In vitro Bactericidal Assay under Simulated Practical Conditions for Comparison of Chlorhexidine Mouthrinses: Chlorhexidine Concentration is only one of the *In vitro* Activity Criteria

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Abstract

Aim: To determine the in vitro bactericidal activity of different chlorhexidine (CHX)-based commercial mouthwash products claiming different chlorhexidine concentrations under conditions similar to their use. Method: Bactericidal assays were performed using four major bacterial species implicated in periodontal disease: *Fusobacterium nucleatum* CIP101130, *Aggregatibacter actinomycetemcomitans* CIP 52.106T, *Prevotella intermedia* CIP 103607, and *Porphyromonas gingivalis* CIP 103683. Seven commercially available mouthwash products were chosen, each containing CHX digluconate (concentrations ranged from 0.1% to 0.2%) as the principle active ingredient. Assays were performed according to European guidelines for antiseptics (with modifications to mimic conditions of use) by exposing bacterial suspensions to the mouthwash solutions for 1 min ± 5 seconds at 32 ± 1°C in the presence of an interfering substance (artificial saliva). The log reduction in bacterial count was determined. Results: Five of the tested mouthwashes were defined as bactericidal to each of the four test strains (log reduction ≥ 5). However, two mouthwashes were not defined as bactericidal to all test strains (log reduction <5). In one case, a 0.12% CHX mouthwash was not bactericidal towards A. actinomycetemcomitans. In the other case, a 0.2% CHX mouthwash was not bactericidal towards two test strains, *A. actinomycetemcomitans* and *P. intermedia*. Conclusions: This study emphasizes that antimicrobial activity of CHX-based mouthwash products is not determined lonely by the CHX concentration, but by all the components of the formulation as a whole. Indeed, interactions between CHX and the different components, and not only alcohol, may affect antibacterial activity positively or negatively.

Key Words: Chlorhexidine, Mouthwash, Antiseptic, Bactericidal, Periodontal pathogen

Introduction

The use of chemical antibacterial agents especially antiseptics is considered an important complement to mechanical oral hygiene practices [1-5]. In this respect, the effectiveness of chlorhexidine digluconate (CHX) in the prevention and treatment of oral disease has been recognized for a number of years [1,6-11]. Indeed, CHX remains the current gold standard oral antiseptic, its efficacy in terms of significantly reducing oral biofilms has been confirmed [1,12-15]. CHX is used primarily in a mouthwash formulation in dentistry and exhibits potent, broad-spectrum antimicrobial activity and has the ability to adsorb to negatively charged surfaces in the mouth (tooth, mucosa, pellicle, restorative materials) which results in prolonged activity [16]. At low concentrations, the activity of CHX is bacteriostatic, while at higher concentrations it is rapidly bactericidal [17-20] according to the species [1], leading to therapeutic and/or prophylactic indications, in agreement to the limitation of topical antibiotic use [1,6,16,21,22] The most common adverse side effect associated with oral use of CHX is extrinsic tooth staining (dental dyschromia) which occurs when CHX combines with dietary chromogens, which are precipitated onto the tooth surface [21,23]. Commercially available CHX based mouthwash products contain different CHX concentrations, ranging from 0.02% to 0.3%. CHX tends to have a dose-dependent effect, in terms of both bactericidal activity and local adverse effects (tooth staining) [1,12]. However, there is evidence that the antibacterial activity of CHX solutions cannot be predicted solely on the concentration of CHX [20, 24]. Other constituents of CHX mouthwash formulations (e.g. alcohol content) as well as environmental parameters (e.g. pH, proteins) may influence antimicrobial activity [25-29].

Aim

The aim of this study was to determine the in vitro bactericidal activity of different CHX- based commercial mouthwash products containing different chlorhexidine concentrations under conditions similar to their use. In this way, assays were performed according to European standards [30,31] taking into account the short contact time (1 min), and the local conditions e.g. 32°C contact temperature and the p resence of artificial saliva as interfering substance.

Methods

Bacterial strains

All bacterial strains used in this study were obtained from the Institute Pasteur Collection (Paris). Testing was performed using four strains: *Fusobacterium nucleatum* CIP 101130, *Aggregatibacter actinomycetemcomitans* CIP 52.106T, *Prevotella intermedia* CIP 103607, and *Porphyromonas gingivalis* CIP 103683. These strains were chosen based on their implication as periodontal pathogens [6]. Bacteria were cultured at 36 ± 1°C under anaerobic conditions (*F. nucleatum, P. intermedia* and *P. gingivalis*) or under 5% CO_2 (*A. actinomycetemcomitans*). The following culture media were used for maintaining and CFU numeration: Columbia agar with 5% sheep blood (*A. actinomycetemcomitans* and *P. intermedia*), Schaedler agar (*F. nucleatum*), and Wilkins-Chalgren agar (*P. gingivalis*).

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Test products

The formulation of seven commercially available mouthwash products (chlorhexidine concentration and list of other claimed active substances and excipients) is presented in *Table1*, along with the usage directions suggested by the manufacturer. The *in vitro* bactericidal activity of these products, each containing chlorhexidine digluconate, was tested according to the usage recommendations (pure or diluted).

Table 1. Composition of the seven commercial mouthwash products tested.

Chlorhexidine digluconate concentration	Other constituents (active substances/ excipients)	Ethanol content	Usage directions (pure/ diluted)
0.20%	Sodium hyaluronate (0.05%) Water, sorbitol, xylitol, sodium citrate, PEG-40 hydrogenated castor oil, glycerin, aroma, sodium lauroyl sarcosinate, polysorbate 20, citric acid, salvia officinalis (sage) oil, sage leaf extract, commiphora myrrtha resin extract, limonene, bisabolol, CI 16035	Alcohol free	Pure
0.20%	Water, xylitol, PEG-40 hydrogenated castor oil, chamomilla recutita extract, bisabolol, potassium acesulfame, aroma, cinnamal, CI 42090	Alcohol free	Pure
0.20%	Glycerol, macrogolglycerol hydroxystearate, sorbitol liquid (non-crystallising), peppermint flavor, purified water	Alcohol free	Pure
0.12%	Water, glycerin, propylene glycol, PEG-0 hydrogenated castor oil, olaflur, aroma, aluminum lactate, zinc sulfate, potassium acesulfame, limonene	Alcohol free	Pure
0.12%	Water, propylene glycol, glycerin, PEG- 0 hydrogenated castor oil, CI 16255, benzyl alcohol, aroma, limonene, potassium acesulfame	Alcohol free	Pure
0.12%	Water, hydrogenated glucose syrup, denatured alcohol, laureth-9, aroma, CI 16255	Alcohol -3.5%	Pure
0.10%	Chlorobutanol (0.5%) Glycerin, alcohol, water, aroma, benzyl alcohol, CI 16255, citral, citronellol, diethylhexyl sodium sulfosuccinate, eugenol, limonene, linalool, menthol	Alcohol - 42.8%	Dilute 1:3

Bactericidal assays

In vitro bactericidal assays were conducted in accordance with the NF EN 13727 standard "Quantitative suspension test for the evaluation of bactericidal activity of chemical disinfectants and antiseptics used in medical area" [31]. Some modifications were made to the procedure in order to test the mouthwash products under conditions similar to their use. The tests were performed as follows.

All reagents were brought to the testing temperature of 32 ± 1°C. Bacterial cells were suspended in tryptone salt broth to a density of approximately1.5×10^8 to 5.0×10^8 CFU/ml. 1 ml of interfering substance (artificial saliva: soy peptone 0.25g/L, yeast extract 0.25g/L, NaCl 0.5961 g/L, KCl 0.7978 g/L, $MgCl_2$ 6H2O 0.0589 g/L, $CaCl_2$ $2H_2O$ 0.1588 g/L, KH_2PO_4 0.2994 g/L, K_2HPO_4 0.7995 g/L and $NaHCO_3$ 0.021 g/L) was added to 1 ml of the Bacterial suspension in a test tube and the mix was incubated for 2 mins ± 10 secs. 8 ml of each test product (neat or diluted in hard water [30°F] to mimic tap water according to Manufacturer's directions for use) were added and the mix was incubated for 1 minute ± 5 seconds. For *F. nucleatum, A. actinomycetemcomitans* and *P. intermedia*, the reaction was stopped by adding 8 ml of neutralizing solution (tween 80 (10%), lecithin (2%), saponin (2%), sodium thiosulfate (0.5%), trypticase soy broth) to 1 ml of the test mix along with 1 ml of water. This mix was incubated for 5 min at 20 ± 1ºC. For *P. gingivalis*, considering the non inocuity of the neutralizing solution, filtration was used to terminate the reaction: 0.1 ml of the test mix was deposited on a 0.45 m membrane with 50 ml of diluent and the membrane was rinsed with sterile distilled water. Viable bacteria were enumerated in duplicate by plating 100 µl of 10^{-6} and 10^{-7} serial dilutions (neutralization method) or by depositing membranes onto agar plates (filtration method). Bacterial colonies were counted after 48 to 72 hours of incubation (7 days for *P. gingivalis*). In accordance with the standards, test products were considered bactericidal if a reduction of $\geq10^5$ CFU (5 log) was recorded. The bactericidal assay was validated by performing control experiments to determine the effect of the following on bacterial counts: experimental conditions, the neutralizing solution (or filtration for *P. gingivalis*), and neutralized (or filtered) test products.

Results

The number of viable *F. nucleatum*, *A. actinomycetemcomitans*, *P. intermedia* or *P. gingivalis* cells was not reduced by a factor greater than two-fold when experimental conditions were applied, including neutralization/filtration validation (*Table 2*). Thus, it was concluded that the bactericidal assay used in this study was appropriate for determining the in vitro bactericidal activity of the seven commercial mouthwash formulations selected. The log reductions in bacterial counts following 1 min incubation of each of the 4 strains with each of the 7 test products are presented in *Table 3*. Solutions 1, 3, 5, 6 and 7 were found to be bactericidal to each of the 4 strains (log reduction in bacterial counts ≥ 5). Solutions 2 and 4 were not bactericidal towards *A. actinomycetemcomitans* (log reduction in bacterial counts <5). Furthermore, solution 2 was also not bactericidal

towards *P. intermedia*. The results of the bactericidal assays performed in this study are summarized together with the key features of each mouthwash product in *Table 4*.

Table 2. *Validation of the bactericidal assay conditions.*

Test organism	Mean bacterial counts (CFU/ml) at 10^{-6} dilutiona									
	Suspension for validation	Experimental conditions	+Neutralizing solution/ filtration[b]	+Neutralized/filtered[b] test products						
				Sol.1	Sol.2	Sol.3	Sol.4	Sol.5	Sol.6	Sol.7
F. nucleatum	107	94	149	149	157	148	154	163	147	143
A. actinomycetemcomitans	142	111	129	145	123	112	155	126	146	118
P. intermedia	57	98	61	38	35	48	39	54	63	53
P. gingivalis[C]	60	159	105	89	92	-	74	-	-	-
	197	215	102	-	-	101	-	104	111	128

[a]Values represent the mean of duplicate counts. [b]Filtration corresponds with the results for *P. gingivalis* only. [c]Two validation experiments were performed for *P. gingivalis*, the first involved testing solutions 1, 2 and 4, the second involved testing solutions 3, 5, 6 and 7.

Table 3. *In vitro bactericidal activity of seven chlorhexidine-based commercial mou*

Test organism	Test suspensiona (log CFU/ml)	Log reduction in bacterial counts[a]						
		Solution 1	Solution 2	Solution 3	Solution 4	Solution 5	Solution 6	Solution 7
F. nucleatum	7.56	>5.41	>5.41	>5.41	>5.41	>5.41	>5.41	>5.41
		(0 – 0)		(0 – 0)	(0 – 0)	(0 – 1)	(0 – 0)	(0 – 0)
A. actinomycetemcomitans	7.72	>5.57	4.36*	>5.57	4.92*	>5.57	>5.57	>5.57
		(0 – 0)	(226– 230)	(0 – 0)	(48 – 78)	(1 – 1)	(0 – 0)	(0 – 0)
P. intermedia	7.38	>5.24	4.08*	>5.24	>5.24	>5.24	>5.24	>5.24
		(0 – 0)	(90 – 203)	(0 – 0)	(0 – 0)	(0 – 0)	(0 – 0)	(0 – 0)
P. gingivalis[b]	7.52	>5.37	>5.37		>5.37			
		(0 – 0)	(0 – 0)		(0 – 0)			
	7.67			>5.53		>5.53	>5.53	>5.53
				(0 – 0)		(0 – 0)	(0 – 0)	(0 – 0)

[a]Values represent the mean of duplicate counts (duplicate values). [b]Two experiments were performed for P. gingivalis, the first involved testing solutions 1, 2 and 4, the second involved testing solutions 3, 5, 6 and 7. *Values are lower than the log reduction cut-off defined as representing bactericidal activity.

Table 4. *Summary of mouthwash product characteristics (composition and bactericidal activity).*

Commercial product	Chlorhexidine digluconate concentration	Other claimed active ingredients	Alcohol content	Usage directions (pure/ diluted)	Final chlorhexidine digluconate concentration	Bactericidal activity
Solution 1	0.20%	Sodium hyaluronate (0.05%)	Alcohol free	Pure	0.20%	Effective against all strains tested
Solution 2	0.20%	None	Alcohol free	Pure	0.20%	Ineffective against two strains tested
Solution 3	0.20%	None	Alcohol free	Pure	0.20%	Effective against all strains tested
Solution 4	0.12%	None	Alcohol free	Pure	0.12%	Ineffective against one strain tested
Solution 5	0.12%	None	Alcohol free	Pure	0.12%	Effective againstall strains tested
Solution 6	0.12%	None	Alcohol -3.5%	Pure	0.12% (final alcohol conc° 3.5%)	Effective against all strains tested

Solution 7	0.10%	Chlorobutanol (0.5%)	Alcohol -42.8%	Dilute 1:3	0.033% (final alcohol conc° 14.3%)	Effective against all strains tested

Discussion

Chlorhexidine is a bisbiguanide antiseptic which has a wide spectrum of bactericidal activity encompassing Gram positive and Gram negative bacteria [32-34]. It is also effective against some fungi and yeast, including Candida, and some lipophilic viruses including HIV and HBV [35]. The bactericidal effect of chlorhexidine is due to the cationic nature of the agent binding to extra microbial complexes and negatively charged microbial cell wall, thereby altering the cells osmotic equilibrium [36]. Lesions of the cell wall and cytoplasmic membrane are then combined with intracellular precipitation of proteins [37-40]. Indeed, the bactericidal activity of CHX is known to be sensitive to interfering substances, thus in vitro tests used to test the efficacy of CHX solutions must mimic the in-use conditions as closely as possible to be clinically relevant [41]. The efficiency of chlorhexidine mouthwashes on plaque control and in reduction of gingivitis and other periodontal diseases is well described and known [12,13,15,23] and to correlate that with in vivo activity, in vitro assays need to be performed according to Phase 2, step 1 tests which are quantitative suspension tests to establish that a product induces an irreversible inactivation of microorganisms (bactericidal and/or other biocidal) under simulated practical conditions appropriate to its intended use [30].

The present results obtained on periodontopathic bacterial species, in the presence of artificial saliva as interfering substance, confirmed a five log reduction by 1 minute of contact at 32°C, for 5 of the 7 containing CHX mouthwashes tested.

The bacterial strains tested in this study have been earlier found to exist as microbial complexes within subgingival plaque and as supragingival biofilms [42,43]. Among these gram negative species, *A. actinomycetemcomitans* appeared as the less sensitive followed by *P. intermedia*. *A. actinomycetemcomitans* has been earlier described as more resistant than other Gram negative species involved in periodontitis to antibiotics and also to antiseptics.

Currently chlorhexidine (CHX) is considered the gold standard for oral antisepsis considering significant clinical and microbiological effects [12,14,44,45]. Therefore, the data obtained in this in vitro study are likely to be directly applicable to the clinical setting. Those products that exhibited a greater spectrum of bactericidal activity are likely to be more effective in the prevention or treatment of periodontal disease. However, the data presented here demonstrated different level of activity among the tested products. The antibacterial activity of CHX is known dosage dependent [9,46] and it is considered that no further benefits can be expected above 0.20%. The main important side effects described are undesirable tooth and tongue staining and taste disturbance [47]. These side effects are also dosage dependent, being accentuated at concentrations above 0.10% [23]. The combination of these two CHX characteristics explains the various marketed formulations with CHX concentrations ranging from 0.1 to 0.2%, associated or not with alcohol or other active compounds. However, the data presented here support the notion that the concentration of CHX is not the sole factor in determining the antimicrobial activity of commercial CHX-based mouthwash formulations. Different bactericidal activity profiles were observed for mouthwashes containing the same CHX concentration. Solutions 1, 2 and 3 contain 0.2% of chlorhexidine digluconate (alcohol free) and bactericidal activity on the 4 tested strains was observed only for solutions 1 and 3. If we considered the claimed composition, solution 1 presents another active ingredient (Sodium hyaluronate: 0.05%) but without described antimicrobial activity. In the same way, solutions 4 and 5 contain the same chlorhexidine concentration (0.12%) without any claim of other active ingredient, but express different level of activity considering *A. actinomycetemcomitans* At last, two tested mouthwashes are characterized by alcohol content (solutions 6 and 7) and are considered here as bactericidal despite different CHX concentrations (0.12% to 0.033% as final concentrations respectively) but also alcohol concentrations (3.5% and 14.3% as final concentrations respectively). The same level of activity considering the high difference in CHX content may be explained by other formulation components, e.g. alcohol but also chlorobutanol in the case of solution 7. Potentiation of bactericidal activity has been described between CHX and chlorobutanol [48]. Solution 7 used in our study contains 0.5% chlorobutanol or rather 0.17% in the test conditions (1/3 dilution) and CHX at a relatively low concentration of 0.1% or rather 0.033% (final concentration after dilution according to manufacturer's instructions). CHX solutions at low concentrations (0.02%-0.06%) have been typically associated with bacteriostatic activity, while solutions at higher concentrations (0.12-0.2%) have been associated with bactericidal activity [1]. So a positive interaction between chlorobutanol and CHX might explain a lower CHX concentration to be used in this solution whilst maintaining bactericidal activity. On another hand, the activity of CHX but also of chlorobutanol was described as dependent of interfering substances like organic matter or divalent cations [49-51], despite of this, solution 7 which is the lonely diluted in artificial saliva presents a bactericidal activity on the 4 tested strains. Differences in activity level between solutions containing the same CHX concentration are difficult to explain if we considered the lack of indication about the concentration of each excipient. As we have previously described, the interaction of sodium dodecylsulfate, an anionic agent, with CHX, a cationic one, mainly considered as antagonist may be synergistic, indifferent or additive according to the respective concentrations or ratio [52]. Another point needs to be underlined; many solutions even considered alcohol free, may include alcoholic solution (i.e. Plant essence or extract) or other compounds known for antimicrobial activity like citric acid or benzyl alcohol (preservative agents present respectively in solutions 1 and 5-7) or aromatic agents like citronellol, eugenol, limonene,

linalool, menthol (some of them present in solutions 1, 5 and 7; even if limonene is also in solution 4).

These results suggest that the mouthwash formulation as a whole, rather than simply CHX concentration, influences antimicrobial activity. Ethylic alcohol content is considered to play a role in the antibacterial activity of mouthwashes by enhancing solubility, and also the biocidal spectrum. In this study the influence of alcohol on mouthwash bactericidal activity was not so obvious; three of the five alcohol-free mouthwashes tested (containing 0.12% or 0.2% CHX) exhibited bactericidal activity towards all test strains; in the same time the two formulations containing alcohol are bactericidal but present different CHX/alcohol ratio. The results of our study seem to indicate those excipients, as well as the presence of other active compounds including alcohol), within the mouthwash formulation are important indetermining bactericidal activity. Synergistic or antagonistic interactions between ingredients occurring within the specific physiological environment of the mouth, replicated in our *in vitro* assay, are likely to play an important role in determining the efficacy of the mouthwashes. Considering active ingredients and co-formulants, interactions might be studied in the proposed assay conditions using checkerboard method as previously described [53-55]. In the same way, the assay conditions might be improved according to specific uses i.e. in presence of blood. In conclusion, this study proved the possibility of validating antiseptic formulation choice in vitro, in current practice conditions. The most unfortunate side effect of CHX-based mouthwash use beyond 1 week is dental and mucosal (lingual) colorations. These side effects can greatly affect patient compliance with respect to the frequency and length of product usage. It is generally accepted that the efficacy of CHX-based mouthwashes is directly proportional with the concentration of CHX and the degree of dental dyschromia [4]. However, we demonstrated in this study that a mouthwash formulation containing 0.033% CHX exhibits equal or greater bactericidal activity compared to those containing 0.12%/0.2% CHX, illustrating the importance of the overall formulation of the product in determining efficacy and perhaps in reducing the probability of dyschromia.

These decreased side effects are likely to result in increased patient compliance and greater overall efficacy of the treatment.

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