# An *in Vivo* Comparison of Antimicrobial Efficacy of Sodium Hypochlorite and Biopure MTAD™ against *Enterococcus Faecalis* in Primary Teeth: A qPCR Study

Tulsani SG*/ Chikkanarasaiah N**/ Bethur S***

***Objectives:*** *Biopure MTAD™, a new root canal irrigant has shown promising results against the most common resistant microorganism, E. faecalis, in permanent teeth. However, there is lack of studies comparing its antimicrobial effectiveness with NaOCl in primary teeth. The purpose of this study was to compare the in vivo antimicrobial efficacy of NaOCl 2.5% and Biopure MTAD™ against E. faecalis in primary teeth.* ***Study design:*** *Forty non vital single rooted primary maxillary anterior teeth of children aged 4-8 years, were irrigated either with NaOCl 2.5% (n=15), Biopure MTAD™ (n=15) and 0.9% Saline (n=10, control group). Paper point samples were collected at baseline ($S_1$) and after chemomechanical preparation ($S_2$) during the pulpectomy procedure. The presence of E. faecalis in $S_1$ & $S_2$ was evaluated using Real time Polymerase Chain Reaction.* ***Results:*** *Statistical significant difference was found in the antimicrobial efficacy of NaOCl 2.5 % and BioPure MTAD™ when compared to saline* ***(p<0.05)****. However, no statistical significant difference was found between the efficacies of both the irrigants.* ***Conclusions:*** *NaOCl 2.5% and BioPure MTAD™, both irrigants are equally efficient against E. faecalis in necrotic primary anterior teeth. MTAD is a promising irrigant, however clinical studies are required to establish it as ideal root canal irrigant in clinical practice.*

***Key words:*** *Sodium hypochlorite, Biopure MTAD, Irrigant, Endodontic treatment, Primary teeth, Pulpectomy.*

## INTRODUCTION

An overwhelming evidence indicates that microbial agents are essential for the pulp pathology which further result in the progression and perpetuation of the periradicular inflammatory diseases.[1-3] Anaerobes, especially black-pigmented anaerobes are implicated in the development of periradicular inflammation, involving signs and symptoms such as pain, swelling, tenderness and exudation.[4] *Enterococcus faecalis,* a facultative anaerobe, is associated with the different forms of periradicular diseases including primary endodontic infections and persistent infections. It has been identified as one of the most difficult bacterial species to eradicate from the infected root canals and is a commonly recovered microbe in the failing root canals.[5] Such microorganisms that are located in the privileged and strategic positions within the root canal containing necrotic pulp tissue are difficult to eradicate in primary teeth; as the root canal morphology is complex and contains numerous ramifications and anatomical irregularities.[6] Therefore, chemical disinfection through irrigation becomes a critical adjunct to mechanical instrumentation.

Sodium hypochlorite (NaOCl), with its antibacterial and dissolving effects on the necrotic tissues, is the most popular and commonly used root canal irrigant. It has excellent antimicrobial and tissue dissolving properties. However, its main disadvantages are unpleasant taste and the high toxicity when injected into the periradicular tissues.[7-9] In the root canal treatment of deciduous teeth, NaOCl can compromise permanent tooth follicles, peripheral tissues and oral mucosa.[10] Hence, various alternatives to NaOCl are being studied. Recently, MTAD (a mixture of tetracycline isomer, an acid and a detergent) has been introduced by Torabinejad and Johnson[11] in 2003,[12] as a final rinse for the disinfection of the root canal system.[13] Studies have reported MTAD to be clinically effective[14] and biocompatible when used as an endodontic irrigant.[15] However, there are very few studies comparing its antimicrobial effectiveness with Sodium hypochlorite under in vivo conditions.

Many studies regarding the effect of irrigants on *E. faecalis* are conducted on permanent teeth, but there is insufficient data available

From the Department of Pedodontics and Preventive Dentistry,
Rajarajeswari Dental College and Hospital.
*Sneha G Tulsani, PostGraduate Student.
**Nagarathna Chikkanarasaiah, MDS, Professor and Head of Department.
*** Shakuntala Bethur S, MDS, Professor.

Send all correspondence to:

Sneha. G.Tulsani, PostGraduate Student,
Department of Pedodontics and Preventive Dentistry,
Rajarajeswari Dental College and Hospital,
Bangalore 560074. Karnataka State. India.

Phone : +91 9535644942

E-mail : dr.sneha_tulsani@yahoo.in

on primary teeth. Most of the studies conducted are in vitro, and have been reported based on culturing techniques, which are time consuming and have low diagnostic sensitivity. Alternative methods for identification of *Enterococci,* such as Polymerase chain reaction (PCR) have been proposed which can overcome the aforementioned shortcomings. Detection of *E. faecalis* by culturing method has shown lower percentages (24-70%), as compared to consistently higher percentage (67-82%), when a PCR detection method is used.[16] Hence, the aim of this *in vivo* study was to compare the efficacy of Sodium hypochlorite 2.5% and Biopure MTAD™ against *E. faecalis* in necrotic primary maxillary anterior teeth undergoing pulpectomy using PCR analysis.

## MATERIALS AND METHOD

The present *in vivo* study was conducted on 40 primary maxillary anterior teeth. The study population consisted of systemically healthy children requiring endodontic treatment with an age range of 4-8 years. All participants were selected randomly from the outpatient section, Department of Pedodontics and Preventive Dentistry, Rajarajeswari Dental College and Hospital, Bangalore, India. The study was approved by institutional ethics and research committee, Rajarajeswari Dental College and Hospital, Bangalore, India. The purpose of the study was explained to the parent of each child and a written informed consent was obtained before inclusion in the study. The selection of the teeth was made according to the clinical and radiographic diagnosis of pulp necrosis. The inclusion criteria consisted of asymptomatic primary maxillary anterior teeth with pulp necrosis, teeth with intact roots or less than 2/3rd of physiological root resorption, teeth with no periodontal pockets or operative intervention of the root canals and patients with no significant systemic conditions. The exclusion criteria comprised of teeth with excessive root resorption and mobility, teeth requiring pulp therapy due to periodontal problems, developmental anomalies or traumatic injuries, children with special health care conditions, patients who have received any antibiotics for at least 3 months before treatment.

### Sample Collection

An aseptic environment was maintained throughout the procedure. Each tooth showing presence of supragingival biofilm was cleansed by scaling and caries was removed with sterile high-speed and low-speed burs. The teeth were isolated under rubber dam and access opening was done with sterile water cooled high speed diamond round bur (#330). After completing access with 0.9% sterile saline irrigation, the operative field, including the coronal pulp chamber was cleaned and disinfected by vigorous swabbing with 2.5% NaOCl and then neutralized with sodium thiosulfate solution.

The first root canal sample **($S_1$)** was taken as follows. The canal was irrigated with 0.9% sterile saline solution and a sterile #20 K file was introduced to a level approximately 1mm short of root apex, based on diagnostic radiographs, and a gentle filing motion was applied. Sterile paper points were placed in the canal for at least 60 seconds to the same level to soak up the fluid in the canal. Paper points were placed into marked sterile Eppendorf tubes which contained transport media, 200µl of 10X TE buffer (Tris-HCl EDTA) and sent for PCR analysis.

Chemomechanical preparation was completed at the same appointment in all the cases. Canal preparation was completed to the working length, with hand nickel titanium files in a back and forth alternating rotation motion using stepback technique up to #40 to #50 size file. The teeth were irrigated with one of the following irrigant.

Control Group (10 teeth) – 0.9% Saline only using 5ml 24-gauge sterile needle and syringe.

Experimental Group I (15 teeth) – 0.9% sterile saline after each instrument size, followed by 5ml final rinse of 2.5% NaOCl solution, for 5 minutes using 24-gauge sterile needle and syringe. The canals were then flushed with 5ml of sodium thiosulphate to neutralize any residual NaOCl.

Experimental Group II (15 teeth) – Biopure MTAD was mixed according to manufacturer's instructions. The canals were irrigated with 0.9% sterile saline after each instrument size followed by final 5ml rinse of MTAD, for 5 minutes using 28 gauge needle with side vented bore which had come along with BioPure MTAD.

The second root canal sample **($S_2$)** was collected using sterile paper points as described previously. The paper points were transferred into transporting medium and sent for PCR analysis. Samples were stored at -70°C until processing in the laboratory. The canals were filled with zinc oxide eugenol paste.

DNA extraction from the paper point samples was done using highly purified Invitrogen DNA isolation kit (Purelink™ DNA extraction kit, Applied BioSystems, India). The standard "Proteinase K" method was followed for DNA isolation.

The extracted DNA was purified using a purification procedure designed for purifying genomic DNA using a spin column based centrifugation procedure on a total time of 10-15 minutes.

Custom SYBR® Green assay reagents for *E. faecalis* (Applied Biosystems, India) were used in this study. The Primer sequence specific to *E. faecalis* selected for the study was as follows:

*E. faecalis* forward – 3'-ATCAAGTACAGTTAGTCT-5'.

*E. faecalis* reverse –5'-ACGATTCAAAGCTAACTG-3'. In brief, a reaction solution composed of SYBR® Green Universal PCR Master Mix (10µl), forward primer (1µl) and reverse primer (1µl) for *E. faecalis*, extracted DNA of unknown sample (1µl) and nucleus free water to make a complete reaction volume of 20µl. The conditions for Real-Time PCR were as follows: Holding stage at 95°C for 10 seconds followed by 40 cycles of shuttle heating at 95°C for 15 seconds and at 60°C for 1 minute. The melt curve stage was at 95°C for 15 seconds, 60°C for 1 minute and 95°C for 15 seconds. 16S rRNA was used as an endogenous control. (SYBR® Green assay reagents, Applied Biosystems, India). Relative Quantification (RQ) for *E. faecalis* was based on the Ct (the number of PCR cycles necessary to obtain the threshold signal of fluorescence) values. All the calculations were done using Applied Biosystems Software.

### Statistical Analysis

The overall comparison of the pre instrumentation values and post instrumentation values in all the three groups were compared using Kruskal Wallis ANOVA test. The pair wise comparisons between the groups was done using Mann-Whitney U test. The comparison of pre instrumentation to post instrumentation values within each group was performed using Wilcoxon matched pairs test. The tests were done using SPSS software version 20.0. The level of statistical significance was set at 5% (□ < 0.05).

## RESULTS

In the present randomized controlled trial, qPCR was used to determine the presence of *Enterococcus faecalis* in the primary root canal infections. qPCR yielded the test organism in 55% of the root canal infections studied. The mean scores of the relative quantification of *E. faecalis* (RQ) in the pre-instrumentation samples analyzed by Kruskal Wallis ANOVA for Group I, II and control group were 1.37±1.59, 1.55±1.54 and 2.13±1.57 respectively (Table 1). No statistical significant difference was found between all the groups. Statistical reduction in the RQ of E. faecalis from pre-instrumentation to post-instrumentation was seen in the Group I and II. No statistical significant difference was found in the control group (Table 2) (Figure 1). Pair-wise comparison of the RQ of *E. faecalis* in the post-instrumentation samples by Mann-Whitney U test showed the least presence in Group II (RQ= 0.19±0.40). A statistical significant difference was found in the post-instrumentation score values between Group I and control group; and between Group II and control group. No significant difference was found between Group I and II (Table 3) (Figure 2).

Table 1: Comparison of three groups (Group I, Group II and control group) with respect to pre-instrumentation scores by Kruskal Wallis ANOVA.

| Groups | Mean | SD | SE | Sum of ranks |
|---|---|---|---|---|
| Group I | 1.37 | 1.59 | 0.41 | 281.00 |
| Group II | 1.55 | 1.54 | 0.40 | 295.50 |
| Control group | 2.13 | 1.57 | 0.50 | 243.50 |
| F-value | | 1.6500 | | |
| P-value | | 0.4380 | | |
| **Pair wise comparison by Mann-Whitney U test** | | | | |
| Group I vs Group II | | P=0.8035 | | |
| Group I vs Control group | | P=0.2555 | | |
| Group II vs Control group | | P=0.3181 | | |

Figure 1: Comparison of pre and post instrumentation scores in three groups (Group I, Group II and control group).

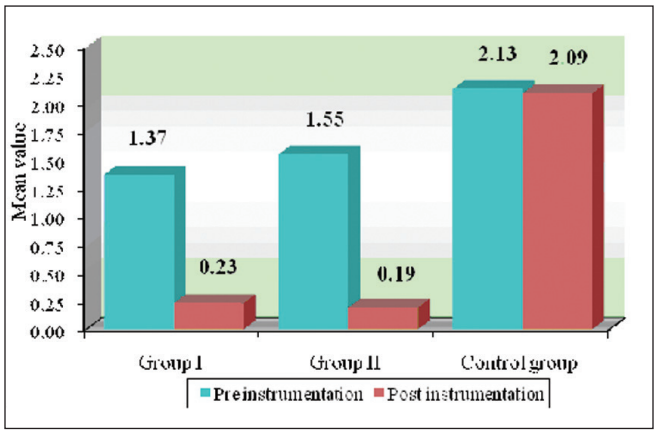


Figure 2:Comparison of three groups (Group I, Group II and control group) with respect to post-instrumentation scores.

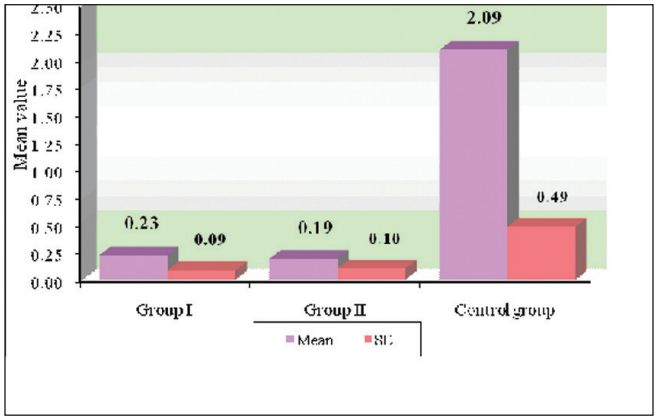


Table 2: Comparison of pre and post instrumentation scores in three groups (Group I, Group II and control group) by Wilcoxon matched pairs test.

| Group | Test | Mean | SD | MeanDiff. | SD Diff. | % of change | Z-value | p-value |
|---|---|---|---|---|---|---|---|---|
| Expt Group I | Pre instrumentation | 1.37 | 1.59 | | | | | |
| | Post instrumentation | 0.23 | 0.34 | 1.14 | 1.31 | 83.41 | 2.3664 | 0.0180* |
| Expt Group II | Pre instrumentation | 1.55 | 1.54 | | | | | |
| | Post instrumentation | 0.19 | 0.40 | 1.36 | 1.36 | 87.55 | 2.5205 | 0.0117* |
| Control group | Pre instrumentation | 2.13 | 1.57 | | | | | |
| | Post instrumentation | 2.09 | 1.54 | 0.04 | 0.07 | 1.69 | 1.3522 | 0.1763 |

*p<0.05

Table 3: Comparison of three groups (Group I, Group II and control group) with respect to post-instrumentation scores by Kruskal Wallis ANOVA.

| Groups | Mean | SD | SE | Sum of ranks |
|---|---|---|---|---|
| Group I | 0.23 | 0.34 | 0.09 | 281.50 |
| Group II | 0.19 | 0.40 | 0.10 | 246.50 |
| Control group | 2.09 | 1.54 | 0.49 | 292.00 |
| F-value | 8.9890 | | | |
| P-value | 0.0110* | | | |
| **Pair wise comparison by Mann-Whitney U test** | | | | |
| Group I vs Group II | P=0.5069 | | | |
| Group I vs Control group | P=0.0198* | | | |
| Group II vs Control group | P=0.0126* | | | |

## DISCUSSION

Successful root canal therapy depends on the chemomechanical debridement of pulpal tissue, dentin debris and infective organisms. The use of irrigants becomes essential as they can augment mechanical debridement thus rendering a successful root canal treatment. Although a number of different *in vitro*, *ex vivo* and *in vivo* approaches have been used in an effort to determine the various disinfecting agents against the involved microorganisms, no irrigant agent has been considered to be the ideal agent. It is therefore understandable that researchers are currently interested in the promising perspectives that the newly developed irrigating agents have to offer.

In the present study, a total number of 40 primary maxillary anterior teeth fulfilling the inclusion and the exclusion criteria were selected. The study assessed and compared the antimicrobial efficacy of Sodium hypochlorite 2.5 % and Biopure MTAD against E. faecalis in necrotic primary maxillary anterior teeth using qPCR analysis. *E. faecalis* was selected as the test organism because of its implication as a possible factor therapy resistant microbial factor in chronic infections. Also, previous in vitro studies conducted on permanent teeth have reported MTAD as an effective final irrigating solution for eradication of *E. faecalis.*[13,17,18] Necrotic primary anterior teeth due to early childhood caries were selected, as *E. faecalis* has been identified as the persistent microorganism present in such conditions.[19,20,21] In our study, *E. faecalis* was detected from 55% of the total root canal infections which was consistent with the other studies that have showed a prevalence up to 63%.[19,20,21] The high detection rate of *E. faecalis* (55%) seen in this study could be because of the exposure of the primary necrotic anterior teeth to the oral cavity due to early childhood caries in most of the patients.

Real Time PCR was used for the detection of *E. faecalis* in our study because of its advantages over culturing, methods and conventional PCR which include the rapidity of the assay, the ability to quantify and identify PCR products directly without the use of agarose gels, and the fact that contamination of the nucleic acids is limited because of avoidance of post-amplification manipulation.[22]

The choice of 2.5% concentration of NaOCl in our study was based on the fact that no significant differences in the intracanal antibacterial effects have been observed when comparing it with the higher concentrations.[23] Copious irrigation with NaOCl may maintain a chlorine reserve that is sufficient to eliminate bacterial cells and compensate for the effect of concentration.[23] MTAD was used as the sole irrigant (without initial use of 1.3% NaOCl) in the experimental group II. This was selected as the *in vivo* studies comparing the antimicrobial efficacy of MTAD alone (without the initial use of 1.3% NaOCl) are lacking. The earlier studies in literature have shown that the cleanest canals were obtained if NaOCl was used before a final rinse with MTAD.[24] But, Tay *et al* found that when MTAD is applied to 1.3% NaOCl-irrigated dentin obtained from third molars, its antimicrobial substantivity is reduced.[25] The authors also reported intrinsic staining of coronal and intraradicular dentin after natural light exposure of NaOCl and MTAD regime.[26] The duration of exposure of the root canals to the irrigant in our study, was five minutes which was decided based on the studies conducted by Torabinejad *et al* [24,13] There was a significant reduction in the mean relative quantification (RQ) of *E. faecalis* from the pre instrumentation to the post instrumentation samples in both the experimental groups thus depicting the antimicrobial efficacy of NaOCl 2.5% and MTAD against *E. faecalis.* This was in accordance with the earlier studies that have proven NaOCl and MTAD significantly effectively against *E. faecalis* in permanent teeth. [13, 23,24]

The results of the present study showed that for group comparison of post instrumentation between groups I and II, the 'p' value is 0.5069, which is not significant. Similarly, 'p' value for comparison of group I vs control group and group II vs control group is less than 0.05, which is statistically significant indicating that the antibacterial efficacy of both MTAD and NaOCl 2.5% is much better than saline. Thus, if the groups were overall compared, it can be concluded that the experimental group II (MTAD) showed maximum efficacy against *E. faecalis*, followed by the experimental group I (NaOCl 2.5%) and the least effective was the control group where only saline was used. Our results were in agreement with those of Kho and Baumgartner who demonstrated no difference in the antimicrobial efficacy for irrigation with the 5.25%NaOCl/15% EDTA versus irrigation with 1.3% NaOCl/MTAD in the roots of permanent infected with *E. faecalis*.[27] Ahangari *et al* reported no significant difference between antimicrobial efficacy of MTAD, NaOCl 2.5% and 2% Chlorhexidine gluconate against *E. faecalis* in the root canals of human extracted single rooted permanent teeth.[28] However, the studies conducted by Torabinejad *et al*, Shabahang *et al*, that compared the antibacterial effects of MTAD with those of NaOCl and EDTA by using standard in vitro microbiological techniques reported that MTAD was significantly more effective against E. *faecalis.* [13,24] The later in vitro studies[27,29] that tried to simulate the clinical conditions have shown a lesser antimicrobial effect of MTAD than NaOCl. This could be because of the difference in methodology and microbial sampling procedures. An *in vivo* study concluded Sodium Hypochlorite to be superior to the sole irrigation regime of MTAD.[30] Thus, in the light of these studies, it can be said that antimicrobial efficacy of MTAD when compared to NaOCl is showing conflicting results.

To the best of our knowledge, this is the first in vivo study comparing the antimicrobial efficacy of NaOCl 2.5% and Biopure

MTAD™ against *Enterococcus faecalis* using PCR analysis in necrotic primary anterior teeth of children aged 4-8 years during pulpectomy procedure. None of the children in all the three groups reported any discomfort or adverse effects during or after the procedure. This study which derives its significance from being conducted under *in vivo* conditions yielded good post operative results; however, certain amount of contamination could be expected due to difficulty to efficiently standardize the sample collection from young patients.

## CONCLUSION

This study concluded NaOCl 2.5% and Biopure MTAD to be equally effective statistically, however further *in vivo* studies with a larger sample size with respect to various clinical scenarios are required. Although Biopure MTAD™ proves to be an effective irrigant for the primary teeth, certain limitations like high cost, reduced shelf life and difficulty in availability makes it much less of a choice than Sodium Hypochlorite in clinical practice.

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