In vitro antimicrobial efficiency of a mouthwash containing triclosan/gantrez and sodium bicarbonate

Eficiência antimicrobiana *in vitro* de um enxaguatório bucal contendo triclosan/gantrez e bicarbonato de sódio

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Abstract: Several antiseptic substances have been used as adjuncts to routine mechanical procedures of oral hygiene, based on their antimicrobial effects. The objective of this study was to assess *in vitro* the antimicrobial efficiency of a mouthwash containing Triclosan/Gantrez and sodium bicarbonate in comparison to both positive and negative controls. Standard strain samples of *Escherichia coli*, *Pseudomonas aeruginosa*, *Actinomyces viscosus* and *Bacillus subtilis* were used. Samples of *Streptococcus mutans* and Gram-negative bacilli were collected from 20 volunteers (10 with a clinically healthy periodontium and 10 presenting biofilm-associated gingivitis). Evaluation of the antimicrobial activity was performed by determining the minimal inhibitory concentration (MIC). The results indicated that the test solution inhibited the growth of both Gram-negative and Gram-positive microorganisms from the volunteers' saliva as well as that of the standard strains at the MIC dilution of 1:20, whereas the MIC dilution of 0.12% chlorhexidine against the same bacteria was 1:80. Thus, even though the tested mouthrinse solution presented an *in-vitro* antimicrobial activity superior to that of a placebo, it was inferior to that of chlorhexidine.
Descriptors: Anti-bacterial agents; Triclosan; *In vitro*.

Resumo: Diversas substâncias antisépticas têm sido utilizadas como adjuntos aos procedimentos mecânicos rotineiros de higiene oral, com base em seus efeitos antimicrobianos. O objetivo deste estudo foi avaliar, *in vitro*, a eficiência antimicrobiana de um enxaguatório bucal contendo Triclosan/Gantrez e bicarbonato de sódio, em comparação a controles positivos e negativos. Linhagens padrão de *Escherichia coli*, *Pseudomonas aeruginosa*, *Actinomyces viscosus* e *Bacillus subtilis* foram utilizadas. Amostras de *Streptococcus mutans* e Bacilos Gram-negativos foram coletadas de 20 voluntários (10 com um periodonto clinicamente saudável e 10 com gengivite associada à presença de biofilme). A avaliação da atividade antimicrobiana foi realizada pela determinação da Concentração Inibitória Mínima (CIM). Os resultados mostraram que a solução teste inibiu o crescimento de microrganismos Gram-negativos e Gram-positivos da saliva dos voluntários, bem como das linhagens padrão na CIM de 1:20, enquanto que a CIM da diluição de clorexidina 0.12% contra as mesmas bactérias foi de 1:80. Desta forma, apesar de o enxaguatório bucal testado apresentar atividade antimicrobiana *in vitro* superior à do placebo, esta foi inferior à da Clorexidina.
Descritores: Agentes antimicrobianos; Triclosan; *In vitro*.

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Introduction

Mechanical control of dental biofilm has a somewhat limited success in part because it is regarded as time-consuming and technically difficult by most patients. This fact, coupled with an increase in the information available on the microbiology of periodontal diseases, has stimulated a great interest in developing topical antimicrobial agents to control dental biofilm. Mouthwashes have been particularly well accepted by patients due to their ease of use.

Among the chemotherapic agents used in mouthwashes, chlorhexidine usually is the "gold-standard" or positive control for comparison to other substances due to its proven efficiency.[4,11] Recently, Triclosan – a low-toxicity, non-ionic phenolic derivative with a wide spectrum of antimicrobial activity – has been successfully incorporated into toothpastes, resulting in moderate but distinct positive effects on both the dental biofilm and marginal inflammation or gingivitis.[9,10]

There is evidence indicating that the ingredients in the formula of triclosan-containing mouthwashes, including vehicle and other active substances, may influence its antimicrobial activity, and consequently its clinical efficiency.[6] The use of copolymers to improve active agent delivery is well established.[7] Methoxyethylene and maleic acid (Gantrez) are copolymers that have been proven to increase triclosan retention in the oral cavity, but the precise mechanism of this enhancement is still unknown.[12,13] Some authors have reported that, when used in combination with Triclosan, Gantrez clearly enhances the uptake of this antibacterial agent by teeth and soft tissues.[14]

Rodrigues *et al*.[12] (1999) reported that the addition of Zinc Citrate or Gantrez copolymer increased the biofilm inhibitory activity of triclosan. This observation is supported by previous studies[13,15] reporting that the addition of the copolymer gantrez may potentiate the activity of triclosan *in vitro*. An *in-vitro* study on biofilm formation in an experimental biofilm model observed that a mouthwash containing triclosan/gantrez provided a 31% reduction in biofilm formation when compared to a placebo solution.[19]

Thus, the purpose of this study was to evaluate the antimicrobial efficiency of a mouthwash containing an association of Triclosan/Gantrez and sodium bicarbonate.

Material and Methods

Volunteers were selected among patients seeking dental care at the Periodontics undergraduate clinic, School of Dentistry of Araraquara, São Paulo State University (UNESP). The protocol was approved by the Research Ethics Committee of that school, and the volunteers that agreed to participate signed an informed consent term after being informed about the nature and purpose of the study.

The inclusion criteria were the following: presence of at least 20 natural teeth; not currently under orthodontic treatment; absence of either fixed or removable prostheses; non-smokers; without any relevant medical alterations, including diabetes, cardiovascular or allergic alterations; no history of therapy with antibiotic, anti-inflammatory or corticosteroid drugs in the previous 2 months.

In order to have microbial samples representative of health- and disease-associated conditions, the 20 volunteers selected included 10 periodontally healthy individuals (less than 10% of the sites with bleeding of gingival margin, plus a maximum of 2 sites with probing depth > 6.0 mm or attachment level > 5.0 mm); and 10 individuals presenting gingivitis (bleeding of gingival margin in at least 10% of the sites), but also no more than 2 sites with probing depth > 6.0 mm or attachment level > 5.0 mm.[5]

These 20 individuals of both genders, including nine males and eleven females, with at least 20 natural teeth and a mean age of 20 ± 3.2 years, showing 24 ± 11.39% of visible plaque and 18.85 ± 12.41% of gingival index, were submitted to a collection of approximately 3 ml of unstimulated saliva. *Streptococcus* of the *mutans* group and Gram-positive filiform bacilli were grown from those samples. Standard strains of bacterial culture from international collections including *Actinomyces viscosus, Bacillus subtilis, Streptococcus mutans, Escherichia coli* and *Pseudomonas aeruginosa* were also used alongside those grown from the volunteers' saliva samples. The strains were numbered as follows:

1. *Bacillus subtilis* (G+) – standard strain 6633;

2. *Actinomyces viscosus* (G+) – standard strain 19246;
3. *Streptococcus* of the *mutans* group – patient 13 (G+);
4. Gram-negative filiform bacillus – patient 14 (G–);
5. *Escherichia coli* (G–) – standard strain 25922;
6. *Streptococcus* of the *mutans* group – patient 12 (G+);
7. *Streptococcus* of the *mutans* group – patient 09 (G+);
8. *Streptococcus* of the *mutans* group – patient 10 (G+).

Preparation of samples

Bacterial samples were put in a sterile vial containing 1 ml of defibrinated blood and then stored frozen at –18°C. To restart the cultures, the samples were thawed and aliquots of 0.1 ml were inoculated in Brain-Heart Infusion medium (DIFCO Laboratories Incorporated, Detroit, MI, USA) and incubated at 37°C for up to 24 hours. To confirm the purity of the cultures, aliquots were plated in Brain-Heart Infusion Agar under the same conditions.

Bacterial cultures were plated in the Brain-Heart Infusion broth for 24 hours at a temperature of 37°C. Purity of growth was checked by performing Gram staining on some culture smears and examining them under an optical microscope (Carl Zeiss ICS/KF2, Oberkachen, Germany). The inoculum prepared was always used within 15 minutes in order to maintain microbial viability.

Assessment of the antimicrobial activity of the solutions

A sensitivity test was carried out according to the agar dilution method described by Sutter *et al*.[17] (1979) and by the National Committee for Clinical Laboratory Standards[8] (1985). Serial dilutions of the different mouthwashes were prepared in artificial saliva (School of Pharmaceutical Sciences of Araraquara, São Paulo State University – UNESP). The solution tested was a mouthwash containing an association of Triclosan/Gantrez and sodium bicarbonate (Kolynos Ah!, Kolynos do Brazil Ind. Com. Ltda., São Paulo, SP, Brazil). The positive control was a 0.12% Chlorhexidine solution (Periogard, Colgate-Palmolive Ind. Com. Ltda., São Paulo, SP, Brazil), while the negative control was a placebo (with no mouthwash dilution added).

After that, 10 µl of the bacterial inoculum was seeded with a disposable sterile loop onto Brain-Heart Infusion Agar plates prepared with dilutions from 1:10 to 1:5120 (v/v) of each mouthwash. The plates were incubated in microaerophilia (pot with candle) at 37°C for 48 hours. After 24 and 48 hours of incubation, colony formation was checked by visual inspection.

The dilution was considered effective only when there was no growth of microorganisms on the plate. Sensitivity tests were carried out in triplicate for each dilution and microbial strain tested. The deal was to determine the Minimal Inhibiting Concentration (MIC) of each mouthwash.[2] The letters "A", "B" and "C" on the headers of the columns of Table 1 represent the experiments performed for each dilution of each mouthwash, according to all bacterial strains used. The results are presented in qualitative form only.

Results

Standardization of the methods and selection of the microbial species to be used were based on pilot experiments using the same experimental protocol described under 'Material and Methods'. The bacterial strains used were Gram-negative filiform bacilli and *Streptococcus* of the *mutans* group, grown from samples of saliva obtained from volunteers with clinically healthy periodontium and also from volunteers with marginal gingivitis. Besides these samples, standard strains from international collection of bacterial cultures were used, including *Actinomyces viscosus, Streptococcus mutans, Bacillus subtilis* and *Escherichia coli*.

The data were tabulated according to the presence or absence of growth of each bacterial strain in the different mouthwash dilutions assessed. Since none of the mouthwashes showed antimicrobial activity in dilutions higher than 1:80, the highest dilution used for the sensitivity test was 1:160.

According to Table 1, the placebo solution (negative control) did not inhibit growth of any bacterial strain used.

Table 1 - Antimicrobial activity of the tested solutions, according to dilution (1:10, 1:20, 1:40, 1:80, 1:160) and bacterial strain (1 through 8) tested.

Strains	Control			Experimental Mouthwash														
				10			20			40			80			160		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
1	+	+	+	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
2	+	+	+	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
6	+	+	+	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
7	+	+	+	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
8	+	+	+	–	–	–	–	–	–	+	+	+	+	+	+	+	+	+
Strains	Control			0.12% Chlorhexidine Solution														
				10			20			40			80			160		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
1	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
6	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
7	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
8	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	+	+	+
Strains	Control			Placebo Solution														
				10			20			40			80			160		
	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C	A	B	C
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

A: 1st assessment, B: 2nd assessment, C: 3rd assessment; 1, 2, 3, 4, 5, 6, 7 and 8: Strains used; (+): positive bacterial growth (no antimicrobial activity of tested dilution); (–): no bacterial growth (effective antimicrobial activity of tested dilution).

The test mouthwash (containing Triclosan/Gantrez and sodium bicarbonate) inhibited the growth of bacterial strain numbers 1, 2, 5, 6, 7 and 8. The inhibitory dilution of the test solution for all these 6 strains was 1:20 (Table 1).

The 0.12% Chlorhexidine solution used as positive control also inhibited 6 bacterial strains, including 5 that were inhibited by the test mouthwash (strains 1, 5, 6, 7 and 8) plus strain number 3 (instead of strain number 2, inhibited by the test mouthwash). The inhibitory dilution for the positive control was 1:80.

Discussion

The data were tabulated according to the presence (positive sign on the table) or absence (negative sign on the table) of growth of each bacterial strain in the different mouthwash dilutions assessed. Since none of the mouthwashes showed antimicrobial activity in dilutions higher than 1:80 in the pilot experiment, the highest dilution used for the sensitivity test was 1:160.

The placebo solution (negative control) did not inhibit growth of any bacterial strain used.

The results indicated that the mouthwash solution tested featured a MIC (Minimal Inhibiting Concentration)[2] of 1:20 against some standard-strains of bacterial culture from international collection used, including *Actinomyces viscosus*, *Bacillus subtilis* and *Escherichia coli*. Moreover, this mouthwash presented efficiency against some strains isolated from the patients' saliva (*Streptococcus* of the *mutans* group – patient 12, *Streptococcus* of the *mutans* group – patient 09, and *Streptococcus* of the *mutans* group – patient 10), but it was not efficient against strains of *Streptococcus* of the *mutans* group isolated from the saliva of patient 13, nor against filiform microorganisms (G–).

The MIC of the positive control mouthwash solution (0.12% chlorhexidine) was 1:80 against 5 of the same microorganisms killed by the test mouthwash (numbers 1, 5, 6, 7 and 8). However, it did not inhibit the growth of a standard-strain of *Actinomyces viscosus* (number 2), which is a microorganism present in dental biofilm associated with clinical periodontal health. Both test and positive control mouthwash solutions did not inhibit growth of Gram-negative filiform bacilli isolated from the saliva of different patients.[14]

In the present study, antimicrobial activity was assessed by the agar method using serial dilutions of the tested mouthwashes. The agar method is considered a standardized and reliable technique, allowing the simultaneous evaluation of many substances

using a large number of bacterial strains. Furthermore, this method involves a direct contact of the tested substances with the microbial cultures, which is important for the evaluation of mouthwash solutions.[18]

There are, however, other methods such as the disc diffusion method, which is also commonly used in this type of study, where the diameter of an inhibition halo of bacterial growth is considered to be directly proportional to the antimicrobial activity of the test solution. The diameter of the halo, however, can be influenced by the thickness and composition of the culture, by the concentration of the antimicrobial agent in the paper disc and by the degree of diffusion of the tested substances, which can be affected by the composition of the mouthwashes and dentifrices.[16]

Chlorhexidine-based formulas are currently the golden standard for antimicrobial mouthwashes, with abundant evidence supporting its effectiveness and safety.[4] Relatively recent information on the literature regarding triclosan[6], however, has generated interest based not only on its antimicrobial and anti-inflammatory activity, but especially because of the absence of undesirable side effects.

It is important to bear in mind that an experiment conducted *in vitro* has limitations, as it is considered a static system compared to *in vivo* tests, which may reflect the influence of various dynamic factors like systemic conditions, salivary flow, diet and dental anatomy.[1] Nevertheless, it might be considered that if the antimicrobial agent does not have activity *in vitro* it most likely will not work *in vivo*.

In this sense, assessments of antimicrobial activity conducted using monocultures *in vitro* enable a direct contact between the bacterial colonies and the chemical substances tested for a period of 24 to 48 hours (time usually demanded for incubation),[3] whereas in experiments conducted *in vivo* there is a greater number of microbial species, including indigenous and even "protective" bacterial species (those species associated with clinical health) which colonize dental biofilm, thus reducing the accuracy of the antimicrobial testing. One way of circumventing the limitations of *in vitro* studies evaluating the antimicrobial activity of mouthwashes against microorganisms of the dental biofilm is to make reference to clinical studies assessing their efficacy.

Conclusion

The present study demonstrated an intermediate level of efficacy of a solution containing Triclosan/Gantrez and sodium bicarbonate against some microbial species found in the oral microbiota, as compared to a placebo solution (negative control) and to a 0.12% Chlorhexidine solution (positive control).

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