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Comparative evaluation of anti-bacterial efficacy of two different chemical irrigation protocols against *Enterococcus faecalis* in an infected root canal model by using polymerase chain reaction- an *in vitro* dental study

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The success of endodontic treatment depends upon the eradication of microbes from the root-canal system and the prevention of reinfection. Optimal irrigation is based upon the combined use of 2 or more irrigating solutions, in a specific sequence in order to achieve the goals of safe and effective irrigation. Materials Andmethods; 20 extracted Human lower premolars stored in 0.5% sodium hypochlorite at 4 ^{0}C were taken. The teeth were instrumented with Protaper rotary system up to (F3) after which the root canals were sterilized by autoclaving for 20 min at 121 ^{0}C. After which Enterococcus Faecalis (MTCC 2129) was incubated into all the samples for three weeks to form biofilm. Root canals were then randomly divided into 2 groups. Group 1(N-10) used ProRinse Endodontic Irrigation Probes (Dentsply). Group 2(N-10) Canal Brush (Coltene Whaledent Germany) After 24hrs the dentin shavings were taken using sterile gates glidden size 3 and were stored in sterile aluminium foils. The dentine chips were kept in a glass bottle containing 2 ml phosphate buffered saline and they were transferred into agar plates and the microbial load was calculated. Semiquantitative densitometry analysis of amplified CDNA bands was used to analyze the antimicrobial activity.

Results; Results of our study showed that maximum antimicrobial activity was achieved in group 2. The microbial load was only 8% after 24 hrs in group 2 while in group 1 the microbial load was 43% after 24 hrs.

Conclusion; During cleaning and shaping procedure a combination of effective irrigation and micro brush activation will effectively remove debris from root canals, thereby it reduces the bacterial load PCR can be used as a valuable tool for microbial identification.

Keyword: *Enterococcus Faecalis*, dentinal shavings, root canal, biofilm.

1. Introduction

The success of endodontic treatment depends upon the eradication of microbes from the root-canal system and the prevention of reinfection [1]. The root canal is shaped with hand and rotary instruments under constant irrigation in order to remove the inflamed and necrotic tissue, microbes, biofilms and other debris from the root canal space [2]. The main

goal of instrumentation is to facilitate effective irrigation, disinfection and obturation. Several studies using advanced techniques such as microcomputed tomography (CT) scanning have demonstrated that proportionally large areas of the root-canal wall remain untouched by instrumentation thereby emphasizing the importance of cleaning and disinfecting all the areas of the root canal space [1, 3].

At present, there is no single irrigating solution that has all the requirements of an ideal irrigant. Optimal irrigation is based upon the combined use of 2 or more irrigating solutions, in a specific sequence in order to achieve the goals of safe and effective irrigation. Irrigants have traditionally been delivered into the root-canal space using syringes and metal needles of different sizes and tip design. Clinical experience and research have shown, however, that this classic approach typically results in ineffective irrigation, particularly in areas such as between the canals, fins and the apical most part of the root canal. Therefore, many of the solutions used for irrigation have been chemically modified and also several mechanical devices have been developed to improve both the penetration and effectiveness of the irrigation solutions [1, 4].

The effectiveness of endodontic files, rotary instrumentation, irrigating solutions and chelating agents to clean, shape and disinfect root canals underpins the success, longevity as well as the reliability of modern endodontic treatment modalities. To prevent endodontic treatment failure irrigation is mandatory for the effective removal of smear layer, pulp tissue and microorganisms. Irrigation dynamics also play an important role, and the effectiveness of irrigation depends upon the working mechanism of the irrigant as well as the ability to bring the irrigant in contact with the microorganisms and the tissue debris present inside the root canal complex [5].

Careful removal of the vital and necrotic remnants of pulp tissues, microorganisms and microbial toxins from the root canal system is essential for endodontic success. This can be achieved through chemo-mechanical instrumentation. However, root canals are irregular and complicated systems that are often difficult to clean. Although, during root canal therapy, we rely primarily on instrumentation for canal debridement, irrigation is also an important adjunct. Irrigation facilitates the cleaning of the root canal system by flushing out debris as well as serving as a bactericidal agent, tissue solvent and lubricant [6].

Debris in the root canal space is usually present in the form of dentin chips, residual vital and necrotic pulp tissue, which is loosely attached to the root canal wall and in most cases is infected [4, 7]. The presence of debris on the prepared root canal surface prevents the efficient removal of microorganisms which is one of the major goals of debridement. Furthermore, after instrumentation a smear layer is formed which is a surface film 1- to 2-mm thick that remains adherent to the root canal wall. This smear layer consists of dentin particles, pulp tissue, bacterial components as well as retained irrigants and it occludes the dentinal tubular openings. The chemo mechanical action of sodium hypochlorite helps to remove loosely attached debris and organic material, whereas chelating agents help to remove the smear layer. Numerous studies have been done to evaluate the effectiveness of instruments and instrumentation techniques as well as irrigants and the methods of irrigation which are used in canal debridement. These studies have all demonstrated that debris remains in the root canal system after instrumentation and irrigation [4, 5, 7]. Recently a rotary hand piece attached micro brush has been used by Ruddle to facilitate debris and smear layer removal from the instrumented root canals [8].

2. Materials and Methods

20 extracted Human lower premolars stored in 0.5% sodium hypochlorite at 4 ^{0}C were taken the teeth were then radiographed to confirm canal patency and complete root formation. The teeth were decoranated to obtain 14 mm length #15 k-type file was inserted into the canal until the tip and the canals were instrumented with Protaper rotary system up to (F3) after which the root canals were sterilized by autoclaving for 20 min at 121 ^{0}C [9]. After which Enterococcus Faecalis (MTCC 2129) was incubated into all the samples for three weeks to form biofilm. 2 samples were randomly selected for bacterial penetration into the dentinal tubules which was then confirmed using a scanning electron microscope.

Root canals were then randomly divided into 2 groups. Group 1(N-10) used Pro Rinse Endodontic Irrigation Probes (Dentsply) The needle was advanced into the canal until it bound to the canal walls and then was retracted by 1 mm to allow easy back flow of the irrigating solution. Group 2(N-10) CanalBrush (Coltene WhaledentGermany) was mechanically activated in an active scrubbing action followed by irrigation with ProRinse Endodontic

Irrigation Probes (Dentsply) in each group one tooth was kept as negative control. Irrigation protocol was done by using 1ml of 5% NaOCl, {Sodium hypochlorite} followed by 1 ml of 17% EDTA (Ethylene diaminete traacetic acid) which was followed by 1 ml of 2% CHX in both study groups. All samples were then placed into sterile microplates and stored at 37 ^{0}C and 100% humidity for 24 hrs. After 24 hrs the dentin shavings were taken using sterile gates glidden size 3 and were stored in sterile aluminium foils. The dentine chips were kept in a glass bottle containing 2 ml phosphate buffered saline and they were transferred into agar plates and the microbial load was calculated.

2.1 Microorganism and inoculum preparation.

A suspension of 50 µl of *E. Faecalis* (ATCC 29212) strain was incubated in 5 ml of Trypticase Soy Agar broth (TSA) culture medium (Difco, Sparks, MD, USA) at 37 ºC in an incubator for 4 hours. The concentration of the inoculation was then adjusted to a degree of turbidity (0.5) according to the McFarland scale (BioMerieux, Marcyl' Etoile, France), which corresponds to a bacterial load of 3×10^8 cells/ml and an optical density of 600 nm

2.2 Real-time Polymerase chain reaction using species specific 16S RDNA primer for *E. faecalis*

The reaction mix was prepared to a final volume of 20 µl and loaded in a 96-well plate, which was then covered with an optical adhesive sheet.

2.3 The Real-time PCR assay was carried out in a thermal cycler (7900 HT Real-Time PCR system).

Components	Volume added (µl)
Milli Q Water	7 µl
Forward primer	1 µl
Reverse primer	1 µl
Template	1 µl
SYBR Green master mix	10 µl
Total volume	20 µl

2.4 Primer sequence

primer	Nucleotide sequence (5'-3')	
EF16 S	*Forward primer 5'-GATTAGATACCCTGGTA GTCCAC-3'* *Reverse primer 3'-TAAGGTTCTTCGCGTTGCTT-5'*	*320bp -*

2.5 PCR Cycling Parameters

Sample volume:- 20 µl

Number of cycles:- 35

Steps	Temperature (ºC)	Duration
Enterococcus faecalis		
Initial denaturation	90	5 minutes
Denaturation	95	1 minute
Annealing	60	15 seconds
Extension	72	1 minute

2.6 Semiquantitative densitometry analysis of amplified CDNA bands

Amplified CDNA were run on a 1.2% agarose/EtBr gel in 1x TAE (Tris-acetate-EDTA) buffer and then visualized under UV light. The density of each band on the agarose gel was measured using Image pixel quantitation software. Background measurements were subtracted, and a relative number was assigned to each band intensity.

2.7 Statistical Analysis

The results were analyzed using one-way analysis of variance (Anova). The percentage change was also statistically analyzed by Fisher exact test.

3. Results

The study showed that maximum antimicrobial activity was achieved in group 2. The microbial load was only 8% after 24 hrs in group 2 while in group 1 the microbial load was 43% after 24 hrs.

Group 1 Maxiprobe

Group 2 Canal Brush

Groups Treated	Microbial load after24hrs
Control	97%
Group 1 (Maxiprobe)	43%(100)
Group 2 (Canalbrush)	8%(100)

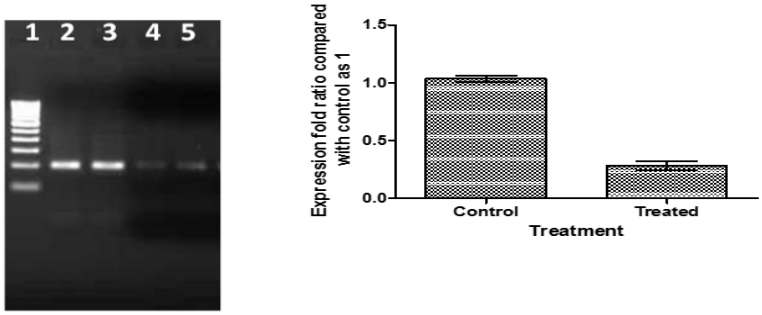

Fig 1: PCR Analysis: Semiquantitative densitometry analysis of amplified CDNA bands showing difference between control and treated samples

4. Discussion

Enterococcus faecalis is the most isolated or detected species from oral infections, including marginal periodontitis, infected root canals, periradicular abscesses and also detected in cases of failed endodontic therapy. *Enterococcus faecalis* are well adapted for the survival and persistence in a variety of adverse environments [11]. The root canal system is a complex structure and accessory features, such as fins, cul de sacs and intercanal communications that are easily colonized by microorganisms once the tooth becomes infected This may explain its survival in root canal infections where the availability of nutrients is scarce and there are limited means of escape from the root canal medicaments used [12, 13].

Sodium Hypochlorite has been the most widely used root canal irrigating solution for several decades due to its excellent properties of tissue dissolution and antimicrobial activity. But, also it has several undesirable characteristics such as tissue toxicity, allergic potential, disagreeable smell and taste. The use of Chlorhexidine as an irrigant is generally restricted because it can discolor the teeth and tongue, can cause loss of taste, burning sensation of the oral mucosa and subjective dryness of the oral cavity [14, 15]. Maxiprobe is uniquely equipped with a lateral vent that gently stirs the irrigant while quickly and safely flushing them up and out of the canal with flexible stainless steel construction and 30-gauge tip, the probe easily delivers the irrigating solution at optimal depths while preventing apical extrusion [4].

Endodontic microbrush is a spiral brush that consists of nylon bristles set in twisted wires with an attached handle which is similar to a rotary file handle. Canalbrush was mechanically activated in an active scrubbing motion in order to increase the efficiency of the canal brush [5, 8]. The microbrush rotates at about 300 rpm, causing the bristles to penetrate into the irregularities of the preparation. This helps to dislodge residual debris out of the canal in a coronal direction, this highly flexible microbrush is made entirely from polypropylene and might be used manually with a rotary action. However, it is more efficient when attached to a contra-angle handpiece.

The results showed that root canals with effective irrigation along with canal brush activation showed a marked reduction in the microbial load, the reason being that because of the rotary action of the brush coupled with the bristles were able to extend into the noninstrumented canal walls and the fins, cul-de-sacs, and isthmus of the canal where by removing the trapped tissue and debris enabling the reduction of the bacterial load. A recent report by Weise et al showed that the use of the small and flexible Canal Brush along with irrigant removed debris effectively from simulated canal extensions and irregularities [8].

The advantages associated with RT-PCR are the detection of not only cultivable species but also of uncultivable microbial species or strains. It has higher specificity and accurate identification of microbial strains with ambiguous phenotypic behavior, including divergent or convergent strains [16]. It detects microbial species directly in the clinical samples without the need for cultivation. It has higher sensitivity and is less time-consuming. RT PCR offers rapid diagnosis, which is particularly advantageous in cases of life-threatening diseases or diseases caused due to slow growing micro-organisms. The technique does not require carefully controlled anaerobic conditions during sampling and transportation, which is advantageous since fastidious anaerobic bacteria and other fragile micro-organisms can lose their

viability during transit. When a large number of samples are to be surveyed in epidemiological studies, the samples can be stored and analyzed all at once [16, 17, 18].

However, there are a few limitations associated with RT- PCR such as the detection of dead cells by a given identification method can be regarded as a double-edged sword. This can be both an advantage and also a limitation of the method. This ability can allow detection of uncultivable bacteria or fast growing bacteria that can die during sampling, transportation or isolation procedures [19]. Also, if the bacteria were already dead in the infected site, they may also be detected and this might give rise to a false assumption about their role in the infectious process. To minimize this potential problem some adjustments in the method for DNA detection or take advantage of the technology derivatives that detect RNA. As for modifications in DNA-based techniques, designing primers to generate large polymerase chain reaction (PCR) amplicons reduces the risks of positive results due to DNA from dead cells, because DNA remnants, if present, are usually fragmented [16, 20, 21].

Within the limitations of this study, we can conclude that during the cleaning and shaping procedure a combination of effective irrigation and micro brush activation will effectively remove debris from root canals, thereby it reduces the bacterial load PCR can be used as a valuable tool for microbial identification

5. References

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