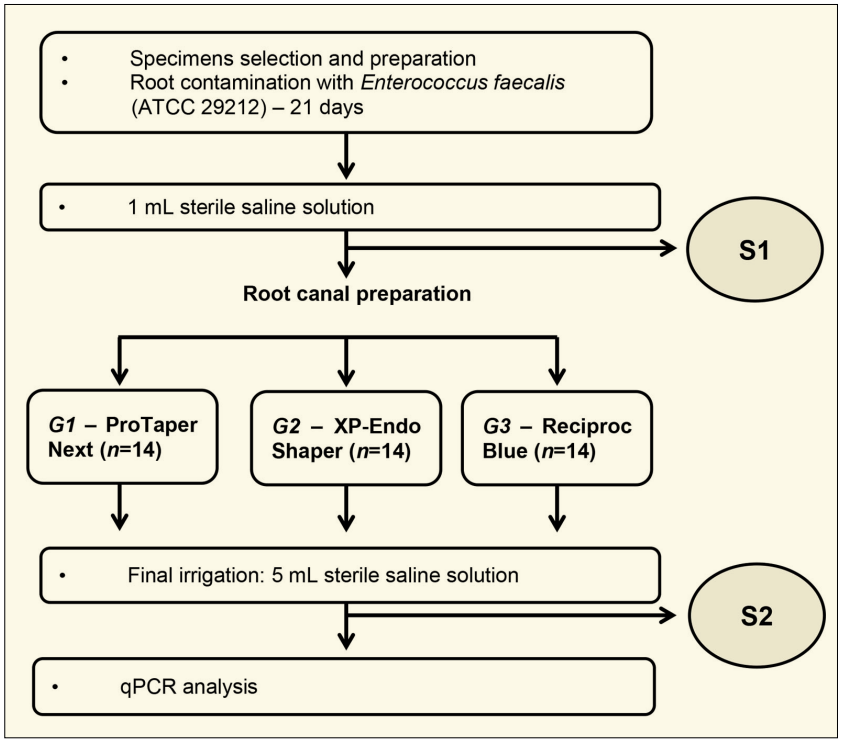

Figure 2
Flow chart of endodontic procedures and bacterial samples for quantitative polymerase chain reaction (qPCR) analysis. Root canal samples were taken before treatment (S1) and after root canal preparation according to each group (S2).

tion stage was included using lysozyme during 30 min. Obtained DNA extracts were stored, frozen at 20 °C, until qPCR analysis.

Afterwards, *E. faecalis* cells levels were quantified in root specimens by a 16S rRNA gene-based qPCR, using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) on an ABI 7500 real-time PCR instrument (Applied Biosystems). A total reaction volume of 20 µL was applied. Species-specific primers for *E. faecalis*, qPCR conditions, standard curve construction, controls, and data analyses were performed as previously described (6, 10). Primers in a concentration of 0.5 µmol/L each and DNA extract volume of 2 µL were added to the PCR master mix in MicroAmp Optical 96-well reaction plates. The conditions for the qPCR amplification cycling were: 95 ºC for 10 min; 40 repeats of the following stages: 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. Double-stranded DNA product was measured at 78 °C. At each cycle, accumulation of PCR products was verified by monitoring the increase in fluorescence of the reporter dye (dsDNA-binding SYBR Green).

All measurements were performed in duplicate for samples and triplicate for standards. In order to detect and exclude any possible contamination or carryover, triplicates of appropriate negative controls containing no template DNA were subjected to the same procedures. After amplification, melting curve analysis of PCR products was performed to determine the specificity of the amplified products.

Melting curve was obtained from 60 to 95 °C, with continuous fluorescence measurements taken at every 1% increase in temperature. Data acquisition and analysis were performed using the abi 7500 software v2.0.4 (Applied Biosystems).

E. faecalis cell counts were accomplished based on obtained standard curves. *E. faecalis* ATCC 29212 was used to create a 10-log-fold standard curve for direct bacterial quantification. DNA was isolated from a fresh pure culture of this strain using the QIAamp DNA Mini Kit (Qiagen) and quantified using a spectrophotometer (BioPhotometer, Eppendorf, Hamburg, Germany). Knowing the genome size of *E. faecalis* (3.2 Mb, http://www.cbs.dtu.dk/services/GenomeAtlas-3.0/) and the average molecular weight of one base pair (660 Da), the measured DNA value could then be converted into target genomic copy levels per microlitre using the formula

$$m=n\ [1\ mole\ /6{\cdot}10^{23}\ (pb)]\ [660\ (g)/mole] = n\ [1.096{\cdot}10^{-21}(g/bp)]$$

where "m" is the genomic mass of a single cell and n the genome size. Genome copy levels were considered as numerically equivalent to bacterial cell levels. The standards were then 10-fold diluted from 10^7 to 10^2 cells in TE buffer and used for standard curve construction (6).

Statistical analysis

Obtained values were transformed to log10 and statistically analyzed using SPSS 23.0 (SPSS Inc., Chicago, IL, USA). Differences in bacterial counts before (S1) and after treatment (S2) were analyzed in each group by the Wilcoxon test. Comparisons between groups were performed by

Table 1

Intracanal *E. faecalis* quantification [Mean (standard deviation - SD)] before (S1) and after root canal preparation (S2) and total bacterial percentage reduction for qPCR analysis, observed in all groups

Groups	Sample		p**	Total bacterial reduction
	S1	S2		
G1 ProTaper Next	$5.96x10^5$ ($1.13x10^6$)[a]	1.52x103 ($1.15x10^3$)[b]	p=0.003	$8.02x10^5$ ($1.36x10^6$)[A]
G2 XP-Endo Shaper	$8.26x10^5$ ($1.02x10^6$)[a]	5.92x104 (1.68x104)[b]	p=0.001	$4.42x10^5$ ($6.19x10^5$)[A]
G3 Reciproc Blue	$9.36x10^5$ ($7.06x10^5$)[a]	8.16x103 (1.23x104)[b]	p=0.009	6.54x105 ($7.81x10^5$)[A]
p*	-	-	-	p=0.176

p: comparison between groups. Different uppercase letters indicate statistically different values (p<0.05) between groups.*
*p**: comparison between S1 and S2 within each group. Different lowercase letters indicate statistically different values (p<0.05) between S1 and S2 samples.*

Kruskal-Wallis test. Significance level for all analyses was p<0.05.

Results

Tables 1 reveals the bacterial percentage reduction qPCR analysis before (S1) and after root canal preparation (S2), observed in all groups. All endodontic systems presented effective bacterial reduction. However, despite that bacterial levels were reduced after preparation with ProTaper Next (p=0.003), XP-Endo Shaper (p=0.001) and Reciproc Blue (p=0.009), all groups still had bacterial growth. Protaper Next was the most effective system, whereas preparation with XP-Endo Shaper showed the highest post-preparation bacterial levels. Nevertheless, no significant difference between the evaluated groups was demonstrated (p=0.176).

Discussion

In the present study, it was revealed similar bacterial decontamination values promoted by ProTaper Next, XP-Endo Shaper and Reciproc Blue systems preparation. Therefore, despite presenting considerable differences (e.g. cross-sectional design, tapers, kinematics, manufacturing process, alloy processing and heat treatment), all systems were individually effective in mechanical preparation of severely curved root canals. In addition to that, although all file systems showed efficient decrease of E. faecalis counts [ProTaper Next (p=0.003), XP-Endo Shaper (p=0.001) and Reciproc Blue (p=0.009)], which can be translated into adequate mechanical action of instruments in dentinal walls, none of them was able to render severely curved mesiobuccal canals of mandibular molars completely free from bacteria. This finding is in accordance with previous researches (1, 2, 4, 15) and highlights the need for the development of new instruments capable of increasing the cleaning of root canal complexities and irregularities.

The comparison in bacterial reduction of ProTaper Next, XP-Endo Shaper, and Reciproc Blue in severely curved canals had not been ranked in literature yet. Nevertheless, this result consists with the findings of recent studies that concluded that Reciproc Blue and XP-endo Shaper instrumentation presents no differences with respect to the bacterial reduction in oval-shaped canals (9, 16). Also, the disinfecting abilities of Reciproc Blue and XP-endo Shaper also showed similar results in an association between micro-computed tomographic and histobacteriologic approaches (11).

ProTaper Next is a multi-file system fabricated with a NiTi M-Wire alloy that also presents a rectangular cross-section design with an increasing and decreasing tapering. This configuration permits to mold root canals asymmetrically by a continuous asymmetrical rotational kinematics, simi-

lar to snake movements, which improves the modeling effectiveness of root canals (17). In XP-Endo Shaper system, the Max-Wire alloy is used so that files can achieve greater flexibility and resistance to cyclic fatigue. In addition to that, this file has a reinforcing tip (Booster Tip) that allows to start shaping the root canals with an initial diameter smaller than the original diameter (5). According to the manufacturer's instructions, at 37 °C, the instrument is capable of expanding from an initial taper of 30/0.01 to a final canal preparation of a minimum of 30/0.04, adapting to the morphology irregularities of the root canal system. Lastly, Reciproc Blue system presents a S-shaped cross-section that allows deeper cutting and favors the removal of smear layer and debris (12). Its NiTi wire processing method uses a visible layer of titanium oxide that results in a distinct blue color that changes its molecular structure to generate greater flexibility and resistance to cyclic fatigue (10). The methodology applied in the current study presents some features that should be addressed. The intrinsic heterogeneity of root canals morphology between specimens is largely known as a notorious biological bias. It is important to emphasize, however, that efforts by radiological analysis and anatomy classification were undertaken to ensure a reliable comparison of groups. This stage reduced anatomical biases and allowed to increase the internal validity of this study (5, 6, 15). Furthermore, severely curved mesiobuccal canals from mandibular molars were selected for this study due to the considerable challenge that they represent for proper cleaning and disinfection (18, 19). The high prevalence of isthmus in mesial root canals of mandibular molars should also be highlighted (20).

The number of bacteria in an endodontic infection is restricted, predominating facultative or strictly anaerobic microorganisms (21). For this study, only one bacteria specie, *E. faecalis*, was used. This isolated standard strain was selected based on its survival characteristics associated with its prevalence in cases of endodontic failure, and for being widely used in previous studies with similar aim (4, 6, 21, 22).

In contrast to bacterial culture-dependent analysis, molecular assays such as qPCR present high sensitivity, since they allow the amplification of bacterial DNA in low amounts (1, 6). Also, qPCR method presents the ability to detect bacteria in their stationary stage (1). Under stress, the resistant bacteria selected for this study, *E. faecalis*, is capable of entering into stationary phase; being viable but undetectable in conventional CFU counts (1, 23). To date, a limited number of works used qPCR for the evaluation of mechanical bacterial reduction of root preparation methods (6-8).

Finally, the limitations of this study should also be pointed. Considering that only mesiobuccal canals were used in this study and the presence, size and volume of isthmus were not considered, this anatomy can influence the results in a certain way. Isthmus represents a real system with connections between roots canal (20), what may reflect in the remaining contamination of each group. Also, aiming to isolate the action of instruments from the chemical action of irrigating solutions, solely sterile saline solution was used during root canal preparation (2, 4, 22). This permitted the observation of isolated mechanical action of endodontic files, but did not reflect the clinical conditions of endodontic treatment. Further studies could use using molecular microbiological analysis for investigating the bacterial decontamination results of other endodontic systems and/or using different irrigating solutions.

Conclusions

ProTaper Next, Reciproc Blue and XP-Endo Shaper presented similar mechanical reduction of intracanal bacteria. No file system was capable of rendering severely curved canals completely free from bacteria.

Clinical Relevance

This study evaluated by a molecular microbiological analysis for the first time the mechanical reduction of intracanal bacteria produced by ProTaper Next, XP-Endo Shaper and Reciproc Blue in severely curved canals. Despite consid-

erable differences, endodontic systems demonstrated similar results.

Conflict of Interest

The authors affirm that this paper has been submitted solely to Giornale Italiano di Endodonzia and it is not published, in press, or submitted elsewhere. The authors deny any other conflict of interest.

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We have no financial affiliation (e.g. employment, direct payment, stock holdings, retainers, consultant ships, patent licensing arrangements or honoraria), or involvement with any commercial organization with direct financial interest in the subject or materials discussed in this manuscript, nor have any such arrangements existed in the past three years of beginning the submitted work that could inappropriately influence, or be perceived to influence, this work.

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