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MAXIMUM INHIBITORY DILUTION OF MOUTHWASHES CONTAINING CHLORHEXIDINE AND POLYHEXAMETHYLENE BIGUANIDE AGAINST SALIVARY *STAPHYLOCOCCUS AUREUS*

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ABSTRACT

Objective: The aim of the present study was to determine the *in vitro* maximum inhibitory dilution (MID) of two chlorhexidine-based oral mouthwashes (CHX): Noplak®, Periogard®, and one polyhexamethylene biguanide-based mouthwash (PHMB): Sanifill Premium® against 28 field *Staphylococcus aureus* strains using the agar dilution method. Materials and Methods: For each product, decimal dilutions ranging from 1/10 to 1/655,360 were prepared in distilled water and added to Mueller Hinton Agar culture medium. After homogenization, the culture medium was poured onto Petri dishes. Strains were inoculated using a Steers multipoint inoculator and dishes were incubated at 37ºC for 24hours. For reading, MID was considered as the maximum dilution of the mouthwash still capable of inhibiting microbial growth. Results: Sanifill Premium® inhibited the growth of all strains at 1/40 dilution and of 1 strain at 1/80 dilution. Noplak® inhibited the growth of 23 strains at 1/640 dilution and of all 28 strains at 1/320 dilution. Periogard® showed inhibited growth of 7 strains at 1/640 dilution and of all 28 strains at 1/320 dilution. Data were submitted to Kruskal-Wallis statistical test, showing significant differences between the mouthwashes evaluated ($p<0.05$). No significant difference was found between Noplak® and Periogard® ($p>0.05$). Sanifill Premium® was the least effective ($p<0.05$). Conclusion: It was concluded that CHX-based mouthwashes present better antimicrobial activity against *S. Aureus* than the PHMB-based mouthwash.

Key words: Bacteria. Anti-infective agents. Chlorhexidine.

INTRODUCTION

Mouthwashes have been used for centuries[19] with the objective of reducing the amount of microorganisms in the oral cavity[14]. These chemical agents have been widely employed in the fields of Preventive Dentistry and Periodontics[2,10,13]. Among the microorganisms present in the oral cavity, the reduction in the number of *Staphylococcus aureus* prior to surgical procedures has been associated with a lower incidence of infective endocarditis and postoperative infections[4].

Chlorhexidine gluconate (CHX) is a cationic biguanide with broad-spectrum antimicrobial action, whose effectiveness in decreasing the formation of dental biofilm (plaque) and gingivitis has been demonstrated in clinical studies[1,5,6,13]. An important characteristic of chlorhexidine is its substantivity or persistence of action, which consists of the ability of this product to bind to oral tissues and remain active for long periods after application[1].

The antimicrobial properties of mouthwashes containing CHX and other antimicrobial agents have been assessed *in vivo* and *in vitro*, with excellent results for CHX-based solutions[3,4,7,11,15,16,20].

Polyhexamethylene biguanide hydrochloride (PHMB) is

a polymeric biguanide with broad antimicrobial spectrum against both Gram-positive and Gram-negative bacteria[8,9]. PHMB has been used for several years as an antiseptic agent in Medicine[12]. Welk, et al.[23] demonstrated in clinical studies that PHMB-based mouthwashes inhibited biofilm formation and reduced contamination in the oral cavity, suggesting that these solutions may be an alternative for prevention of dental biofilm.

The purpose of this study was to determine *in vitro* the maximum inhibitory dilution (MID) of two mouthwashes containing 0.12% chlorhexidine gluconate and one mouthwash containing 0.35% PHMB and to compare their action against 28 *Staphylococcus aureus* field strains using the agar dilution method.

MATERIAL AND METHODS

The following mouthrinses were evaluated: Periogard® (Colgate-Palmolive, Ind. Brasileira, Osasco, SP, Brazil), Noplak® (Laboratório Daudt Oliveira Ltda., Rio de Janeiro, RJ, Brazil) and Sanifill Premium® (Facilit Odontológica e Perfumaria, Brazil) (Table 1).

Determination of the maximum inhibitory dilution (MID) was performed in duplicate by double serial dilution (from 1/10 through 1/655.360) in test tubes (20x200mm) with 2.0 mL of sterile distilled water. After dilutions were made, 18.0 mL of Mueller Hinton Agar culture medium (Difco®) were added to each tube, and the resulting solutions were poured onto Petri dishes (20x100mm).

The microbial inoculum (~10^8UFC/mL) with turbidity equivalent to a 0.5 McFarland standard was prepared in test tubes (15x125 mm) with saline, using 28 young *S. aureus* field strains previously incubated at 35°C for 24 h. *S. aureus* strains were collected from the oral and nasal cavities of volunteer undergraduate students.

Microorganisms were seeded using a Steers multipoint inoculator[21]. The Steers inoculator consists of two metallic plates: one of these plates has 25 wells into which 200 µL of each standardized microbial inoculum were transferred. The other plate has 25 metallic needles that fit into the wells. Using these needles, the inoculi were seeded onto the surface of the culture medium in Petri dishes containing different dilutions of the mouthwashes. Since the Steers inoculator has 25 wells and 28 strains were evaluated, three inoculi (5.0 µL) were seeded equidistantly from each other, approximately one centimeter from the periphery of each Petri dish, using an automatic pipette.

The dishes were then incubated at 37°C for 24 hours, and readings were performed considering the MID as the greatest dilution of mouthwash capable of inhibiting growth of all evaluated strains, following the methodology by Wade and

TABLE 1- Formulation of the antiseptic solutions evaluated

Product	Composition
Periogard®	0.12% chlorhexidine gluconate, water, glycerin, ethanol, polysorbate 20, flavoring agents, - sodium saccharin, FD&C Blue n°1
Noplak®	0.12% chlorhexidine gluconate, water, 3.5% ethyl alcohol, hydroxyethylcellulose, sodium cyclamate, glycerin, menthol, hydrogenated castor oil, sodium saccharin, sorbitol, CI-19.140 and 42.090, flavoring agents, demineralized water
Sanifill Premium®	0.05% sodium fluoride (226ppm), sorbitol 70%, sodium benzoate, 0.35% polyhexamethylene biguanide chlorhydrate, methylsilanol, sodium cocoanphoacetate, disodium monophosphate, disodium phosphate, sodium saccharin, CI-19.140 and 42.090, deionized water

TABLE 2- Percentage of *S aureus* strains inhibited (from a total of 28 strains) by each dilution of the mouthwashes tested

	Sanifill Premium				Noplak				Periogard			
	Inhibited strains		Cumulative data		Inhibited strains		Cumulative data		Inhibited strains		Cumulative data	
Dilution	No.	%	No.	%	No.	%	No.	%	No.	%	No	%
1/640	0	0.0	0	0.0	23	82.1	23	82.1	7	25.0	7	25.0
1/320	0	0.0	0	0.0	5	17.9	28	100.0	21	75.0	28	100.0
1/160	0	0.0	0	0.0								
1/80	2	7.1	2	7.1								
1/40	26	92.9	28	100.0								

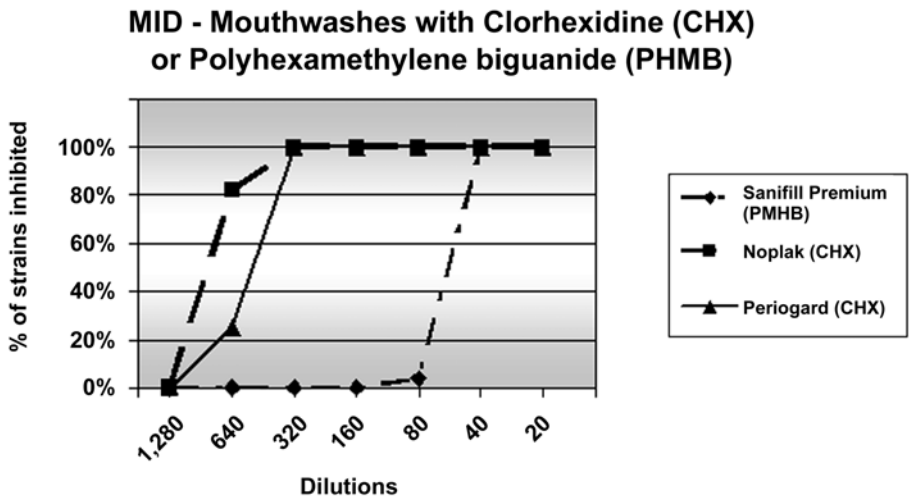

FIGURE 1- Graph depicting MID values obtained for each solution evaluated

Addy[22].

Statistical analysis

Results were expressed as scores determined from minimum to maximum dilution and comparison between all groups was performed using Kruskal-Wallis nonparametric test. When this test showed significant difference between the groups, Dunn's multiple comparison test, which allows two-by-two comparison between groups, was applied. Significance level was set at 5% ($p<0.05$).

RESULTS

The mouthwashes evaluated in this study presented different MIDs (Table 2). No significant difference was found between Noplak® and Periogard® ($p>0.05$). Sanifill Premium® was the least effective agent ($p<0.05$).

DISCUSSION

Chlorhexidine gluconate mouthwashes are available in the market at concentrations ranging from 0.12% and 0.2%. Smith, et al.[20], using the plaque formation index, compared *in vivo* the dental biofilm-inhibiting properties of mouthwashes containing 0.12% and 0.2% chlorhexidine gluconate. Both formulations presented better results than other mouthwashes without CHX. The CHX-based mouthwashes evaluated in this study contain 0.12% CHX.

Albuquerque Jr, et al.[4] assessed the MID of a mouthwash containing 0.12% chlorhexidine gluconate against 25 *S. aureus* strains, and found that all of them were inhibited up to a 1/80 dilution of the mouthwash. In the present study, the *S. aureus* strains were inhibited by Periogard® and by Noplak® up to a 1/320 dilution. This difference in the results of both studies may be related to the different sources of the microorganisms. Albuquerque Jr, et al.[4] used *S. aureus* strains collected from the oral cavity, while in the present study the microorganisms were retrieved from the oral and nasal cavities of the patients.

A study by Herrera, et al.[11] demonstrated that four mouthwashes containing the same active ingredient (0.12% chlorhexidine) but different formulations demonstrated statistically significant differences in their antimicrobial activity, both *in vitro* and *in vivo*. For the *in vitro* antimicrobial activity test, 20 selected bacterial species were evaluated. The *in vivo* test consisted of salivary bacterial count. The samples were cultured both aerobically and anaerobically. The formulation with alcohol was more active than those without alcohol, except for the formulation with chlorhexidine and cetylpyridinium chloride, which presented better antimicrobial activity. The addition of other active components should be further evaluated because chlorhexidine is highly cationic and may be inactivated by anionic substances[11].

Our results demonstrated that the CHX-based mouthwashes (Noplak® and Periogard®) were more effective in inhibiting bacterial growth in comparison to the PHMB-based product (Sanifill Premium®).

The antimicrobial activity of mouthwashes containing PHMB has been evaluated in several *in vivo* studies[17,18,23]. In one of these works, Rosin, et al.[17], using a method that measured bacterial counts on the tooth surface and oral mucosa, demonstrated that a mouthwash with 0.04% PHMB was more capable of inhibiting biofilm/dental plaque formation, compared to a negative control (placebo); however, the PHMB-based product was not as efficient as a mouthwash containing 0.12% chlorhexidine. This result is in agreement with our study, in which the PHMB-based mouthwash was not as effective as the CHX-based mouthwashes.

In another *in vivo* study in which a method to measure bacterial counts on tooth surface and mucosa was used, Rosin, et al.[18], observed that a mouthwash containing 0.12% PHMB was more capable of inhibiting biofilm/dental plaque, in comparison to a negative control (placebo), but no statistically significant difference was observed between the

PHMB-based mouthwash and a product with 0.12% chlorhexidine. Regarding the ability to reduce the number of intraoral bacteria, the chlorhexidine-based product performed better than the PHMB-based mouthwash. In our study, the evaluated mouthwash contains 0.35% PHMB, and presented lower MID values than CHX-based mouthwashes containing 0.12% CHX.

An *in vivo* study[23] using a method to determine bacterial counts on tooth surface and mucosa demonstrated that a solution containing 0.12% chlorhexidine was more capable of inhibiting and reducing biofilm/dental plaque than a mouthwash containing 0.2% PHMB. However, the PHMB-based mouthwash presented similar results to the chlorhexidine solution in reducing the number of bacteria present on the oral mucosa.

The mouthwashes evaluated by Rosin, et al.[17,18] and Welk, et al.[23] had a lower concentration of PHMB than the product tested in the present study (Sanifill Premium®). Despite its higher concentration of PHMB, Sanifill Premium® showed lower MID than the chlorhexidine-based mouthwashes.

CONCLUSIONS

The mouthwashes containing 0.12% chlorhexidine (Noplak® and Periogard®) presented higher MID values than that containing 0.35% PHMB (Sanifill Premium®) against salivary *S. aureus*. It was concluded that CHX-based mouthwashes have better antimicrobial activity than the PHMB-based mouthwash.

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