Research Article

"*In vitro*" comparative experimental study of antimicrobial action of mouth washing products

Lidia Ruiz[1*], César Escribano[1], Patricia Veiga-Crespo[2],Tomas G. Villa [2] and Teresa Vinuesa[1],
[1] Dept. Pathology and Experimental Therapeutics, Microbiology Division. [2] Dept. Microbiol & Parasitol. University of Santiago de Compostela

Abstract
Regular use of mouth rinses modifies the oral habitat, since bacterial populations are submitted to a high selective pressure during the treatment exercised by the active presence of the disinfectant.. Mostly mouth rinses are based on the antibacterial effect of Chlorhexidine, Triclosan, essential oils and other antibacterials although other pharmaceutical characteristics can also affect their effectiveness. In this paper we compare "in vitro" the antibacterial effect of different oral rinsing solutions. Minimal Inhibitory Concentrations (MIC) and Minimal Bactericidal Concentrations (MBC) were determined as well as the kinetics of bacterial death in the presence of letal concentrations of the mouth rinses. MIC values expressed as Maximal Inhibitory Dilution (MID) of the mouth rinse ranged from 1 to 1/2048 depending on the microorganism and product, whereas Minimal Biocidal Concentration (MBC), expressed as Maximal Biocidal Dilution (MBD) ranged from 1 to 1/1024, being in general one dilution less than MIC.Maximal Biocidal Dilution is a good tool to measure the actual efficiency of mouth washing solutions. However, kinetics of death seems to be better in our work killing curves demonstrate that bacterial populations are mostly eliminated during the first minute after the contact of bacterial suspension and the mouth-washing solution. In all tested bacterial species mouth-washing solutions tested were able to reduce until undetectable number of viable bacteria the suspension treated except 1 and 5.
Key words: Mouth rinsing, Minimal Inhibitory Concentrations, Minimal Biocidal Concentrations, Kinetics of death.

Introduction
Dental plaque is found on enamel and is involved in the etiology of the most prevalent oral diseases: caries and periodontal disease. Prevention of these pathologies involves mechanical removal of plaque and chemical adjunctive measures that significantly contribute to oral health. There is therefore considerable interest in the use of antiplaque and/or antimicrobial agents in the prevention and treatment of these diseases[1].
Chlorhexidine (CHX) is the most extensively used biocide in periodontology, and prevents the colonization of the mouth by *Streptococcus mutans* [2,3] . The daily application of mouthwash with CHX reduces dental plaque, gingivitis, and caries in the oral cavity [4-6] . CHX is the agent that is used more frequently against *S. mutans*. Natural susceptibility to CHX varies, being more potent on Gram-positive than on Gram-negative microorganisms [7-9] although is highly variable depending on the isolates used. Chlorhexidine alters the permeability of the bacterial cell membrane. However, the chlorhexidine-induced alterations of the biofilm only affected a minor part of it being unable to cause its disintegration. These suggest the insufficient efficiency of chlorhexidine against oral biofilm.
Because of antibacterial properties phenolic compounds are used in antiseptics and disinfectants, in mouth rinse solutions.

Short- and long-term clinical studies have indicated that the daily use of Listerine® a mouth rinse that contains different phenolics such as thymol, eucalyptol, menthol, as well as methyl salycilate, retard plaque buildup and reduce gingivitis 10, and their low toxicity 11-17. The effect of Listerine on plaque was ascribed to its bactericidal properties that were documented in vitro as well as in vivo 18,19, Phenolic compounds, however, are also known to interfere with the inflammatory process 20,21. In addition, the presence of ethanol in Listerine preparations is a source of disagreement since most dentists are reluctant to use alcoholic preparations in the mouth. Propolis (bee glue) a natural resinous hive product, collected from various plant sources, manipulated by honeybees and extensively used in folk medicine, has been also studied as a possible active principle to be used in oral decontamination due to its antibacterial and antifungal activities probably originated from the flavonoid presence 22. The antibacterial and antifungal properties of propolis have been extensively investigated, although its chemical composition is linked to the phytogeographic origin, the activity of bee glue has been reported 23,24.

Another common ingredient for plaque inhibition found in mouth rinses is cetylpyridinium chloride (CPC). It has moderate chemical plaque inhibitory properties when used without mechanical tooth cleaning [25-30]. Longer, home use studies employing CPC mouth rinses as adjuncts to tooth brushing have mostly failed to prove a benefit on gingivitis. Several explanations have been reported [31,32].

The large variety of ingredients used in mouth rinse preparations, as well as a certain puzzle one can see when analyzing data of bacterial susceptibility was on the origin of this paper. In it we have determined the susceptibility of several bacteria obtained from culture collections as well as clinical isolates belonging to species known to play active role in oral infections.

Finally it should be emphasized that the regular use of mouth rinses modifies the oral habitat, since bacterial populations are submitted to a high selective pressure during the treatment exercised by the active presence of the disinfectant. Moreover many oral rinsing solutions, namely those including chlorhexidine and Triclosan, have been defined as oral rinses with a high degree of the so-called substantivity (i.e. local sustained-release delivery of active principle). This property originates new conditions since the strong selective pressure tends to diminish along time because decreasing concentrations of antimicrobial are generated.

Materials and Methods

Microbial strains

In this work we have used several strains from collections belonging to different species involved in oral pathologies: *Prevotella intermedia* DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen) 20706; *Porphyromonas gingivalis* DSMZ 20709; *Capnocytophaga ochracea (Bacteroides)* DSMZ 7271 ;*Micromonas micros (Peptostreptococcus)* DSMZ 20468; *Fusobacterium nucleatum* DSMZ 20482; *Streptococcus mutans* DSMZ 20523 ; *Aggregatibacter actinomycetemcomitans* CUB (Colección Universitat de Barcelona) O526; *Staphylococcus aureus* CECT (Colección Española de Cultivos Tipo) 4146¸ *Escherichia coli* CECT 101

We also use bacterial isolates from clinical specimens belonging to the following species: *Prevotella intermedia, Porphyromonas gingivalis, Micromonas micros, (Peptostreptococcus), Fusobacterium nucleatum, Streptococcus mutans, Aggregatibacter actinomycetemcomitans.* These isolates were obtained from patients of the "Clínica Odontologica Universitaria de la Universitat de Barcelona" and identified in our laboratory. Finally clinical isolates of the oral pathogenic yeast *Candida albicans* was also tested. *Candida* isolates were also obtained from clinical specimens in our dental clinic and characterized by us.

Mouth rinses

Eight different mouth rinses were used. All of them were purchased in a pharmacy; the ensemble represented the most widely used by Spanish population. Five of them contained Chlorhexidine digluconate as main antibacterial component, differences between them were based on the rest of the formula or on the chlorhexidine concentration. One was a triclosan-based rinsing solution whereas another contained fluorides and castor oil and finally one

contained cetylpiridinium as main active component. Formulae of the 8 oral rinsing solutions tested are indicated in table 1.

Inhibitory and biocide effect

Minimal Inhibitory Concentrations (MIC) were determined as the maximal dilution of the mouth rinsing solutions inhibiting visible growth. Thus we called this MID (**M**aximal **I**nhibitory **D**ilution). Serial dilutions of the mouth rinses studied were prepared in appropriate bacteriological media in tubes. Inocula of approximately 10^4 ufc/ml were added to each tube and subsequently incubated at 37 ºC in aerobic or anaerobic conditions depending on the microorganism for periods between 24 hours and four days also depending on the microorganism. Growth was observed visually after incubation.

Minimal Biocide concentrations (MBC) were determined as the maximal dilution preventing growth after inoculation on appropriate solid media (MBD, **M**aximal **B**iocide **D**ilution). 100 µl of the content of all tubes used in the determination of MID were transferred to plates of appropriate media (table 21) and plates incubated and visible growth of colonies scored after incubation.

	Composition (as indicated by manufacturer)
1	Cetylpiridinium chloride 5 mg/100 g; Chlorobutanol hemihydrate 50 mg/100 g; Eugenol 4 mg/100g
2	Chlorhexidin digluconate solution 0.5 ml/100 ml, chlorobutanol, alcohol 42.8 %, Glycerol, sodium docusate, ethanol, levomenthol, essential oils, E-124
3	Triclosan 0.15g/100ml; Zinc chlorhide 0.10g/100 ml; Vitamin E acetate 0.04 g/100 ml; Xylitol 1.00 g/1000 ml; Alcohol free excipient
4	Chlorhexidine digluconate; Cetylpiridinium chloride; Water, propylene glycol, Glycerin, PEG-40, hydrogenated Castor; oil, arome, potassium acesulfame,
5	Amine fluorides (olafluor) 0.1641 %; Stannous-Fluoride 0.0523 % Water, xylitol, PVP, PEG-40, hydrogenated Castor oil, Olaflur,; Sodium saccharin, CI42051
6	Chlorhexidine digluconate0.05g/100g; Sodium fluoride 0.05g/100g Water, Glycerin, Polysorbate 20, Aroma, Methylparaben, sodium saccharin, sodium benzoate, Propylparaben, CI 42051, CI 47005.
7	Chlorhexidine digluconate0 0.12g/100g; Water;propylene glycol, glycerin, PEG-40 hydrogenated Castor; oil, arome, potassium acesulfame, C.I.14720
8	Chlorhexidine digluconate 0.05g/100g
	Table 1 Composition of mouth-washing solutions used

Species	***Medium***	***Incubation***	***Time of incubation***
Escherichia coli	**MH**	**aerobic**	**24 h**
Staphylococcus aureus	**MH**	**aerobic**	**24 h**
Aggregatibacter actinomycetemcomitans	**TSA**	**CO_2**	**3 days**
Streptococcus mutans	**MH**	**CO_2**	**24 h**
Fusobacterium nucleatum	**Supplemented *Brucella* broth**	**anaerobic**	**4 days**
Micromonas micros (Peptostreptococcus)	**Supplemented *Brucella* broth**	**anaerobic**	**4 days**
Capnocytophaga ochracea (Bacteroides)	**Supplemented *Brucella* broth**	**anaerobic**	**4 days**
Porphyromonas gingivalis	**Supplemented *Brucella* broth**	**anaerobic**	**3 days**
Prevotella intermedia	**Supplemented *Brucella* broth**	**anaerobic**	**3 days**
Candida albicans	**Sabouraud**	**aerobic**	**24 h**

Table 2. Bacterial strains and culture conditions

Kinetics of action

In order to determine the kinetics of antimicrobial action of the rinsing solutions, sets of experiments were performed as follows: microbial suspensions were mixed with rinsing solutions at concentrations twice the MID value as previously determined. At intervals of 15 seconds aliquots were obtained and immediately diluted 10,000 times to inhibit the antibacterial effect of the rinsing solution. Viable count of surviving bacteria were made, plates incubated in appropriate conditions and bacteria scored after incubation. Results were plotted in order to compare death kinetics. Slopes can be directly related with antibacterial effect.

Results

Maximal dilutions able to inhibit visible microbial growth (MID) of the products tested are shown in Table 3. Table 4 shows the values of MBD which were in almost all cases just one level of dilution less than MID. Figures 1.1 to 1.4 show death kinetics of four different bacteria when contacted with the 9 mouth-rinses tested.

	1	***2***	***3***	***4***	***5***	***6***	***7***	***8***
Aggregatibacter actinomycetemcomitans	1/32	1/128	1/2048	1/2048	1/16	1/1024	1/2048	1/2048
Candida albicans	¼	1/32	1/16	1/583	¼	1/4048	1/583	1/512
Capnocytophaga ochracea	1/512	1/64	1/256	1/2048	1/32	1/2048	1/2048	1/2048
Escherichia coli	>1	1/1024	1/512	1/2048	>1	1/512	1/2048	1/1024
Fusobacterium nucleatum	1/32	1/256	1/2048	1/1024	1/16	1/1024	1/2048	1/2048
Micromonas micros (Peptostreptococcus)	1/32	1/128	1/2048	1/512	1/16	1/1024	1/4096	1/2048
Porphyromonas gingivalis	1/16	1/256	1/2048	1/2048	1/16	1/1024	1/2048	1/2048
Prevotella intermedia	1/32	1/128	1/2048	1/2048	1/8	1/512	1/2048	1/1024
Staphylococcus aureus	1/4	1/512	1/1024	1/1024	1/4	1/64	1/1024	1/1024
Streptococcus mutans	1/256	1/8	1/16	1/512	1/256	1/128	1/512	1/256

Table 3 Values of Maximal Inhibitory Dilutions (MID) of the different mouth-washing solutions tested for the strains used in the study

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Aggregatibacter actinomycetemcomitans	1/8	1/64	1/1024	1/1024	¼	1/1024	1/1024	1/1024
Candida albicans	¼	1/32	1/16	1/512	¼	1/1024	1/512	1/512
Capnocytophaga ochracea	1/32	1/32	1/64	1/1024	1/16	1/512	1/1024	1/512
Escherichia coli	>1	1/512	1/512	1/256	>1	1/8	1/16	1/64
Fusobacterium nucleatum	1/256	1/32	1/64	1/1024	1/8	1/1024	1/1024	1/512
Micromonas micros (Peptostreptococcus)	½	1/8	1/32	1/64	½	1/128	1/128	1/256
Porphyromonas gingivalis	1/8	1/8	1/32	1/1024	1/8	1/64	1/256	1/512
Prevotella intermedia	¼	1/2	1/64	1/1024	¼	1/8	1/256	1/512
Staphylococcus aureus	>1	1/256	1/256	1/256	>1	1/32	1/256	1/256
Streptococcus mutans	1/128	1/8	1/8	1/256	1/128	1/128	1/256	1/256

Table 4 Values on Maximal Biocidal Dilutions (MBD) of the different mouth-washing solutions tested for the strains used in the study

Discussion

Although we have seen slight differences between clinical and collection strains they were of one level dilution when existing. It is apparent that mouth washers based on chlorhexidine were the most active in inhibiting microbial growth. In principle it is widely accepted that values of minimal inhibitory concentrations (MID in this case) constitute a good parameter to describe the antimicrobial action of either antibiotics or disinfectants, although it is commonly behaved that the parameter is not accurate enough for disinfectants. In fact, values of MID can be strongly affected by long incubation periods (up to 18 hours when testing aerobic and facultative bacteria and more than 48 hours when anaerobic microbes are studied) this is the origin of significant limitations, since concentration of the antimicrobial can became altered along the incubation period. Moreover, prolonged time of contact between bacteria and disinfectant is far from conditions of use.

Thus, maximal biocidal dilution (MBD) would be a much better parameter to measure the actual efficiency of mouth

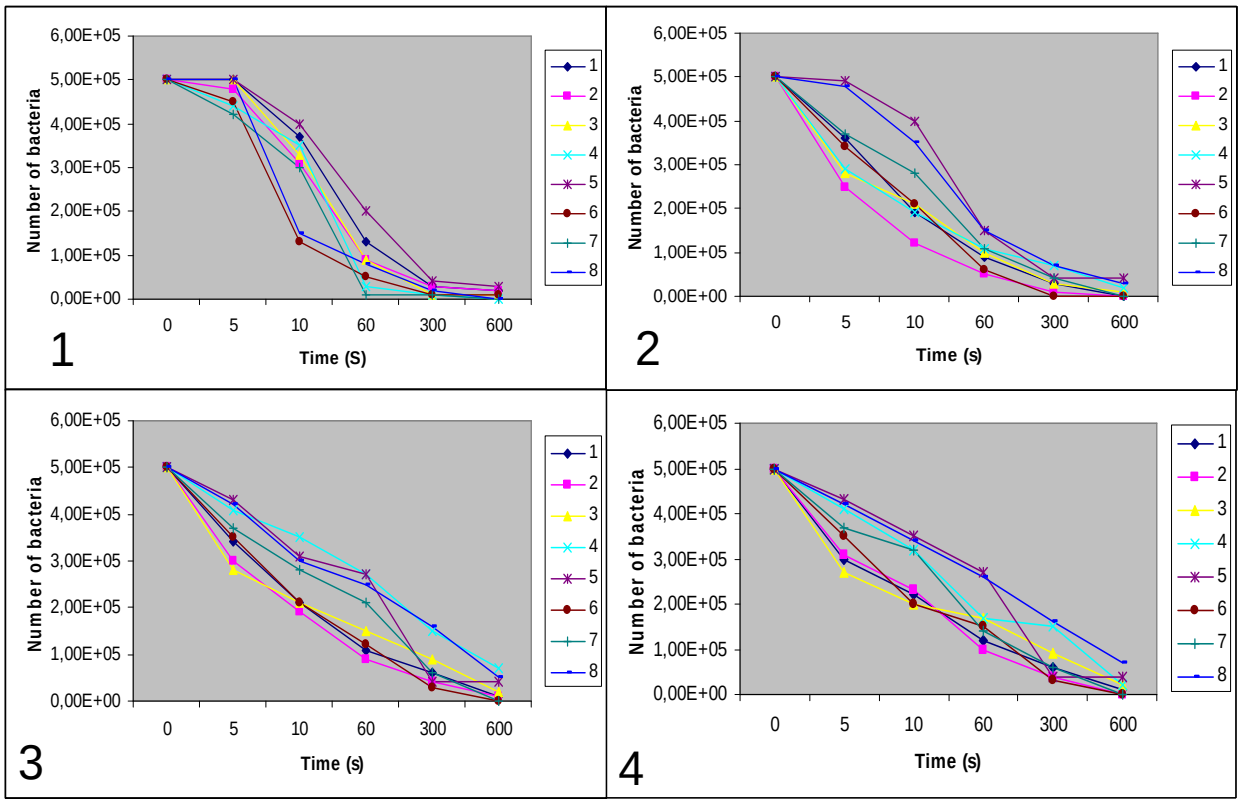

Figure1. 1.- Death curve of *Streptococcus mutans* when contacted with mouth rinses at concentrations two times the MIC; 2.- Death curve of *Porphyromonas gingivalis* when contacted with mouth rinses at concentrations two times the MIC; 3.- Death curve of *Prevotella intermedia* when contacted with mouth rinses at concentrations two times the MIC; 4.- Death curve of *Micromonas micros* when contacted with mouth rinses at concentrations two times the MIC.

washing solutions. In our results, as can be seen in the table 4 values of MBD were in almost all cases just one level of dilution less than MID.

The kinetics of killing effect is in fact a much better parameter to evaluate the actual efficiency of disinfectants.. *S. mutans* (Fig 1) was completely killed by mouth rinses 4, 7 and 8 (less than 10 ufc/ml). The rest of mouth washing products were able to eliminate most of bacteria although bacterial population was still detectable after 10 min of contact. *P gingivalis* (Fig 2) was effectively killed by all of the mouth-washing solutions studied. After 5 min contact mouthwash 7 already have killed the whole population. Moreover, after 10 min a few survivors were still detectable in 1, 3, 5, 8. In the rest of studied products no bacteria was detected after 10 min. When *P. intermedia* were tested, 4 and 7 were able to kill 100 % of bacteria after 5 min contact. The rest of mouthwashes were significantly slower (Fig 3). Similar results were obtained in the case of *M. micros,* again 4, 6 and 7 were the most active killing the whole population after 5 min contact the rest were again slower in eliminating bacterial population (Fig. 4).

In principle it is assumed that MIC values constitute a good tool to determine the antimicrobial action of either antibiotics or disinfectants, although the parameter is not accurate enough for disinfectants. Values of MIC can be strongly affected by long incubation periods (up to 18 hours when testing aerobic and facultative bacteria and up to 48 hours when anaerobic microbes are used). In principle the results herein presented show that all (but 1 and 5) had remarkable antimicrobial activity.

It seems clear that microbicidal concentration (MBC) or even better the kinetics of killing effect are in fact much better parameters to evaluate the actual efficiency of mouth washing solutions . It should be noted that in general, values of MBC were just one dilution less than MIC.

In summary, 4 and 7, were the most active mouth-washing solutions, followed by 8, 3 and 6, (in this order from higher to lower antimicrobial action), whereas the

antimicrobial action of the rest was clearly lower. Killing curves demonstrate that bacterial populations are mostly eliminated during the first minute after the contact of bacterial suspension and the mouth-washing solution. In all tested bacterial species mouth-washing solutions tested were able to reduce until undetectable number of viable bacteria the suspension treated except 1 and 5.

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Corresponding author
lidiaruizm@ub.edu
phone +34 934024265
fax +34 934029082