

Comparative evaluation of biofilm formation ability of *E. faecalis* in alkaline conditions and its susceptibility to endodontic irrigant regimens – An In vitro microbiological study.

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Abstract: The present study aimed to evaluate the alkaline tolerance ability of *Enterococcus faecalis*; one of the most commonly isolated bacterium from failed root canal treatments. *E. faecalis* was grown in Brain heart Infusion (BHI) broth and maintained at different alkaline conditions (pH); its ability to form biofilm in polystyrene plates was assayed by O'Toole method. Intracanal irrigants sodium hypochlorite (NaOCl), chlorhexidine digluconate (CHX) and MTAD were used to evaluate the suppression of *E. faecalis* biofilm. The data obtained was statistically analysed by Anova and Kolmogorov-Smirnov test. *E. faecalis* was able to survive and form biofilm at all tested pH range (7.3-12.3). 2% NaOCl and 1% CHX were highly effective in suppressing *E. faecalis* biofilm whereas MTAD had limited activity.

Keywords: alkalinity, biofilm, *Enterococcus faecalis*, intracanal irrigants, pH.

I. Introduction

Enterococcus faecalis a gram positive facultative anaerobe is seen in primary and failed root canal treatments. *E. faecalis*, have been frequently found in obturated root canals exhibiting signs of chronic apical periodontitis, isolated upto 70% of the positive cultures and often occur in monoculture [1-3].

E. faecalis possesses certain characteristics and virulence factors that enable them to survive for long periods of time in the root canal. These include the production of proteolytic enzymes, aggregation substances and adhesions [4]. Additionally *E. faecalis* has the ability to survive long periods of starvation [5, 6] and can form biofilms [7] and can invade and live within the dentinal tubules [8]. *Enterococcus faecalis* can grow in harsh environment and can survive in extremes. It can grow at 10⁰C - 45⁰C at pH 9.6 and in 6.5%NaCl broth survive at 60⁰C for 30minutes. Other important feature of this organism is that it can colonise and survive as a single organism without the support of other bacteria [9].

Sodium hypochlorite (NaOCl) and chlorhexidine digluconate (CHX) are the most commonly used irrigants. Sodium hypochlorite is well known for its tissue solubilising property and antimicrobial property [10]. Chlorhexidine digluconate is popular for its low toxicity, broad antimicrobial property and substantivity [11]. Biopure MTAD (Dentsply Tulsa Dental) commercially available root canal irrigant contains antibiotic, surfactant and acid; found to be effective in smear layer removal [12].

The aim of this study is to evaluate the tolerance of *E. faecalis* to alkaline condition and it's susceptibility to various concentrations of NaOCl, CHX and MTAD in vitro.

II. Materials And Methods

2.1 Source of Microorganism and maintenance

The study involved *Enterococcus faecalis* ATCC 29212, procured from Himedia. The stock culture was maintained at-80⁰C. The bacterium was subcultured in Brain Heart Infusion Broth (Himedia).

2.2 Evaluation of alkaline tolerance of *E. faecalis* biofilm

E. faecalis was grown in Brain heart Infusion Broth of different pH's and assayed by its ability to form biofilm. Growth of biofilm in 96 well microtiter plate (Himedia) was done as per O'Toole method [13].

The pH of media prepared as per manufacturer's instruction was found to be 7.3. Media of pH 8.3, 9.3, 10.3, 11.3 and 12.3 were prepared by adding 1mol/L sodium hydroxide to media drop by drop. To 5ml media of different pH taken in separate tubes; 20µL of *E. faecalis* culture (optical density was adjusted to 0.5 Mc Farland constant) was added. After 18hrs of incubation 10µL from each culture was added to fresh 1ml containing

medium of respective pH. Then 100µL of culture was added to the wells of the microtitre plates. Six replicates were kept for each pH and two independent tests were performed.

After inoculation of wells, culture plates were incubated for 24hrs. Then contents were dispelled and washed with distilled water and blotted. Staining of cells was done using 0.1% crystal violet (Himedia) followed by 15 min incubation. After incubation plates were washed thrice and set upside down. After the water content completely drained off, stain was removed using 30% acetic acid. Quantification of stain was done at 630nm using ELISA reader (Lisa plus). Data obtained was analysed by Anova and Kolmogorov-Smirnov test.

2.3 Preparation of irrigants

Sodium hypochlorite and chlorhexidine digluconate were prepared at 5%, 2.5%, 2% and 1% concentration [14]. The different concentrations of irrigants were prepared by diluting 5% NaOCl (Prevest Denpro Limited) and 20% aqueous solution of chlorhexidine digluconate (SIGMA). MTAD was prepared as per the manufacturer's instruction. All prepared irrigants were stored in sterile bottles.

2.4 Susceptibility of *E. Faecalis* biofilm to endodontic irrigants

E. faecalis biofilm was grown in Brain Heart Infusion broth (pH 7.3). The susceptibility of *E. faecalis* cells in biofilm was evaluated by adding 50 µl of each irrigant to the wells followed by quantification of viable cells by staining with crystal violet. *E. faecalis* biofilm (not subjected to any irrigants) served as control group. The data obtained was analysed by Anova and Bonferroni multiple comparison tests.

III. Results

The growth of *E. faecalis* biofilm at different alkaline pH is summarised in Table 1 in terms of mean optical density. At pH 7.3 the mean optical density was 0.193 ± 0.044 as compared to pH 12.3 (0.139 ± 0.037). The results showed no statistical difference in growth of the *E. faecalis* due to pH change (pH 7.3 – 12.3). We found that increase in pH caused decrease in adherence capacity of *E. faecalis* biofilm to the polystyrene wells (Fig 1).

Table 2 shows the susceptibility of *E. faecalis* to NaOCl, CHX at concentrations of 1% to 5%. In NaOCl group; 5% NaOCl (0.089 ± 0.03) was statistically significant over 1% NaOCl (0.156 ± 0.01). No other concentrations were found statistically significant; thus 2% NaOCl (0.128 ± 0.03) was taken for intergroup comparison. In CHX group there was no statistical significance between any two concentrations tested and hence 1% CHX (0.146 ± 0.01) was considered for intergroup comparison. MTAD showed a mean optical density value of 0.204 ± 0.03 .

Thus intergroup comparison was done with 2% NaOCl (0.128 ± 0.03), 1% CHX (0.146 ± 0.01) and MTAD (0.204 ± 0.03) with control (0.254 ± 0.02) as summarised in Table 3. 2% NaOCl was statistically significant over control and MTAD, but not with 1% CHX. 1% CHX was statistically significant with control and not with 2% NaOCl, MTAD. MTAD was not statistically significant in suppressing *E. faecalis* biofilm. The mean optical density values obtained are summarized in Fig 2.

IV. Discussion

Enterococcus faecalis survives root canal treatment and adapts to extreme conditions. It has been shown to invade dentinal tubules [15], survive nutrient deficiency and resistant to medicaments. These characteristics explain its survival adaptability under stress which accounts for its viable but non-cultivable state [16]. Starving *E. faecalis* cells maintain their viability for a long period of time and become resistant to UV irradiation, heat, NaOCl, hydrogen peroxide, ethanol and acids [17, 18].

Biofilm formation by *E. faecalis* cells in various growth phases (exponential, stationary and starvation phase) has been demonstrated by Liu et al [19]. Aggregation substance produced by *E. faecalis* has been found to mediate binding to extracellular matrix proteins. Enterococcal gene “esp” is associated with promotion of primary attachment to dentinal collagen type I and biofilm formation on abiotic substances [20]. Further, biofilm formation by *E. faecalis* has been observed in medicated dentinal walls, and this form of organization could allow the bacteria to resist the bactericidal effect of calcium hydroxide medication in infected root canals [21].

The purpose of this study was to evaluate the growth of *E. faecalis* biofilm on polystyrene plates under different alkaline conditions and its susceptibility to various concentrations of endodontic irrigants. The endodontic irrigants used were NaOCl, CHX at various concentrations (1%, 2%, 2.5% and 5%). Biopure MTAD which is a commercially available irrigant was prepared as per manufacturer's instructions.

The results of our study showed that *E. faecalis* has the ability to form biofilm and survive at all alkaline pH's tested (7.3 to 12.3). The biofilm formation decreased with increasing alkalinity (pH). The data obtained in our study was in accordance with Kayaoglu et al., [22] where they found increasing pH above 8.5 caused decrease in adherence capacity of *E. faecalis* on Bovine Serum Albumin and Collagen type I coated wells.

E. faecalis biofilm cells were grown in stationary phase. As regarding to the phase of the cells and their susceptibility towards dental irrigants different opinions have reported [19, 23]. Dunavant et al [24] study design showed that 1% and 6% NaOCl both were significantly effective in killing the *E. faecalis* biofilm than 2% CHX and MTAD. NaOCl at 0.00625% concentration effectively removed *E. faecalis* biofilm within 1 min than CHX [25] and other study demonstrated that 5.25% NaOCl was capable of disintegrating *E. faecalis* biofilm only after 5 min and MTAD after 30 min [26]. The different study designs may be complicating to conclude regarding the exact percentage of irrigants required for complete elimination of *E. faecalis* biofilm.

With respect to antimicrobial effect on *E. faecalis* biofilm; we found that NaOCl and CHX were equally effective (P= 0.000) on *E. faecalis* biofilm. Intergroup comparison of 2% NaOCl, 1% CHX and MTAD showed no significant difference between the 2% NaOCl and 1% CHX (p= 1.000). MTAD had limited activity on *E. faecalis* biofilm.

V. Tables & Figures

Table 1: Showing mean optical density given by *E. faecalis* biofilm cells with standard deviation at different alkaline pH's.

pH	Optical density
7.3	0.193±0.044
8.3	0.183±0.029
9.3	0.171±0.039
10.3	0.161±0.040
11.3	0.141±0.036
12.3	0.139±0.037

Table 2: Showing the susceptibility of *E. faecalis* to different irrigants. Data shown are mean ± sd value of optical density and their significance level to control.

Irrigant	mean+sd	P value	Irrigant	mean +sd	P value
1 % NaOCl	0.156±0.01	0.000 *	1% CHX	0.146±0.01	0.000 *
2% NaOCl	0.128±0.03	0.000 *	2% CHX	0.132±0.03	0.000 *
2.5% NaOCl	0.122±0.03	0.000 *	2.5% CHX	0.134±0.01	0.000 *
5% NaOCl	0.089±0.03	0.000 *	5% CHX	0.134±0.01	0.000 *

* level of significance at 0.05

Table 3: Intergroup comparison between 2%NaOCl, 1% CHX, MTAD with Control.

(I) VAR00001	(J) VAR00001	Mean Difference (I-J)	Std. Error	p	Sig
Control	2%NaOCl	.12580	.01997	.000*	HS
	1%CHX	.10840	.01997	.000*	HS
	MTAD	.05000	.01997	.141	NS
2%NaOCl	1%CHX	-.01740	.01997	1.000	NS
	MTAD	-.07580	.01997	.010*	sig
1%CHX	MTAD	-.05840	.01997	.060	NS

* level of significance at 0.05

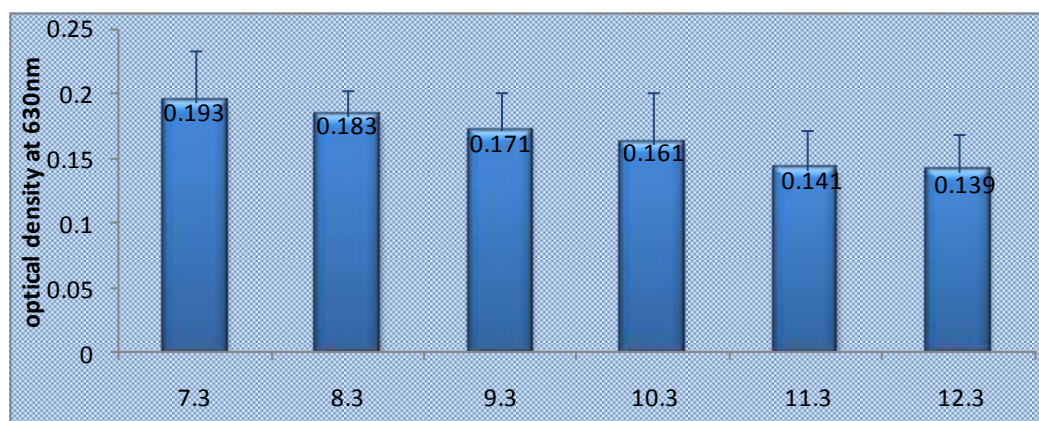


Fig 1. Graph showing mean optical density of *E. faecalis* biofilm at different pH (7.3-12.3)

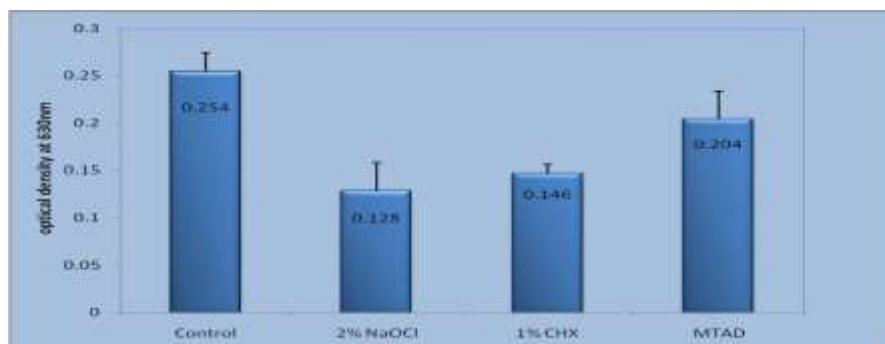


Fig 2. Graph showing mean optical density of *E. faecalis* biofilm suppression by 2% NaOCl, 1% CHX, MTAD with control.

VI. Conclusion

Within the limitations of our study we conclude that *E. faecalis* forms biofilm at various pH (7.3-12.3) and 2% NaOCl and 1% CHX are equally effective and have greater antimicrobial effect on *E. faecalis* biofilm. MTAD has limited antimicrobial effect on *E. faecalis* biofilm.

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