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MOUTHRINSES: A COMPARATIVE MICROBIOLOGICAL STUDY

G. GAUTIER[1,4], M. NOGUER[1,3], N. COSTA[1], J. CANELA[2], M. VIÑAS[1]

1 Unitat de Microbiologia
2 Unitat de Epidemiologia Clinica
3 Unitat de Pato logia Dental
Dental School - Campus de Bellvitge. Universitat de Barcelona. Spain..
4 Laboratoire de bacteriologie. Faculté de Pharmacie. Université de Montpellier. France.

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ABSTRACT

This study was performed in order to evaluate the efficacy of different mouthrinses whose use is extended in Spain. Six different antiseptic mouthrinses were studied by means of determination of Minimal Inhibitory Concentration (MIC) values against KIebsiella pneumoniae, Serratia marcescens, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhimurium, Bacilus subtilis, Streptococcus mutans, Prevotella intermedia, Porphyromonas gingivalis and Actinobacillus actinomycetemcomitans. Also in vivo experiments were carried out in volunteers by the use of mouthrinses and evaluation of bacterial populations before and after the treatment.. Finally, the kinetics of bacterial deatb was determined. Results suggested that the determination of MIC values is not a reliable method to evaluate tbe antibacterial effect of sucb products. *Gn* the other hand those rinsing solutions based on tbe effect of oxygen, such as tbose containing carbamide peroxide have a greater efficacy against anaerobic bacteria compared with rinses whose active molecule is a disinfectant.. Finally, the kinetics of bacterial death demonstrates that the essential oil rinse kills bacteria much faster.. AlI tested mouthrinses were active as antibacterial although tbose based on oxygen production or essential oils were more active than solutions based on chlorhexidine and Triclosan.

RESUME

Cette étude a été réalisée dans le but d'évaluer l'efficacité des divers bains de bouche dont l'usage est répandu en Espagne. Six types de bains de bouche antiseptiques ont été étudiés en déterminant les valeurs de Concentration Minimum d'Inhibition (CMI) contre la KIebsiella pneumoniae, Serratia marcescens, Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Salmonella typhimurium, Bacilus subtilis, Streptococcus mutans, Prevotella intermedia, Porphyromonas gingivalis et Actinobacillus actinomycetemcomitans. Des expériences in vivo ont également été effectuées sur des volontaires utilisant des bains de bouche, en évaluant les colonies de bactéries avant et apres le traitement.. Enfin, la cinétique de la mort des bactéries a été déterminée. Les résultats ont suggéré que la détermination des valeurs de CMI n'est pas une méthode fiable pour évaluer l'effet antibactérien de ces produits. D'autre part, ces solutions pour rinser basées sur l'effet de l'oxygene, comme celles qui contiennent du peroxyde de carbamide, sont beaucoup plus efficaces contre les bactéries anaérobiques que celles dont la molécule active est un désinfectant.. Finalement la cinétique de la mort des bactéries démontre qu'un bain fait avec une huile essentielle tue les bactéries beaucoup plus rapidement.. Tous ces bains testé s étaient actifs en tant qu'antibactériens bien que ceux qui étaient basés sur la production d'oxygene ou sur des huiles essentielles étaient plus actifs que les solutions à base de chlorexidine et triclosan.

INTRODUCTION

Antiseptic mouthrinses solutions made and commercialized by pharmaceutical companies can be complementary to dental hygiene, and thereby, in preventive dentistry. These kind of products are often used as preventives by populations and occasionally prescribed by dentists. Chemical composition of rinses is extremely variable. Antibacterial effects of antiseptic mouthrinse can be reached through the use of different antimicrobial agents (Brecx M. et al. 1990; Ross N.M. et al. 1989; Newman M.G. et al. 1990).

The intraduction of chlorhexidine-containing mouthrinses has introduced new perspectives in preventive dentistry. Chlorhexidine has been used to prevent dental caries and it has been assumed that its effect take place through a reduction in the levels of bacterial populations(mainly those of Streptococcus mutans) (Lindhe 1. 1987). Other rinsing solutions used in this study contain triclosan , esential oils or peroxides as antibacterials.

Six different antiseptic mouthrinses currently commercialized in Spain either in drugstores and/or supermarkets were selected. This selection included most kinds of mouthrinses. The antibacterial activity was first measured by the determination of minima! inhibitory concentrations against different bacteria! species. Experiments in vivo were carried out in volunteers to determine the microbial elimination. Finally kinetics of bacterial death was studied.

MATERIAL AND METHODS

Rinsing solutions.

In order to avoid the use of commercial name rinsing solutions were called with numerals following a C. The composition of the different rinses are indicated in table 1.

Tab. 1 - *Composition of the rinsing solutions used.*

Rinsing solution	Components	Concentration
CI	Benzoic acid	0.125 mg/ml
	methyl salycilate	0.06 mg/ml
	Essentials	0.196 mg/ml
	Ethanol	27,2%
C2	Chlorhexidine digluconate	1,20 mg/ml
	Sodium fluoride	0,5 mg/ml
	saccharin	0.6 mg/ml
C3	Chlorhexidine digluconate	120 mg/ml
	Saccharin	0.1 mg/ml
C4	Carbamide peroxide	100 mg/ml
	menthol	0.5 mg/ml
	U1C	NI
CS	Potassium nitrate	lO mg/ml
	Triclosan	1,5 mg/ml
	Zinc chloride	1 mg/ml
	Sodium fluoride	2 mg/ml
	Pantenol	5 mg/ml
	Vitamin E	0,4 mg/ml
	Xilitol	10 mg/ml
	UIC	NI
C6	Triclosan	1,5 mg/ml
	Zinc chloride	2 mg/ml
	Alantoin	2 mg/ml
	Saccharin	0.2mg/ml
	Ethanol	NI

(NI: Not Indicated)

Bacterial strains and culture conditions.

Several collection bacterial strains were used. There are indicated in table 2. On the other hand a set of strains isolated from patients were also tested. Among them strains of Streptococcus mutans, Prevotella oralis, Actinomyces odontolyticus and Porphyramonas gingivalis were isolated from patients. Isolates were identified by the use of API20A system. Strains were maintained at -80ºC in culture media supplemented with 10% glycerol until used.

Appropriate bacteriological media were used. Most of them were obtained *fram* ADSA Scharlau Barcelona Spain. Some media were prepared in the lab follbwing the recommendations of the VPI manual (Virginia Politechnic Institute). Anaerobic jars using either Gaspack system or the anaerobic system for the generation of anaerobic conditions (Oxoid, Hampshire, England) were used.

MIC determination

MICs of aerobic and facultative bacteria were determined in Muller-Hinton broth by the broth microdilution method recommended by the National Committee for Clinical Laboratory standards (NCCLS). Microdilution wells were inoculated with 0.5 x 106 cfu/ml of the microorganism to be tested and incubated 24 hours.

MICs for anaerobic microorganisms were determined by a similar procedure but incubation was performed in anaerobic jars.

Tab. 2 - *Bacterial strains used in this study.*

Bacrerial species	*Source*	*Gnaracteristics*	*Reference*
Klebsiella pneumoniae	Our lab	wild type	Viñas *el al., 1983*
Se"utia marcescens	Our lab	Wild type Pigll)ell1ecL strain	Viñas *el al., 1983*
Escherichia coli	Ourlab	Laboratory strain	Leranoz *el al., 1989*
Pseudomonas aeruginosa	CETC	CETC	CETC' manual
Slaphylococcus aureus	CETC	CETC	CETC' manual
Salmonellá typhimurium	CETC	CETC	CETC' manual
Bacillus subtilis	CETC	CETC	CETC' manual

• Manual ofthe Spanish Type Cuhure Collection

Kinetics of action

In order to determine the kinetics of antibacterial action of the rinsing solutions, sets of experiments were carried out as follows: bacterial suspensions were mixed with rinsing solutions at concentrations twice the MIC value previously determined. At intervals of 15 seconds aliquots were obtained and immediately diluted 10,000 times to inhibit the antibacterial effect. Viable count of surviving bacteria were made, plates incubated and bacteria scored after 24 hours incubation. Results were plotted. Slopes resulted to be directly related with antibacterial effect.

In vivo experiments

In order to determine the actual efficiency of rinsing solutions a total of 30 volunteers were selected. The experiment was carried out as follows. Once a week a set of three flasks labeled with numbers 1, 2 and 3 were distributed to the volunteers with a sheet of instructions. Volunteers rinsed with the content of flask 1 (saline) for one minute. Then, volunteers rinsed with the content of flask 2, also for one minute. This second flask contained the rinsing solution to be tested. Finally after two minutes, a new rinse with saline was made. Content of bacteria in the three solutions was estimated both by optical density measurement (560 nm wavelength) and by viable count determination. Results were expressed as percentage of remaining bacteria (UFC/ml in flask 3 x 100/ cfu/ml in flask 1)

Statistical methods

Experiments made in volunteers generate two series of data: before and after treatment. A t-student paired test was applied. The normal distribution of data was tested by the use of Kolmogorov-Smirnov test. Significant level was 5 %.

RESULTS

Table 3 summarizes the results of MIC determination against the different bacteria tested. The concentration of rinsing solutions are indicated in percentage vol/yo!..

Bacterial species	C1	C2	C3	C4	C5	C6
Serratia marcescens	12	0.3	0.3	1.5	25	25
Salmonella typhimurium	12	0.3	0.3	0.3	25	50
E~cherichia coli	12	0.09	0.09	0.39	0.18	0.18
Klebsiella pneumoniae	25	1.5	1.5	0.05	0.05	0.05
Pseudomonas aeruginosa	25	0.7	0.3	3.1	25	50
Bacillus subtilis	12.5	0.04	0.04	1.5	1.5	1.5
StreptococC1lS mutans	25	0.04	004	0.04	0.09	0.09
Porphyromonas gingivalis	0.06	0.0004	0.00048	0.00097	0.00097	0:00009
Prevotella ora/is	0.062	0.00048	000048	0.0039	0.0039	0.00024
Actinob.Actinomyc etemcomitans	12	0.5	0.5	0.025	1.2	1.2

Tab. 3 - *MIC values 01 the six different tested mouthrinses.*

Experiments carried out In volunteers gave extremely variable results as can be seen in figure 1. When a Kolmogorov-Smirnov test was applied a normal distribution was observed. If normal distribution is assumed t-student paired test can be used. Results are indicated in table 4. In all cases differences between pairs of groups were significant. . Finally killing kjnetics is indicated in figure 2 using E. coli as an example although in all cases kinetics of heath was very similar (data not shown).

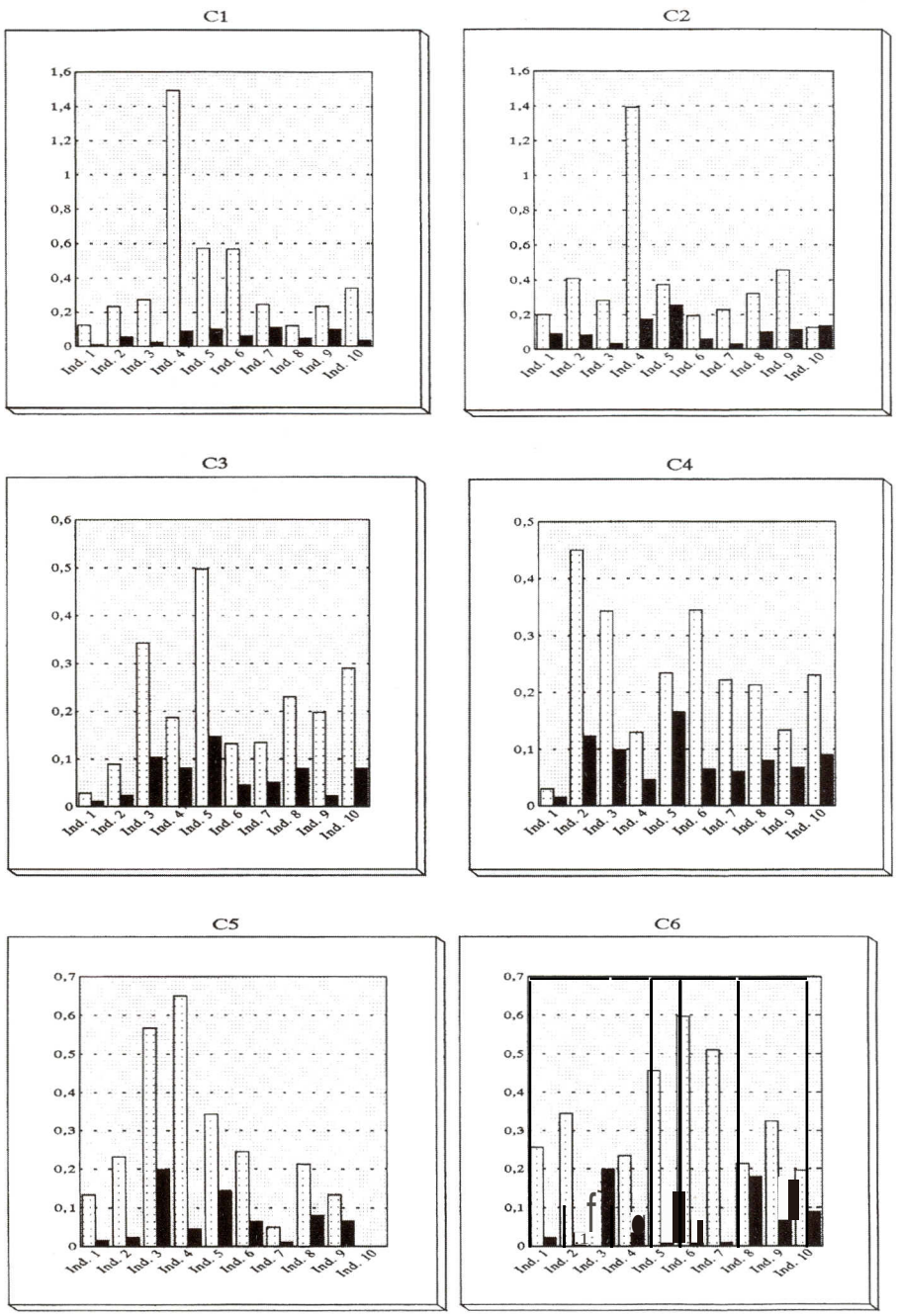

Fig. 1 - *Diagrams showing 10 randomized individuals. Comparison between bacterial recovery before (dotted bars) and after (black bars) treatment.*

Tab. 4

Mouthwashing solution	T-student	P	Significancy
C1	*2.8S*	0,19	YES
C2	2.71	0.024	YES
C3	4.16	'O.003	YES
C4	4.26	0.003	YES
CS	3.65	0.006	YES
C6	3.85	0.004	YES

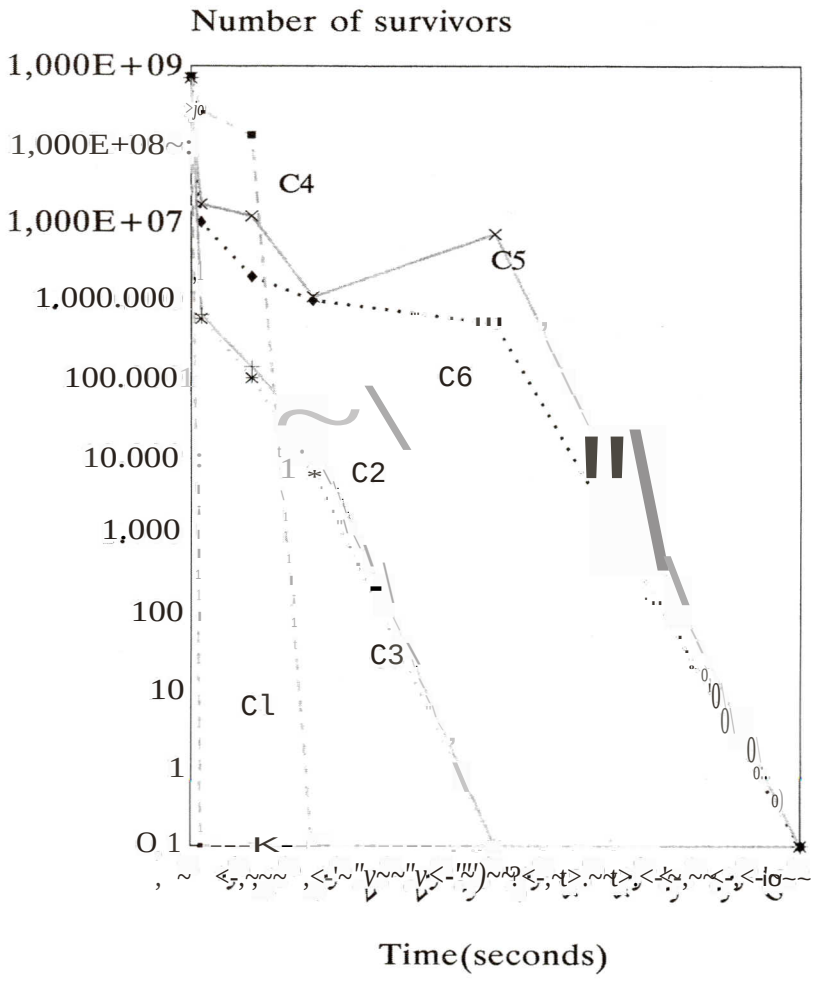

Fig. 2 -*Death kinetics when bacterial cultures were mixed with the different mouthrinses:* Cl *(.); C2(+); C3(*);* C4 (.); *CS (x);* Có (.)

DISCUSSION

Bacterial colonization of the human mouth is strongly correlated with the appearance of most dental diseases such as caries and periodontitis. Bacterial attack take place mainly through acid production and endotoxin activity (Fine D.H. et al. 1992). Both supragingival and subgingival bacterial plaques play important roles in bucco-dental infectious diseases(Hellstrom M.K.. et al. 1996). Several works dealing with comparison of chlorhexidine and non-chlorhexidine containing mouthrinses have been published (Mendieta C. et al. 1994; Walker C. et al. 1994; Rubinstein L. 1994; Elworthy Al et al. 1995). In Spain the use of mouthwashes has increased largely in the last few years.

Determination of bactericidal effect by means of MIC determination demonstrates that in all cases an important antibacterial action could be detected. In all cases C1 was the rinsing solution giving higher MIC.. In other words, based on MIC values, one could conclude that C1 was less powerful as antibacterial solution. However when kinetics of action was analyzed, C1 exhibited the fastest action among all rinsing solutions tested. This is in ¡ine with the results obtained in volunteers.

From experiments of MIC determinations some other conclusions could be drawn. We observed an exceptional resistance to triclosan in Salmonella as well as in Pseudomonas. Obviously neither Salmonella or Pseudomonas are species significant in dentistry , however they are frequently used in antimicrobial studies due to the frequency of resistant phenotypes in these species. We did not investigate the mechanism leading to this resistance but data obtained suggested a specific mechanism. If this is so, and taking into account the frequency of genetic exchange among bacteria it is feasible that the continuous use of triclosan based rinsing solutions could lead to the emergence of resistant strains in bacteria able to produce oral diseases.

As far as gram-positive bacteria values of MIC are concerned, they were slightly lower than in gram negative. This is consistent with the existence of outer membrane in Gram negative bacteria. In principIe this outer membrane prevents the direct contact between rinsing agent and bacterial cytoplasmic membrane. Strep. mutans is considered as the main cariogenic microorganism (primary cariogen) although some authors postulated than the diet is much more important than the prevailing bacterial species, even suggesting that Streptococci (including mutans species) should be considered as belonging to the autochtonous microfiora of the human mouth (van Palenstein W.H. et al., 1996). MIC values obtained with Strep. mutans were low for all tested mouthwashes except C1 (tab. 3).

Activities against anaerobic pathogenic bacteria were also excellent.. In this case carbamide peroxide based rinsing solution was the most active. Since, strict anaerobic bacteria lack enzymes to resist oxygen and reactive forms of oxygen like peroxides and superoxide, oxygen is the best antimicrobial for such group of bacteria.

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Corresponding Author:
Miguel Viñas
Laboratory of Microbiology
Dental School, Campus of Bellvitge
University of Barcelona
08907 Hospitalet (Barcelona). Spain..
Tel.: 34 93 *402 4250*
Fax: 34 93 *402 4258*
email: mvinyas@bell.ub.es