

ANTISEPTIC MOUTHWASHES: *IN VITRO* ANTIBACTERIAL ACTIVITY

Evandro Watanabe¹, Andresa P. Nascimento², Juliane M. Guerreiro-Tanomaru³, Ana M. Razaboni¹, Denise de Andrade⁴, Mário Tanomaru-Filho³

¹ Department of Restorative Dentistry, University of São Paulo (USP), School of Dentistry of Ribeirão Preto, Ribeirão Preto, SP, Brazil.

² Apis Flora Industrial e Comercial, Ribeirão Preto, SP, Brazil.

³ Department of Restorative Dentistry, São Paulo State University (UNESP), Araraquara School of Dentistry, Araraquara, SP, Brazil.

⁴ Department of General and Specialized Nursing, University of São Paulo (USP), Ribeirão Preto Nursing School, Ribeirão Preto, SP, Brazil.

ABSTRACT

Mouthwashes are used as an adjunct to tooth brushing for improving breath and preventing oral diseases. The aim of this study was to compare the *in vitro* Maximum Inhibitory Dilution (MID) of 3 mouthwashes with different active ingredients against mutans streptococci (MS). The products analyzed were Periogard®, Cepacol® and Plax® Fresh Mint. Their antibacterial activity was assessed in duplicate in 96-well microtiter plates against 36 clinical isolates of MS. Each mouthwash was submitted to a serial two-fold dilution (1/2.5 to 1/5120) using double concentration of Tryptose Soy Broth with 1.0% yeast extract. The final volume in each well was 100 mL plus 5 mL of a bacterial suspension, equivalent to 10⁷

CFU/mL. They were incubated microaerobically at 37°C for 48 hours and the MIDs determined. MID was 1/320 for Periogard® and Cepacol®, and 1/20 for Plax®. Statistical analysis revealed that the MID of Periogard® MID did not differ from that of Cepacol® ($p > 0.05$), and was higher than that of Plax® ($p < 0.05$). In conclusion, the antiseptic mouthwashes containing chlorhexidine (Periogard®) and cetylpyridinium chloride (Cepacol®) had higher *in vitro* antibacterial activity (MID) against MS than the antiseptic mouthwash containing triclosan (Plax®), according to microbiological method employed.

Keywords: Microbial Sensitivity Tests, Mouthwashes, Streptococcus.

ANTISSÉPTICOS BUCAIS: ATIVIDADE ANTIBACTERIANA

RESUMO

Os antissépticos bucais são utilizados mundialmente como adjuvantes da escovação para melhoria do hálito e prevenção de doenças bucais infecciosas. O objetivo deste estudo foi comparar *in vitro* a Diluição Inibitória Máxima (DIM) de 3 antissépticos bucais com diferentes princípios ativos contra estreptococos do grupo mutans (EGM). Os produtos analisados foram Periogard®, Cepacol® e Plax® Fresh Mint. A atividade antibacteriana foi avaliada em duplicata em placas de microtitulação de 96 poços contra 36 isolados clínicos de EGM. Cada antisséptico bucal foi submetido a diluição dupla seriada (1/2,5 a 1/5120) com o emprego de concentração dupla de Tryptose Soy Broth com adição de 1,0% de extrato de levedura. O volume final em cada poço foi de 100 mL mais 5

mL da suspensão bacteriana equivalente a 10⁷ UFC/mL. A incubação foi realizada em microaerofilia a 37°C por 48 horas e a DIM determinada. Periogard® e Cepacol® apresentaram DIM de 1/320, e Plax® de 1/20. Os resultados submetidos a análises estatísticas revelaram que a DIM do Periogard® não foi diferente do Cepacol® ($p > 0,05$) sendo maior que do Plax® ($p < 0,05$). Em conclusão, os antissépticos bucais contendo clorexidina (Periogard®) e cloreto de cetilpiridínio (Cepacol®) demonstraram maior atividade antibacteriana *in vitro* (DIM) contra os EGM do que o antisséptico bucal contendo triclosan (Plax®) de acordo com o método microbiológico utilizado.

Palavras-chave: Testes de Sensibilidade Microbiana, Antissépticos Buciais, Streptococcus.

INTRODUCTION

Mouthwashes have been widely employed in the fields of Preventive Dentistry and Periodontics¹⁻³. The use of antimicrobial mouthwashes has been proposed as a means for reducing the levels of oral bacteria, specifically *Streptococcus mutans*. A wide range of mouthwashes containing different active ingredients is available in the market.

Periogard® is a mouthwash which contains 0.12% chlorhexidine gluconate and other ingredients (Table 1). Chlorhexidine is a cationic biguanide with broad-spectrum antimicrobial action, effective against dental biofilm and gingivitis³⁻⁶. Chlorhexidine has an important feature, substantivity, due to its ability to bind to oral tissues and act for extended periods after application⁴.

Cepacol[®] is a cetylpyridinium chloride-based (CPC) mouthwash (Table 1). CPC is a quaternary ammonium compound included in the group of the cationic surface-active agents⁷. It acts primarily by penetrating the cell membrane, causing leakage of cell components, disruption of the bacterial metabolism, inhibition of cell growth, and finally, cell death⁸.

Plax[®] contains 0.03% triclosan (2,4,4'-trichloro-2'-hydroxydiphenyl ether), 0.20% polyvinyl-methyl ether/maleic acid (PVM/MA) copolymer which is used jointly with triclosan to increase its antimicrobial activity,⁹ and other ingredients (Table 1).

Due to their ability to form biofilms on teeth, mutans streptococci (MS) are considered major etiological agents of human dental caries¹⁰. Thus, mouthwashes containing active ingredients against them may help prevent caries. Mouthwashes are expected to maintain their antimicrobial activity even after dilution in oral fluids, and their antimicrobial activity against different strains of *S. mutans* can be evaluated *in vitro*,

The aim of this study was to compare *in vitro* Maximum Inhibitory Dilution (MID) of 3 mouthwashes containing different active ingredients against 36 clinical isolates of MS.

MATERIAL AND METHODS

The following products were evaluated: Periogard[®] (Colgate-Palmolive, São Bernardo do Campo, SP, BR), Cepacol[®] (Aventis Pharma, Suzano, SP, BR) and Plax[®] Fresh Mint (Colgate-Palmolive, São Bernardo do Campo, SP, BR) as shown in Table 1. Antibacterial activity was assessed in duplicate in 96-well microtiter plates against 36 clinical isolates of mutans streptococci (MS).

Each mouthwash was submitted to a serial two-fold dilution (1/2.5 to 1/5120) using double concentration of Tryptose Soy Broth with 1.0% yeast extract. The final volume in each well was 100 mL plus 5 mL of a bacterial suspension equivalent to 10⁷ CFU/mL. They were incubated microaerobically at 37°C for 48 hours and the MIDs determined (the highest dilution of each product that inhibited the bacterial growth).

Statistical analysis

Results were expressed as scores determined from MID. Groups were compared using the Kruskal-Wallis nonparametric test. When this test showed significant difference between groups, Dunn's multiple comparison test, which allows two-by-two comparison between groups, was applied. The significance level in the statistical testing was 5% (p<0.05).

Table 1: Chemical composition of the mouthwashes.

Mouthwash	Composition	
Periogard [®] (Colgate-Palmolive, São Bernardo do Campo, SP, BR)	Chlorhexidine gluconate (0.12%) Water Glycerin Ethanol Polysorbate 20 Flavoring agents Sodium saccharin FD&C Blue n°1	
Cepacol [®] (Aventis Pharma, Suzano, SP, BR)	Cetylpyridinium chloride (0.05%) Disodium EDTA Sodium saccharin Polysorbate 80 Glycerin Water Sodium phosphate monobasic anhydrous	Disodium phosphate anhydrous Eucalyptol Menthol Methyl salicylate Mint oil Chinese cinnamon flavor Yellow tartrazine Ethyl alcohol 96GL
Plax [®] Fresh Mint (Colgate-Palmolive, São Bernardo do Campo, SP, BR)	Triclosan (0.03%) Sodium fluoride (227ppm fluoride) PVM/MA copolymer (0.20%) Ethanol Disodium phosphate Glycerin Sodium hydroxide	Sodium lauryl sulphate Sodium methyl taurate Sodium saccharin Sorbitol Blue CI42090 Yellow CI47005 Flavoring agents Water

RESULTS

The mouthwashes had different MIDs. Statistical analysis revealed that the MID of Periogard® did not differ from that of Cepacol® ($p > 0.05$) but was higher than that of Plax® ($p < 0.05$) against 36 clinical isolates of MS (Table 2).

DISCUSSION

A wide range of mouthwashes containing different active ingredients is available on the market. It is important to know their antimicrobial activity because they are mainly employed to control microorganisms. This study conducted a comparative analysis among three different mouthwashes - chlorhexidine gluconate, cetylpyridinium chloride and triclosan-based solutions – which were effective against MS from the initial dilution of 1/2.5.

Chlorhexidine gluconate mouthwashes have been available on the market for a long time with concentrations ranging from 0.12% to 0.2%. The chlorhexidine-based mouthwash evaluated in this study (Periogard®) contains 0.12% chlorhexidine gluconate.

In this study, Periogard® had the best *in vitro* antibacterial activity against MS, because it inhibited all the clinical isolates of MS at the 1/320 dilution. However, our results disagree with MIDs found in other studies, of 1/16¹¹, 1/80¹² and 1/160¹³.

Other studies found that Cepacol® was able to inhibit MS at 1/20¹² and 1/40¹³ dilutions. In contrast, our study found that all clinical isolates of MS were inhibited by Cepacol® at 1/320 dilution.

In our study, Plax® had lower MID than Periogard® and Cepacol® ($p < 0.05$) and inhibited the growth of all MS at the 1/20 dilution, in agreement with André et al.¹³. Thus, triclosan used with PVM/MA copolymer had *in vitro* antibacterial activity against the different clinical isolates of MS.

McMurry, Oethinger and Levy¹⁴ demonstrated in a study with *Escherichia coli* that the antibacterial activity of triclosan is due to its ability to block the synthesis of fatty acids by inhibiting the enoyl-acyl carrier protein reductase enzyme. This specific action affects several lipid synthesis-dependent processes, resulting in secondary effects on the cytoplasmic membrane¹⁵.

Giertsen¹⁶ analyzed mouthwashes containing triclosan (3.5mM) associated with sodium lauryl sulphate (17.4mM), which were found to have a synergic effect against *S. mutans* (NTCC 10449). The MIC of triclosan and sodium lauryl sulphate against *S. mutans* was 13.49µM. Moreover, sodium lauryl sulphate is an anionic surfactant that has often been used in mouthwashes and toothpastes and it is included in the Plax® formula (Table 1). In addition, Plax® contains a sodium fluoride

Table 2: Data of 36 clinical isolates of mutans streptococci inhibited by each dilution of the mouthwashes.

Dilution	Periogard®				Cepacol®				Plax®			
	Inhibited strains		Cumulative data		Inhibited strains		Cumulative data		Inhibited strains		Cumulative data	
	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
1/5120	1	2.8	1	2.8	0	0	0	0	0	0	0	0
1/2560	1	2.8	2	5.6	2	5.6	2	5.6	0	0	0	0
1/1280	25	69.4	27	75.0	21	58.3	23	63.9	0	0	0	0
1/640	8	22.2	35	97.2	12	33.3	35	97.2	2	5.6	2	5.6
1/320*	1	2.8	36 ^a	100.0	1	2.8	36 ^a	100.0	25	69.4	27	75.0
1/160	-	-	-	-	-	-	-	-	1	2.8	28	77.8
1/80	-	-	-	-	-	-	-	-	0	0	28	77.8
1/40	-	-	-	-	-	-	-	-	0	0	28	77.8
1/20*	-	-	-	-	-	-	-	-	8	22.2	36 ^b	100.0
1/10	-	-	-	-	-	-	-	-	-	-	-	-
1/5	-	-	-	-	-	-	-	-	-	-	-	-
1/2.5	-	-	-	-	-	-	-	-	-	-	-	-

*Kruskal-Wallis's nonparametric test and Dunn's multiple comparison test ($p < 0.05$). Different letters indicate statistically significant difference.

concentration of 270 ppm (270 µg/mL), which is less than that required for the MIC of 600 µg/mL against *S. mutans*¹⁷.

Herrera et al.¹⁸ evaluated the antimicrobial activity of four commercially available mouthwashes, three containing 0.12% chlorhexidine (one containing alcohol and two alcohol-free) and one containing 0.12% chlorhexidine and 0.05% CPC (alcohol-free). They observed that the product containing alcohol was more effective than the alcohol-free rinses, except for the formulation including chlorhexidine and CPC, which had greater antimicrobial activity.

The difference in the MID results of these studies may be related to the different sources of the MS (ATCC strain¹¹, saliva¹³ and dentures¹²).

According to Carlinet al.¹⁹ Periogard[®] and Plax Whitening[®] can induce genetic damage. DNA damage is considered to be the prime mechanism

during chemical carcinogenesis, and these data may be relevant in risk assessment for protecting human health and preventing carcinogenesis. Moreover, the ethanol concentration present in Cepacol induced mitotic recombination between homologous chromosomes in the *Drosophila* SMART assay involved in the genesis of numerous diseases, including cancer²⁰.

In conclusion, the mouthwashes containing chlorhexidine (Periogard[®]) and cetylpyridinium chloride (Cepacol[®]) had higher *in vitro* antibacterial activity (MID) against MS than the mouthwash containing triclosan (Plax[®]), according to microbiological method employed. Further studies should be conducted to evaluate the *in vitro* and *in vivo* antimicrobial activities of Periogard[®], Cepacol[®], Plax[®] and other mouthwashes against MS and a wide range of oral microorganisms, as well as their cytotoxicity.

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CORRESPONDENCE

Prof. Dr. Evandro Watanabe
 Faculdade de Odontologia de Ribeirão Preto
 Universidade de São Paulo.
 Departamento de Odontologia Restauradora.
 Avenida do Café s/ nº, Monte Alegre, Ribeirão Preto, SP, Brasil.
 CEP: 14.040-904
 E-mail: evandrowatanabe@gmail.com

REFERENCES

1. Addy M. Chlorhexidine compared with other locally delivered antimicrobials. A short review. *J Clin Periodontol* 1986; 13:957-964.
2. Gunsolley JC. A meta-analysis of six-month studies of antiplaque and antigingivitis agents. *J Am Dent Assoc* 2006; 137:1649-1657.
3. Lorenz K, Bruhn G, Heumann C, Netuschil L, Brex M, Hoffmann T. Effect of two new chlorhexidine mouthrinses on the development of dental plaque, gingivitis and discoloration. A randomized, investigator-blind, placebo-controlled, 3-week experimental gingivitis study. *J Clin Periodontol* 2006; 33:561-567.
4. Adams D, Addy M. Mouthrinses. *Adv Dent Res* 1994; 8: 291-301.
5. Bascones A, Morante S, Mateos L, Mata M, Poblet L. Influence of additional active ingredients on the effectiveness of non-alcoholic chlorhexidine mouthwashes: a randomized clinical trial. *J Periodontol* 2005; 76:1469-1475.
6. Charles CH, Mostler KM, Bartels LL, Mankodi SM. Comparative antiplaque and antigingivitis effectiveness of a chlorhexidine and an essential oil mouthrinse: 6-month clinical trial. *J Clin Periodontol* 2004; 31:878-884.
7. Mandel ID. Chemotherapeutic agents for controlling plaque and gingivitis. *J Clin Periodontol* 1988; 15:488-498.
8. Merianos JJ: Surface-active agents. In: Block SS: Disinfection, sterilization, and preservation. Philadelphia, USA: Lippincott Williams & Wilkins, 2001:283-320.
9. Nabi N, Mukerjee C, Schmid R, Gaffar A. *In vitro* and *in vivo* studies on triclosan/PVM/MA copolymer/NaF combination as an anti-plaque agent. *Am J Dent* 1989; 2 Spec:197-206.
10. Okada M, Soda Y, Hayashi F, Doi T, Suzuki J, Miura K, Kozai K. Longitudinal study of dental caries incidence associated with *Streptococcus mutans* and *Streptococcus sobrinus* in pre-school children. *J Med Microbiol* 2005; 54:661-665.
11. Da Silva NB, Alexandria AK, De Lima AL, Claudino LV, De Oliveira Carneiro TF, Da Costa AC, Valença AM, Cavalcanti AL. *In vitro* antimicrobial activity of mouth washes and herbal products against dental biofilm-forming bacteria. *Contemp Clin Dent* 2012; 3:302-305.
12. Albuquerque RF Jr, Head TW, Mian H, Rodrigo A, Müller K, Sanches K, Ito IY. Reduction of salivary *S. aureus* and *mutans* group streptococci by a preprocedural chlorhexidine rinse and maximal inhibitory dilutions of chlorhexidine and cetylpyridinium. *Quintessence Int* 2004; 35:635-640.
13. André RF, Andrade IM, Silva-Lovato CH, Paranhos HdeF, Pimenta FC, Ito IY. Prevalence of *mutans streptococci* isolated from complete dentures and their susceptibility to mouthrinses. *Braz Dent J* 2011; 22:62-67.

14. McMurry LM, Oethinger M, Levy SB. Triclosan targets lipid synthesis. *Nature* 1998; 394:531-532.
15. Schweizer HP. Triclosan: a widely used biocide and its link to antibiotics. *FEMS Microbiol Lett* 2001; 202:1-7.
16. Giertsen E. Effects of mouthrinses with triclosan, zinc ions, copolymer, and sodium lauryl sulphate combined with fluoride on acid formation by dental plaque in vivo. *Caries Res* 2004; 38:430-435.
17. Liu J, Ling JQ, Zhang K, Huo LJ, Ning Y. Effect of sodium fluoride, ampicillin, and chlorhexidine on *Streptococcus mutans* biofilm detachment. *Antimicrob Agents Chemother* 2012; 56:4532-4535.
18. Herrera D, Roldán S, Santacruz I, Santos S, Masdevall M, Sanz M. Differences in antimicrobial activity of four commercial 0.12% chlorhexidine mouthrinse formulations: an in vitro contact test and salivary bacterial counts study. *J Clin Periodontol* 2003; 30:307-314.
19. Carlin V, Matsumoto MA, Saraiva PP, Artioli A, Oshima CT, Ribeiro DA. Cytogenetic damage induced by mouthrinses formulations *in vivo* and *in vitro*. *Clin Oral Investig* 2012; 16:813-820.
20. Rodrigues F, Lehmann M, do Amaral VS, Reguly ML, Andrade HH. Genotoxicity of three mouthwash products, Cepacol, Periogard, and Plax, in the *Drosophila* wing-spot test. *Environ Mol Mutagen* 2007; 48:644-649.