Original research article

Antibacterial effect of mouthwashes against Streptococcus mutans, Staphylococcus aureus and Enterococcus faecalis

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Abstract

Introduction: Mouthwashes are one of the resources for controlling oral microbiota. They can reduce microorganisms, because they have antibacterial action and can access microorganisms even in areas of greater difficulty in the oral cavity. **Objective:** Antibacterial effect of some mouthwashes commonly used–0.12% chlorhexidine gluconate, 0.07% cetylpyridinium chloride, a solution based on essential oils (Listerine Zero), a solution of 1.33 mg benzethonium chloride / 25 mg hydrochloride lidocaine, a solution-based essential oils (Malvatricin) and 0.12% chlorhexidine + cetylpyridinium (Noplax)–was evaluated by means of agar diffusion and direct exposure tests. **Material and methods:** Strains of *Streptococcus mutans* (ATCC 27853), *Staphylococcus aureus* (ATCC 6538) and *Enterococcus faecalis* (ATCC 29212) were used in this study. For the agar diffusion test, Petri dishes with brain heart infusion agar (BHIA) were inoculated with the microbial suspensions. Sterile paper disks were immersed in the experimental for 1 min and then placed on the surface of BHIA. After incubation at 37ºC for 48 h, the diameters of the microbial inhibition halos were measured. For direct exposure test, no. 50 sterile absorbent paper points were immersed in the bacterial suspension for 5 min and then placed in Petri dishes and covered with 10 mL

of one of the tested solutions. At intervals of 1, 5, 10 and 30 min, absorbent paper points were removed from contact with substances and immersed in 7 mL of Letheen Broth and incubated at 37°C for 48 h. Microbial growth was assessed by the turbidity of the culture medium. **Results:** The agar diffusion test showed inhibition halos greater than 10 mm for all substances against bacteria. In the direct exposure test, 0.12% chlorhexidine gluconate, 0.07% cetylpyridinium chloride, and 0.12% chlorhexidine associated with cetylpyridinium showed antibacterial effect on all the tested microorganisms after 10 min. **Conclusion:** The tested solutions showed antimicrobial effect on the biological indicators. The solutions of 0.12% chlorhexidine gluconate, 0.07% cetylpyridinium chloride and 0.12% chlorhexidine associated with cetylpyridinium showed antimicrobial effect on all biological indicators after 10 min.

Introduction

The oral cavity presents a wide variety and number of microorganisms. More than 700 different species of microorganisms have been identified, including bacteria, fungi, protozoa, and viruses [6, 25, 42].

Colonization of the oral cavity occurs through contact with the maternal microbiota and objects. Gradually, this microbiota becomes abundant and diversified and may contain from 10^8 to 10^{11} bacteria/mL [41, 47]. Loesche [27] highlights that the oral microbiota presents a series of changes throughout the individual's life. This colonization is specific and involves a process of bacterial interaction with the receptors of host tissues. Microorganisms adhered to epithelial tissues can provide the site for binding of another species, contributing to the maintenance of an abundant and diversified microbiota [19].

Oral microbiota co-exists harmonically with the host keeping homeostasis. This microbiota can express its pathogenic potential when there is an imbalance, leading to the development of different pathologies [6, 16-18, 30, 31, 38, 48], with high prevalence for dental caries, periodontal diseases, and endodontic infections.

Dental caries is considered an infectious disease characterized by solubilizing enamel minerals. Among the main microorganisms, which increasing in number in microbial plaque leads to its emergence, *Streptococcus mutans*, *Lactobacillus sp.* and *Actinomyces sp.* stand out [27]. In endodontic microbiota, *Enterococcus faecalis* proved to be potentially important [33, 43, 51]. Another significant microorganism, but which has been quietly studied by dentistry, is *Staphylococcus aureus*. This microorganism preferentially colonizes the nasopharynx, skin, and mucosa, especially the nasal mucosa, and may also be related to several other pathological conditions [10, 20].

One of the resources for controlling the oral microbiota are the mouthwashes, which are used as antibacterial agents in order to reduce microorganisms and prevent their dissemination. They are easy to use, refreshing, and have access to microorganisms from the oral cavity even in areas of greater difficulty [5, 12, 13]. In this sense, several substances have been recommended for this antisepsis. Among them, chlorhexidine is a substance whose antibacterial effect has been widely studied and it is, therefore, indicated for this purpose. Other substances also recommended for oral antisepsis are quaternary ammoniums, which are also shown to be substances with antimicrobial effect [2, 7, 9, 18, 22, 23, 26, 27, 35, 39]. Considering the importance of the role of bacteria in frequent diseases that occur in the mouth and the possible influences of these microorganisms on other infections, including systemic infections, the analysis of the antibacterial activity of antiseptic solutions available on the market represents aspects of microbial relevance.

Material and methods

For the present study, three samples of microorganisms obtained from the American Type Culture Collection were used:
- *Enterococcus faecalis* (ATCC 29212);
- *Staphylococcus aureus* (ATCC 6538);
- *Streptococcus mutans* (ATCC 27853).

The strains were inoculated in 7 mL of brain heart infusion (BHI) (Difco Laboratories, Detroit,

MI, United States) and incubated at 37ºC for 24 h. The indicator microorganisms were grown on the surface of the brain heart infusion agar (BHIA) (Difco Laboratories, Detroit, MI, United States), following the same incubation conditions. Microbial cells were suspended in saline solution to reach the final concentration of about 3×10^8 cells/mL, similar to the tube in one of the MacFarland scale.

Experimental solutions

The solutions tested in this experiment were:
- 0.12% chlorhexidine gluconate (Colgate PerioGard®, Colgate-Palmolive, São Bernardo do Campo, SP, Brazil);
- 0.07% cetylpyridinium chloride (Oral B Pro Saúde Clinical Protection®, Procter & Gamble Company, Iowa City, IA, United States);
- essential oils-based solution (Listerine Zero®, Johnson & Johnson, São José dos Campos, SP, Brazil);
- 1.33 mg of benzethonium chloride / 25 mg of lidocaine hydrochloride solution (Hertz antiseptic spray®, Kley Hertz, Porto Alegre, RS, Brazil);
- essential oil-based solution (Malvatricin®, Laboratório Daudt Oliveira, Rio de Janeiro, RJ, Brazil);
- 0.12% chlorhexidine solution + cetilpiridinium (Noplak Max®, Laboratório Daudt Oliveira, Rio de Janeiro, RJ, Brazil).

Agar diffusion test

For the agar diffusion test, 18 Petri dishes with 20 mL of brain heart infusion agar (BHIA) (Difco Laboratories, Detroit, MI, United States) were inoculated with 0.1 mL of microbial suspension. The inoculum was spread on the surface of the culture medium, in order to obtain a confluent growth. Fifty-four paper discs with 9 mm of diameter were immersed in the experimental solutions for 1 min. For each plate containing the culture medium, three paper discs were placed. The plates were kept for 1 h at room temperature, and then incubated at 37ºC for 48 h. The diameters of the microbial inhibition zones were measured with digital caliper. Positive and negative controls were made, keeping three inoculated plates and three plates without inoculation, with identical incubation periods and conditions. All experiments were carried out under aseptic conditions and in triplicate.

Direct exposure test

For the direct exposure test, 216 no. 50 sterile absorbent paper points (Tanari, Tanariman Industrial, Manacaru, AM, Brazil) were immersed in the suspensions of microorganisms for 5 min and then placed in Petri dishes and covered with one of the six tested solutions. At intervals of 1, 5, 10 and 30 min, 54 absorbent paper cones were removed from contact with the substances individually transported and immersed in 7 mL of Letheen Broth (LB) (Difco Laboratories, Detroit, MI, United States), a medium that contains neutralizers or addicted by sodium thiosulfate (P.A., Art Laboratories, Campinas, SP, Brazil), and Tween 80 at appropriate concentrations, and subsequently incubated at 37ºC for 48 hours.

The negative control group consisted of 7 mL of sterile LB (Difco Laboratories, Detroit, MI, United States), while the positive control group consisted of test tubes containing 7 mL of LB (Difco Laboratories, Detroit, MI, United States) added with 0.1 mL of each microbial suspension. Microbial growth was analyzed by the turbidity of the culture medium. Next, 0.1 mL inoculum obtained from LB was transferred to 7 mL of BHI (Difco Laboratories, Detroit, MI, United States) under identical incubation conditions. Gram staining was used in BHI cultures to verify contamination and growth, being examined macro and microscopically. All experiments were carried out in triplicate and under aseptic conditions.

Results

Agar diffusion test

The tested solutions showed antimicrobial effect on the biological indicators. Inhibition halos varied according to the tested solution or the biological indicator. The results of the agar diffusion test are presented in Table I.

Table I – Average diameter of the microbial inhibition halos of the tested solutions (mm)

Solutions / microorganisms	Enterococcus faecalis	Staphylococcus aureus	Streptococcus mutans
0.12% chlorhexidine gluconate	15	20	30
0.07% cetylpyridinium chloride	13	13	25
Solution based on essential oils (Listerine Zero®)	10	10	11
1.33 mg of benzethonium chloride / 25 mg of hydrochloride lidocaine solution	17	18	18
Solution-based essential oils (Malvatricin®)	10	25	10
0.12% chlorhexidine + cetylpyridinium (Noplax®)	15	19	30

Direct exposure test

The solutions of 0.12% chlorhexidine gluconate, 0.07% cetylpyridinium chloride and 0.12% chlorhexidine associated with cetylpyridinium showed antimicrobial effect on all biological indicators after 10 min. The results of the direct exposure test are presented in Table II.

Table II – Antimicrobial effect of the tested solutions in the direct exposure test

Solutions / microorganisms	1 min	5 min	10 min	30 min
0.12% chlorhexidine gluconate				
Enterococcus faecalis	+ + +	+ + +	- - -	- - -
Staphylococcus aureus	+ + +	- - -	- - -	- - -
Streptococcus mutans	- - -	- - -	- - -	- - -
0.07% cetylpyridinium chloride				
Enterococcus faecalis	+ + +	+ + +	- - -	- - -
Staphylococcus aureus	+ + +	- - -	- - -	- - -
Streptococcus mutans	+ + +	- - -	- - -	- - -
Solution based on essential oils (Listerine Zero®)				
Enterococcus faecalis	+ + +	+ + +	+ + +	- - -
Staphylococcus aureus	+ + +	+ + +	- - -	- - -
Streptococcus mutans	- - -	- - -	- - -	- - -
1.33 mg of benzethonium chloride / 25 mg of hydrochloride lidocaine solution				
Enterococcus faecalis	+ + +	+ + +	+ + +	+ + +
Staphylococcus aureus	+ + +	+ + +	+ + +	+ + +
Streptococcus mutans	+ + +	+ + +	+ + +	- - -
Solution-based essential oils (Malvatricin®)				
Enterococcus faecalis	+ + +	+ + +	+ + +	+ + +
Staphylococcus aureus	+ + +	+ + +	+ + +	+ + +
Streptococcus mutans	+ + +	+ + +	+ + +	- - -
0.12% chlorhexidine + cetylpyridinium (Noplax®)				
Enterococcus faecalis	+ + +	+ + +	- - -	- - -
Staphylococcus aureus	+ + +	- - -	- - -	- - -
Streptococcus mutans	- - -	- - -	- - -	- - -

+ + +: microbial growth; - - -: lack of microbial growth

Discussion

Oral cavity is a septic environment of the organism, with the presence of a complex microbiota from a quantitative and qualitative point of view. This microbiota is distributed in the four main oral ecosystems – oral epithelium, back of the tongue, supragingival dental surface, and dental surfaces –, and epithelial subgingival, in addition to saliva, which, although it does not have its own microbiota, presents microorganisms from all buccal ecosystems and even transient microorganisms, that is, microorganisms that are not part of this microbiota and are there temporarily [6, 27].

The control of microorganisms in the oral cavity can be achieved through the hygiene of the oral cavity and also through the use of mouthwashes, which allows considerable reduction of the microbial population [1, 28]. Several solutions have been suggested for these purposes, and the most recently studied rinses include chlorhexidine gluconate and cetylpiridium chloride [1, 2, 4, 7, 9, 15, 22, 23, 26, 28, 32, 34, 40, 45, 46, 49].

Prior to the discussion of the results obtained by this study, it is necessary to clarify the methodology. For the evaluation of the antimicrobial activity of some agents, in general, there are three techniques: the dilution method, which produces a quantitative result; the agar diffusion method, which gives an inhibition zone around the agent; and the direct exposure method, which provides qualitative information [6, 16, 17]. The methodology was based on previous studies [16, 17]. Factors such as agar concentration, temperature, pH, absence of pre-incubation, dryness of the culture medium, maintenance for periods that exceed those allowed for the correct analysis, thus favoring the achievement of debatable results, were controlled in this study. This method is widely used in microbiology, and also standard for antibiogram. The choice for these techniques occurred because they were simple, reproducible, and effective. In addition, they allow to reach microorganisms with different morpho-red-respiratory characteristics [17].

The microorganisms selected for this study are present in some of the situations, respectively: dental caries, infected root canals, and hospital infections. They have been studied previously: *S. mutans*, a gram-positive coccus; *E. faecalis*, a facultative gram-positive bacterium that is highlighted as a high pathogenic agent; and *S. aureus* [6, 8, 10, 11, 16-18, 27, 29, 36, 43, 52, 53]. These microorganisms may also be responsible for infections of skin lesions, abscesses, wound infections, pneumonia, toxic shock syndrome, endocarditis, osteomyelitis, etc. [3, 14, 20, 24, 25, 37, 44, 48, 52].

Mouthwashes are recommended to aid the reduction of the oral microbiota, especially when mechanical methods of plaque removal are not effective, so they can act as auxiliaries [1, 30, 31, 46]. Garrote *et al.* [21] evaluated the antibacterial effect of some mouthwashes on facultative bacteria. The solutions tested were 0.07% cetylpyridinium chloride, 0.075% cetylpyridinium chloride, 0.12% chlorhexidine gluconate, and 0.13% benzalkonium chloride. The authors verified that the antiseptic solutions studied presented antibacterial effect by direct contact against *S. mutans*, *E. faecalis* and *P. aeruginosa*. Estrela *et al.* [16] determined the minimum inhibiting concentration of chlorhexidine at 2%, to inhibit *S. aureus*, *E. faecalis*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *Candida albicans*, and a mixture of these microorganisms, from a series of dilutions in the ratio of 1:10. The results demonstrated that 2% chlorhexidine showed minimum inhibitory concentration of 0.000002% for *S. aureus*; 0.002% for *P. aeruginosa*; and 0.02% for *E. faecalis*, *B. subtilis*, *C. albicans*, and for the mixture.

Cetylpyridinium chloride is also widely used in mouthwash due to its antimicrobial properties. Moreira *et al.* [36] demonstrated in vitro its effectiveness on some bacteria present in the oral cavity and saliva, confirming its main target of action on the gram-positive bacteria. Andrade *et al.* [4] determined the minimum inhibitory concentration of some oral antiseptics on the following microorganisms: *S. mutans*, *E. faecalis*, *Escherichia coli*, *S. aureus*, *C. albicans*, and *P. aeruginosa*. The authors verified that for *S. mutans*, *S. aureus*, *E. faecalis*, *E. coli*, and *C. albicans* cetylpiridium chloride presented minimum inhibitory concentration of 0.0125%, while for *P. aeruginosa* it was equal to 0.0333.

Listerine Zero®, a product based on essential oils, is accepted by the American Dental Association (ADA) for the control of plaque and gingivitis. This product is a phenolic compound whose antimicrobial action occurs through damage to the bacterial cell wall, which causes inhibition of enzymatic systems and reduction of lipopolysaccharides [32, 40, 45]. The result obtained in this study in the agar diffusion test may have occurred as a function of the diffusion of this substance in the culture medium, a phenomenon that depends on the physical-chemical characteristics of the analyzed substance, as already reported in the literature [36]. In the direct exposure test, it was verified that in the period of 30 min these solutions presented antimicrobial action on all the analyzed microorganisms.

Malvatricin® is a compound that presents in its formulation mauve, thyrotricin and quinosol. Its antimicrobial effect has been reported by different studies [15, 34, 36, 53]. Moreira *et al.* [36] observed the isolated effect of thyrotricin. It inhibited the growth of *Lactobacillus sp.*, but did not present antimicrobial effect on *S. mutans* and on a pool of microorganisms from the oral cavity. Regarding quinosol, the authors verified that this substance presented high antimicrobial activity, similar to the effect of chlorhexidine.

Benzethonium chloride, a cationic quaternary ammonium compound, has antimicrobial activity. The cation of the molecule stimulates the binding with anionic compound on the bacterium surface, allowing the alteration of the integrity of the cytoplasmic membrane and the inactivation of enzymes on this membrane, which, as a consequence, can result in denaturation of proteins [9, 18]. However, there are studies that highlight its toxic action [50].

The control of oral microorganisms is relevant when considering the possibility of these microorganisms invading and colonizing distant organs and causing systemic infections. The analysis of the antibacterial activity of mouthwashes available on the market is justified in an attempt to maintain harmony between the host and the complex microbiota of the oral cavity.

The results of this study showed that the analyzed solutions pointed out antimicrobial effect on the biological indicators tested. In the agar diffusion test, the inhibition halos varied according to the antiseptic solution or the biological indicator. In the direct exposure test, the solutions 0.12% chlorhexidine gluconate, 0.07% cetylpyridinium chloride, and the association of 0.12% chlorhexidine with cetylpyridinium showed antimicrobial effect on most microorganisms only after 10 min.

Conclusion

Based on the results of this study and respecting the employed methodology, it is possible to conclude that in the agar diffusion test the inhibition zones were greater than 10 mm for all substances and bacteria. In the direct exposure test, 0.12% chlorhexidine gluconate, 0.07% cetylpyridinium chloride, and 0.12% chlorhexidine associated with cetylpyridinium showed antibacterial effect on all microorganisms tested after 10 min.

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