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Molecular Analyses of Bacterial Elimination in Infected Root Canals Using Reciprocating Single File and Rotary Instrumentation-A Clinical Study

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Abstract

Objectives: The aim of this study was to quantify the bacterial taxa and P. micra before and after RCT by using Real Time (RT)-PCR assay and to compare the bacterial reduction promoted by two instrumentation techniques i.e reciprocating single file and rotary multi-file systems.

Methods: A total of 32 patients with single rooted teeth were selected and divided into 2 groups of 16 patients each. After following standardized protocol of administering local anesthesia and rubber dam isolation, access cavity preparation was done and tooth were instrumented using either single file (Reciproc) and multiple file (Pro-taper Next) system. The irrigants used in both groups were 2.5% sodium hypochlorite. DNA extracts for samples taken before and after instrumentation were subjected to quantitative analysis by Real time Polymerase Chain Reaction.

Results: Intragroup analysis showed that both protocols promoted a highly significant bacterial reduction ($p<0.001$). Intergroup analysis showed significant differences between two instrumentation systems ($p<0.05$).

Keywords: *P. Micra;* Polymerase Chain Reaction; Reciprocating Instruments; Rotary Instruments

Introduction

Endodontics has become an increasingly routine aspect of patient care in general dental practice. The knowledge and understanding of the nature of the endodontic microbiota depends upon the recognition of the microorganism present in the root canal system. The presence of bacteria is the main cause for the development of periodontal infection and apical periodontitis [1]. Modern endodontic treatment procedures aim to eliminate these microorganisms during root canal preparation and disinfection. It is of utmost importance to disclose the main bacterial taxa that can endure endodontic antibacterial steps so as to understand their role in treatment outcome [2]. Parvimonas micra previously called peptostreptococcus micros or Micromonas micros is a gram positive obligate anaerobic coccus isolated more frequently in patients with periodontitis and endodontic abscesses [3,4]. P.micra is the most common bacteria, the hyaluradinase produced by it plays an important role in damage of periapical tissues. At the same time, it generates a lot of hydrogen sulfide and is closely related to stink of the root canal [5].

Molecular methods and other research techniques have helped detect and identify many endodontic pathogens. The 16S ribosomal RNA (r RNA) gene sequencing approach has emerged as a more effective, precise and reliable means for the identification of cultivable bacteria that cannot be accurately identified by phenotype-based tests [6,7]. Chemo mechanical preparation promoted a highly significant reduction in intracanal bacterial counts. Many rotary instrumentation systems are available to achieve these goals [8,9]. Recently, single file techniques for root canal instrumentation have been proposed because of convenience and alleged simplification [10,11]. These files are made of a special Ni-Ti alloy called M wire that is created by innovative thermal treatment process. However, there are only a few studies identifying the bacteria persisting after treatment procedure as well as comparing the effectiveness of single file instrumentation technique over multifile systems [12,13]. Hence, the aim of the study was to quantify and compare the bacterial taxa before and after RCT by using Real Time (RT)-PCR assay

and to compare the percentage of bacterium P. micra reduction, promoted by two instrumentation techniques i.e single file and multifile systems. The null hypothesis states that, there will be no significant difference in intracanal bacterial reduction promoted by the different instrumentation techniques.

Materials and Methods

Patient Selection

Samples were selected from 32 patients, age ranging from 18 to 60 years that had been referred for root canal treatment to the department of Endodontics. The study protocol was approved by the college ethical committee and informed written consent was obtained from the patients.

Inclusion Criteria

a) Single rooted and single canal teeth with intact pulp chamber walls with fully formed apex.

b) Teeth with carious lesions, necrotic pulps (confirmed by clinical evidence) and sensibility test with or without radiographic evidence of asymptomatic apical periodontitis.

Exclusion Criteria

i. Grossly destructed teeth.

ii. Teeth with root or crown fracture.

iii. Teeth subjected to previous endodontic treatment.

iv. Patients who received antibiotic therapy within the previous 3 months.

v. Symptomatic teeth and patients with periodontal pockets deeper than 4mm.

vi. Patients with complicating systemic diseases.

Microbial Sampling

Endodontic treatment and sampling procedures were performed by the same endodontic specialist.

A total of 32 patients, fulfilling the above inclusion criteria were divided into two groups of 16 patients each –

a) Group I- Single file system – Reciproc (VDW, Munich, Germany).

b) Group II- Multi-file system – ProTaper Next (Dentsply/ Maillefer).

The standard procedure includes administration of local anaesthesia with 2% xylocaine with 1:200000 epinephrine, using 27 gauze needles. Teeth were isolated using rubber dam and caries/defective restorations were removed. An endodontic access cavity was established using a sterile round carbide bur and Endo Z bur(Dentsply International, York, PA). A sterile K-file of size #15 (Mani, Inc, Japan) was used to determine the working length by radiograph and apex locator (Ray-Pex 5, Dentsply, UK) standardized 0.5-1mm short of the apex. Root canal samples were taken following strict aseptic measures, which included rubber dam isolation and two-step disinfection protocol of the operative field with sequential use of 6% Hydrogen Peroxide, 2% Iodine, 6% Hydrogen Peroxide and then 2.5% Sodium Hypochlorite (NaOCl). This disinfection protocol was applied before and after completing the access preparation. A solution of 5% sodium thiosulphate was used to inactivate residues of both iodine and NaOCl. A microbiologic sample was taken from the root canal immediately before instrumentation (S1sample). Sterile saline solution was placed in the pulp chamber without overflowing and a small instrument # 10 was used to carry the solution into the canal. The root canal was gently filed with #15 instrument, so as to suspend the canal contents in saline. Three sterile paper points of size 25 were consecutively placed in the canal to a level approximately 1mm short of radiographic root apex. Each paper points were left in the canal for about 1 min.

Root canal instrumentation was completed in the same appointment in all cases. Root canals were instrumented with reciprocating system upto R40 (40 with 6% taper) and in ProTaper Next group upto X4 (i.e 40 with 6% taper). Patency of the apical foramen was confirmed with a #10 file throughout the procedure. For irrigation, 2ml of 2.5% NaOCl was delivered using disposable syringes and NaviTip needles (Ultradent, South Jordan, UT, USA) which was inserted up to 4mm short of the working length. Smear layer was removed by rinsing the canal with 2ml of 17% EDTA and 5ml of 2.5% NaOCl. The total volume of NaOCl irrigation for each tooth was approximately 15ml. The canal was dried using sterile paper points and then flushed with 5ml of 5% sodium thiosulphate to inactivate NaOCl. Following which, a post preparation sample (S2) was taken from the canal as outlined for S1 sample. The samples were transferred in vials containing reduced transport fluid to microbiology laboratory (Credora Life Sciences, laboratory, Bangalore) within 24hrs and were subjected to 16S r RNA gene sequencing and Syber Real Time (RT) PCR. The root canals were obturated in the same appointment with .06 taper guttapercha (Dentsply, Maillefar, India) and accessory cones .02 taper (Dentsply, Maillefar, India) using AH Plus sealer (Dentsply, Konstanz, Germany)

DNA Isolation Protocol

The samples in the saline buffer were vortexed. 600 µl of the extraction buffer (Tris-EDTA) was added to the sample and incubated at 55 °C for 3 hours. Equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to the tube and vortexed for 30 seconds. The tubes were centrifuged at 12,000 rpm for 10 minutes. The aqueous phase was removed and taken in a sterile microcentrifuge tube, to which. 0.6 volumes of isopropyl alcohol were added and incubated at -200 C for 1hour. The tubes were centrifuged at 10000 rpm for 10 min, the supernatant was discarded. The pellet was washed in 500µl of 70% ethanol and centrifuged at 10000 rpm for 10 min. The pellet then was dried and dissolved in 20 µl sterile distilled water and were stored at 0 -200 C.

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Primer Synthesis and Validation

The primers for quantification analysis were designed using Perkin Elmer Primer Express® software (Massachusetts, USA). Primers used for quantification and P. micros evaluation included Forward Primer 5' ACTCCTACGGGAGGCAGCAGT 3' and 5' TGAGCAACCTACCTTACACAG 3'and a reverse primer 5' TAATTACCGCGGCTGCTGGC 3', 5' GCCCTTCTTACACCGATAAATC 3'. A gradient PCR was performed to standardize the optimum annealing temperature of the designed primer using 50 ng of synthesized cDNA keeping the temperature range at 50 – 60 oC.

Real-time PCR (qPCR) Analysis

The primer amplified specific amplicon size of 200bp from all DNA samples using P. micra specific primers. No nonspecific amplification was present in any of the sample tested. All reactions were performed in duplicates against a serially diluted standard. 16S ribosomal RNA (r RNA) gene sequencing analysis was done using BLAST (Basic Local Alignment Search Tool) analysis. BLAST similarity search in NCBI revealed 99% similarity to P. micra.

Statistical Analysis

Statistical analysis was done using IBM SPSS statistics 20.0 software (IBM Corporation, Armonk NY, USA). The Wilcoxon signed rank test was used to compare the intragroup reduction in bacterial counts from S1 to S2. In addition, intergroup comparison was accomplished using the Mann Whitney U test. Baseline (S1) bacterial counts differed between the groups, which is by large expected in clinical studies and this variable is virtually impossible to control. Transformation in log numbers did not succeed in normalizing S1counts. Therefore, the proportion of S1 represented by S2 was calculated and the percentages were used for comparison between groups. Significance level for all tests was set at P<0.05.

Results

Intragroup quantitative analysis evaluating the bacterial reduction from S1 to S2 in both groups demonstrated that chemomechanical preparation promoted a highly significant bacterial reduction (p<0.001). The mean bacterial count reduction (S1-S2) for Pro Taper Next and Reciproc group was 12,486235.28 and 5685827.75 respectively. The mean percentage of total bacterial reduction between groups was 95.7 % and 43.63%. Significant reduction of P.micra was seen in Pro Taper Next group (70%) compared with Reciproc (65%). Quantitative data are summarized in (Table 1).

Table 1: Comparison of the bacterial count and P micros values in terms of {Mean (SD)} before and after root canal treatment in Protaper Next &Reciproc group using Mann Whitney U test. (p< 0.05 - Significant*, p < 0.001 - Highly significant**).

	Group	N	Mean	Std. Deviation	Z value	P value
Pre RCT	Reciproc	16	13029048	±12775215.461	2.751	0.006*
	Protaper next	16	13046684	±40826130.550		
Post RCT	Reciproc	16	7343221	±7201130.993	3.694	<0.001**
	Protaper next	16	560448.6	±1147428.222		
P.micros Pre RCT	Protaper next	16	11.1008	±9.52553	3.505	<0.001**
	Reciproc	16	52.4494	±54.99732		
Post RCT	Protaper next	16	3.2288	±2.97545	3.769	<0.001**
	Reciproc	16	18.2531	±18.56838		

Discussion

An optimal outcome of root canal treatment will depend on maximal reduction in the bacterial load to levels compatible with periradicular tissue healing [12]. It is important to use highly sensitive methods to quantify reduction in intracanal bacterial populations. In this study quantitative intragroup comparison between rotary and reciprocating Ni-Ti instrumentation showed significant difference between them in reducing bacterial counts. This finding demonstrated that regardless of the type of instrument, bacterial elimination will be comparable providing both enlargement and irrigation parameters of the canal are similar. This is in agreement with other studies showing that chemo-mechanical debridement is of paramount importance to reduce the infectious bioburden in the main root cana [l4,15]. However, this in vivo study also supports previous studies showing that a large proportion of cases still harbour detectable cultivable bacteria after instrumentation/irrigation in S2 samples in q-PCR analysis. These results confirm the need highlighted by other studies for supplementary disinfection after chemomechanical preparation [15]. Parvimonas micra is a gram-positive anaerobic coccus involved in the pathogenesis of primary periradicular diseases. The virulence traits of P.micra include strong proteolytic enzymatic activity, hyaluronidase, capsule and volatile sulphur compounds (hydrogen sulphide) [16,17]. It also plays an important role in the ecosystem, which can make amino acids and peptides available from serum glycoproteins to be used not only in own metabolism but also in the metabolism of other bacterial species with little or no proteolytic activity in serum. In infected canal, P.micros has been found positively associated with other predominantly found anaerobic bacteria [18].

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Ni-Ti instruments produced a different dentine surface on root canal walls. SEM analysis revealed that conventional rotating files associated with EDTA and NaOCl irrigation leave dentine surfaces free from smear layer [19-21]. The combination of NaOCl and EDTA was probably responsible for the removal of smear layer and for the removal of great portion of circumferential dentine collagen and mineralized dentine wall from most of the tubules, as confirmed by Foschi [19]. This means that an absence of smear layer and the presence of clean dentine walls reduces the bacterial counts. This study showed that both the systems reduced the number of bacteria within the root canal. Similar results were observed by Singla [22] when canals were debrided using conventional technique, rotary ProTaper, or Profile instruments. Therefore, all instrumentation systems seem to have similar effectiveness, making it reasonable to choose debridement instruments and preparation techniques based on other factors or considerations, such as flexibility, or on the dexterity or preferences of endodontic professionals. All Ni-Ti rotary instruments have been shown to produce a moderate to heavy smear layer that needs to be removed with the use of irrigating solutions. Chelating agents like EDTA are currently used to remove the smear layer formed during preparation of root canals [23].

Comfortable results are shown also by innovative single-use Reciproc files, which are designed for root canal shaping with only one instrument. This means that the goal of simplifying the technique and reducing the overall duration of the treatment is respected. But in the literature, there are few studies on the effectiveness of reciprocating files in removing the smear layer and bacteria from root canal walls. Only a study by Burklein [24] has investigated the cleaning effectiveness of reciprocating single –file systems and he never obtained completely cleaned canals. One concern about the single file instrumentation techniques refers to their ability to disinfect the root canal. This is because of the claimed simplification and expediting of the preparation process, which may result in less of antibacterial irrigant being used, the perceived short time of antibacterial irrigant presence in the canal [25]. In this study, though the apical preparation and taper (40 ,6%), volume of irrigants were standardized for both the groups, rotary (Protaper Next) performed better than Reciproc group. This must be because, the frequency of irrigation was difficult to standardize because of different numbers of instruments used in this group. The other reason could be the longer duration of the antibacterial irrigant presence in the canal.

The present study describes development of PCR primers specific for P.micra and their application in a PCR assay for detection of this organism. Use of PCR in the field of molecular diagnostics is now accepted as the standard method for detecting nucleic acids from a number of sample and microbial types [26]. RT-PCR is also a particularly attractive alternative to conventional PCR for the study of microbial load because of its low inter-assay and intra-assay variability, its equivalent or improved sensitivity and ability to detect and quantitative not only cultivable bacteria but also culture-difficult species, bacteria in a viable but noncultivable (VNBC) state and as yet uncultivable species [27]. The 16S ribosomal RNA (r RNA) gene sequencing approach has emerged as a more effective, precise and reliable means for the identification of cultivable bacteria that cannot be accurately identified by phenotype- based tests [28]. To the best of our knowledge, only one study has identified cultivable bacteria in endodontic infections by means of 16S rRNA gene sequencing analysis, but it evaluated only five samples. This study has its own limitations. Only single-rooted single canal teeth were included, because asepsis during sample taking is easier to control from the main large root canal when compared with narrow canals. However, it is likely that in molars with more complex canal anatomy or in teeth with oval canals, the magnitude of bacterial reduction might have been different. Also, the recognized limited ability of paper points to collect a representative sample from the root canal systems makes the information on bacterial counts restricted to the main canal [29].

Conclusion

Both the Ni-Ti instrumentation efficiently reduced the bacterial counts and significant differences were found between them. Single use files can offer antibacterial results.

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