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Comparison of the *in vivo* and *in vitro* antibacterial properties of antiseptic mouthrinses containing chlorhexidine, alexidine, cetyl pyridinium chloride and hexetidine

Relevance to mode of action

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Abstract. A study was carried out to compare the antibacterial properties of four cationic antiseptics, three of which are available as commercial mouthrinse preparations. Minimum inhibitory concentrations for alexidine, cetyl pyridinium chloride, chlorhexidine gluconate and hexetidine against a range of standard test organisms, were determined by tube dilution. Similar values for *Oxford staphylococcus* were then obtained in Dubos medium to which protein as yeast, or food extract, or serum was added in doubling dilutions to 16%. Salivary bacterial counts after a single rinse with the antiseptics or water throughout the day were measured in 10 subjects together with the duration of any residual antiseptic activity in the saliva. All antiseptics were effective at low concentrations against the organisms tested but the minimum inhibitory concentration values for hexetidine were the highest. Food extract and serum markedly increased the minimum inhibitory concentration values of all antiseptics, although alexidine and hexetidine were the least affected in percentage terms. The activity of a 1% povidone iodine preparation, used for comparison, was almost completely vitiated. An immediate significant fall in salivary bacterial counts was produced by the cationic antiseptics. Return to pre-rinse levels was seen for hexetidine after 90 min, cetyl pyridinium chloride after 3 hours, alexidine after 5 hours and chlorhexidine gluconate after 7 hours. Residual salivary antibacterial activity remained to 90 min for cetyl pyridinium chloride, to 3 hours for hexetidine and alexidine and to 5 hours for chlorhexidine gluconate. The antibacterial properties measured, in particular the duration of effect *in vivo*, may be relevant to the anti-plaque activity of cationic antiseptics.

Chlorhexidine, cetyl pyridinium chloride and hexetidine are examples of the relatively few cationic antiseptics which are available commercially as mouthrinse preparations. Furthermore, chlorhexidine is the only bisbiguanide antiseptic in routine clinical use, although alexidine has been studied fairly extensively in clinical and laboratory investigations (Carlson & Porter 1973, Lobene & Soparkar 1973, Zinner & Duany 1975, Spolsky et al. 1975, Formicola et al. 1978, Slee & Tanzer 1979, Baker et al. 1979). Prior to the observations concerning the anti-plaque activity of chlorhexidine (Löe & Schiött 1970) interest in the oral antiseptics appears to have been directed towards their antibacterial action against salivary and mucosal flora (Cawson & Curson 1959). Antibacterial action against oral flora per se, however, does not correlate well with anti-plaque activity (Gjermo et al. 1970) parti-

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cularly when assessed by routine *in vitro* antibacterial tests (Tanzer et al. 1972, Tanzer & Johnston 1976). Comparable *in vitro* and *in vivo* anti-plaque activity to chlorhexidine appears only to have been achieved by related bisbiguanide antiseptics or analogues of chlorhexidine (Gjermo et al. 1973). Cetyl pyridinium chloride and hexetidine either by direct comparison with chlorhexidine or by inference from active placebo studies were considerably less effective in reducing plaque (Gjermo et al. 1970, Bergenholtz & Hanström 1974, Bonesvoll & Gjermo 1978, Lobene et al. 1979). Alexidine also, when compared directly with chlorhexidine, possessed somewhat less anti-plaque activity (Gjermo et al. 1973, Roberts & Addy 1980).

Explanations as to the differences in action of the cationic antiseptics are as yet incomplete and this may reflect a lack of understanding of the exact mode of action of the extensively studied bisbiguanide chlorhexidine. Nevertheless, it is generally agreed that the prolonged action of chlorhexidine within the oral cavity resulting from adsorption to surfaces, plays a major role in plaque inhibition (Rölla et al. 1970, 1971). However, many variables affect the initial adsorption of this antiseptic (Bonesvoll et al. 1974, Bonesvoll & Olsen 1974). Thus differences in anti-plaque action may depend upon many factors and include the amount and duration of retention of antiseptics at antibacterial levels (Bonesvoll & Gjermo 1978). Furthermore, the antibacterial activity of the adsorbed antiseptics and the effects upon activity of material in the oral environment, including salivary proteins, may be of relevance. The importance of such factors may be reflected in the duration of any reduction in salivary flora or antibacterial activity detectable in the saliva. Such differences were suggested (Addy & Wright 1978) as possible explanations for the apparent lack of anti-plaque activity of povidone iodine when compared with chlorhexidine (Saxén et al. 1976, Addy et al. 1977).

The aim of this study was to compare and contrast the bactericidal properties of three commercially available antiseptic mouthrinses containing chlorhexidine, cetyl pyridinium chloride and hexetidine together with an alexidine preparation under investigation for possible commercial use. How protein and saliva affected the activity of the rinses was determined and moreover the duration of any effect upon salivary flora *in vivo* was measured.

Material and Methods

Minimum inhibitory concentration determinations for test organisms

The minimum inhibitory concentrations of chlorhexidine gluconate, alexidine, hexetidine and cetyl pyridinium chloride for the test organisms listed (Table 1) were determined using a tube dilution method. Thus, initially, 10-fold dilutions of an 0.2% chlorhexidine gluconate mouthwash (ICI Pharmaceutical Division, Alderley Edge, Macclesfield, England), an 0.035% alexidine mouthwash (Beecham Products, Leatherhead, England), an 0.1% hexetidine mouthwash (William Warner & Co. Ltd., Eastleigh, Hampshire, England) and an 0.05% cetyl pyridinium chloride mouthwash (R. Merrell Ltd., Slough, Berkshire, England) were made up in brain heart infusion broth. Aliquots (2 ml) of each solution were placed in sterile tubes and each tube inoculated with a single (1/300th ml) loop of an overnight (12-hour) culture of the test organism in plain brain heart infusion broth. The tubes were incubated at 37°C for 24 hours and examined for growth by observing the presence or absence of turbidity in the solution. Positive and negative control cultures were also prepared. Confirmation of growth inhibition was obtained by subculturing two loops (1/150th ml) from each broth culture onto blood agar and incubating aerobically at 37°C for 24 hours. The minimum inhibitory concentration range was then considered to lie between the last non growth concentration and the first growth concentration. These ranges were then subdivided into 10 equal dilutions and the experiment repeated.

Table 1. Range of minimum inhibitory concentrations (μg/ml) of chlorhexidine gluconate, alexidine, hexetidine and cetyl pyridinium chloride for test organisms

Die Streuungsbreite minimaler, gegenüber Testorganismen inhibitatorischer, Konzentrationswerte (μg/ml), für Chlorhexidinglukonat, Hexetidin und Cetyl Pyridinium Chlorid

Fourchette des concentrations minimales inhibitrices (μg/ml), pour le gluconate de chlorhexidine, l'alexidine, l'hexétidine et le chlorure de cétylpyridinium, à l'égard des micro-organismes test

	Streptococcus Mutans NCTC 10832	Streptococcus Sanguis NCTC 7864	Escherichia Coli NCTC 10418	Oxford Staphylococcus NCTC 6571	Candida Albicans LSHTM 3153
Chlorhexidine Gluconate	1·0 - 2·2 (2·0)	6·7 - 20·0 (10·0)	3·3 - 5·0 (4·0)	2·2 - 2·9 (2·5)	3·3 - 5·0 (4·0)
Alexidine	0·6 - 0·9 (0·7)	1·2 - 3·5 (1·75)	7·0 - 11·7 (8·75)	0·9 - 1·75 (1·2)	3·5 - 4·4 (3·9)
Hexetidine	10·0 - 12·5 (11·1)	10·0 - 12·5 (11·1)	100 - 125 (111)	11·1 - 14·3 (12·5)	14·3 - 20·0 (16·7)
Cetyl Pyridinium Chloride	5·0 - 6·25 (5·6)	1·25 - 2·5 (1·7)	62·5 - 83·3 (71·4)	2·5 - 5·5 (5·0)	5·0 - 6·25 (5·6)

The lowest concentration of antiseptic agent inhibiting growth of the respective organism was taken as the minimum inhibitory concentration for that agent. The tube dilution method was repeated for all of the organisms for both mouthwashes on three separate occasions.

The test organism, *Oxford staphylococcus* (NCTC 6571), was used to study the effects of protein on the minimum inhibitory concentrations of the antiseptic agents. A non cationic antiseptic mouthrinse containing 1% povidone iodine (Napp Laboratories Ltd., Watford, Hertfordshire, England), which is known to be sensitive to protein, was used in these experiments for comparison purposes. Tube dilution determinations were carried out employing the low protein medium Dubos broth base to which three different forms of protein were added. These were yeast extract (Oxoid Ltd., Basingstoke, Hants., England), a food extract (Marmite Ltd., Burton on Trent, England) and pooled human serum. The proteins were added to the Dubos broth base at concentrations from 0.5% to 16% w/v. The pooled human serum was also employed as the culture medium of the organism without the addition of Dubos broth. Employing the same tube dilution method described, the minimum inhibitory concentration values for the four cationic antiseptic mouthrinse preparations and the 1% povidone iodine mouthrinse were determined in the Dubos broth base alone and in the different protein enriched media.

Finally, employing pooled human saliva collected from 10 volunteers as the culture medium, the minimum inhibitory concentration for *Oxford staphylococcus* for the four cationic antiseptics and povidone iodine was determined. The method used was as before except doubling dilutions were used instead of 10-fold without further subdivision. *Oxford staphylococcus* was inoculated as a single 1/300th ml loop of an overnight culture in Dubos broth base into 2-ml aliquots of the

saliva antiseptic solutions. The end point for inhibition of growth was determined by subculturing the saliva medium onto manitol salt agar which was incubated aerobically at 37°C for 24 hours. This experiment was carried out in duplicate.

In vivo salivary bacterial study

A group of 10 volunteers (five male and five female) took part in a study to examine the duration and effect of the mouthwash preparations on salivary bacterial counts following a single rinse with each mouthwash or distilled water. A resting saliva sample of at least 2 ml was taken from each volunteer between 9.00 and 9.30 a.m. The volunteers were then supervised whilst they rinsed with 10 ml of sterile water for a timed minute. Following expectoration, samples of saliva were obtained at intervals of 2 min, 30 min, 90 min, 3 hours, 5 hours, 7 hours and 24 hours following the rinse. All volunteers subsequently repeated the experiment using the commercial preparations of alexidine, hexetidine, chlorhexidine and cetyl pyridinium chloride. A minimum of a 7-day rest period following the use of the respective preparations was allowed in an attempt to minimise any carry-over effects. Saliva samples in each case were mixed thoroughly on a Vortex mixer and 1-ml aliquots removed and serially diluted to 1/10000 and 100000 in quarter strength Ringer's solution. Two 0.1-ml aliquots from each of the dilutions were spread over the surface of two blood agar plates using a sterilized glass spreader. One plate was incubated aerobically and the other anaerobically for 48 hours at 37°C. The resulting colonies were counted using a Gallenkamp illuminated colony counter (A. Gallenkamp & Co. Ltd., Christopher Street, London). The number of counts were then expressed as counts in millions per millilitre of saliva.

Employing the same samples the duration of any residual antiseptic activity within the saliva was assessed by means of a well diffusion technique. Aliquots (0.1 ml) of saliva from each sample from each volunteer were placed in 11-mm diameter wells cut in nutrient agar, previously inoculated with a 12-hour culture of *Oxford staphylococcus*. The plates were standardized by using 40 ml of nutrient agar to which 0.2 ml of culture broth was added, giving an average depth of 4 mm of agar in each plate. These plates were then incubated aerobically for 24 hours at 37°C. After incubation the diameter of any zone of bacterial inhibition was measured using a rule. A statistical analysis of the results was carried out using Student's 't' test for paired and unpaired data.

Results

Minimum inhibitory concentrations

Tube dilution in brain heart infusion broth. The ranges of microbial minimum inhibitory concentrations of each antiseptic for the various organisms in brain heart infusion broth are shown (Table 1). The figures in brackets are the precise end points obtained. Essentially the results for chlorhexidine gluconate and alexidine were similar, being effective against all organisms tested and at low concentrations. However, alexidine was effective at lower concentrations for four out of five organisms, the exception being *E. coli*. Cetyl pyridinium chloride was also effective at low concentrations but was required at considerably higher concentrations than chlorhexidine and alexidine against *E. coli*. Hexetidine was less effective than all the other antiseptics for all five organisms tested. Nevertheless, the dilution of the hexetidine preparation required was still relatively high except for *E. coli*.

Tube dilution in Dubos broth medium containing increasing protein concentrations

Yeast extract. The ranges of minimum inhibitory concentration of the cationic antiseptics against *Oxford staphylococcus* cultured in Dubos medium containing yeast extract, are shown in Table 2. The minimum inhibitory concentration values in Dubos medium without added

Table 2. Effect of protein in the form of yeast extract on the minimum inhibitory concentration values for chlorhexidine gluconate, alexidine, hexetidine and cetyl pyridinium chloride against *Oxford Staphylococcus* in Dubos medium (μg/ml)

Die Einwirkung von Proteinen als Hefeextrakt auf die, gegenüber dem Oxfordstaphylokokkus im Dubosmedium (μg/ml) minimal wirksamen, inhibitatorischen Konzentrationswerte für Chlorhexidinglukonat, Alexidin, Hexetidin und Cetyl Pyridinium Chlorid

Action des protéines sous forme d'extrait de levure sur les valeurs de la concentration minimale inhibitrice du gluconate de chlorhexidine, de l'alexidine, de l'hexétidine et du chlorure de cétylpyridinium envers Staphylococcus Oxford dans le milieu de Dubos (μg/ml)

Dubos Medium % Yeast Extract	Chlorhexidine Gluconate	Alexidine	Hexetidine	Cetyl Pyridinium Chloride
0	0·50 - 1·0 (0·67)	0·35 - 0·45 (0·39)	5·0 - 11·1 (10·0)	0·17 - 0·50 (0·25)
0·5	0·40 - 0·67 (0·50)	0·18 - 0·39 (0·35)	5·0 - 11·1 (10·0)	0·25 - 0·55 (0·50)
1·0	1·0 - 2·2 (2·0)	0·12 - 0·35 (0·18)	3·3 - 10·0 (5·0)	0·70 - 1·0 (0·83)
2·0	1·0 - 2·2 (2·0)	0·35 - 0·44 (0·39)	3·3 - 10·0 (5·0)	0·25 - 0·55 (0·5)
4·0	2·2 - 2·9 (2·5)	0·70 - 1·2 (0·88)	5·0 - 11·1 (10·0)	0·55 - 0·71 (0·63)
8·0	3·3 - 5·10 (4·0)	0·87 - 1·75 (1·2)	10·0 - 12·5 (11·0)	0·83 - 1·25 (1·0)
16·0	4·0 - 6·7 (5·0)	1·2 - 3·5 (1·75)	5·0 - 11·0 (10·0)	1·25 - 2·5 (1·67)

yeast extract (*Hefeextrakt, extrait de levure*)

protein were all lower than for *Oxford staphylococcus* in brain heart infusion broth. For alexidine, chlorhexidine gluconate and cetyl pyridinium chloride, increasing concentrations of yeast extract increased the minimum inhibitory concentration values, although even at 16% yeast extract all were still effective against *Oxford staphylococcus* at low concentrations. Hexetidine was almost unaffected by the addition of yeast extract, showing only small variations, nevertheless the minimum inhibitory concentrations were always higher than for the other antiseptics. The minimum inhibitory concentrations for povidone iodine (Table 6) markedly increased with increasing yeast extract additions. Thus, at 4% yeast extract, the mouthwash preparation was effective only at a 1 in 3 dilution and at 8% yeast extract was effective at greater than a 1 in 2 dilution.

Table 3. Effect of protein in the form of food extract on the minimum inhibitory concentration values for chlorhexidine gluconate, alexidine, hexetidine and cetyl pyridinium chloride against *Oxford staphylococcus* in Dubos medium (μg/ml)

Der Effekt von Protein als Nahrungsmittelextrakt auf die, gegenüber dem Oxfordstaphylokokkus im Dubosmedium (μg/ml), minimalen inhibitatorischen Konzentrationswerte für Chlorhexidinglukonat, Alexidin, Hexetidin und Cetyl Pyridinium

Action des protéines sous forme d'extrait alimentaire sur les valeurs de la concentration minimale inhibitrice du gluconate de chlorhexidine, de l'alexidine, de l'hexétidine et du chlorure de cétylpyridinium à l'égard de Staphylococcus Oxford dans le milieu de Dubos (μg/ml)

Dubos Medium % Food Extract	Chlorhexidine Gluconate	Alexidine	Hexetidine	Cetyl Pyridinium Chloride
0	0·5 - 1·0 (0·67)	0·35 - 0·44 (0·39)	5·0 - 11·0 (10·0)	0·17 - 0·50 (0·25)
0·5	2·0 - 2·5 (2·22)	0·87 - 1·75 (1·2)	10·0 - 12·5 (11·0)	1·0 - 1·7 (1·25)
1·0	3·3 - 5·0 (4·0)	0·7 - 1·2 (0·9)	20·0 - 33·3 (25·0)	0·5 - 0·62 (0·55)
2·0	6·7 - 20·0 (10·0)	1·2 - 3·5 (1·75)	20·0 - 33·3 (25·0)	0·83 - 1·25 (1·0)
4·0	28·6 - 40·0 (33·3)	5·8 - 8·75 (7·0)	25·0 - 50·0 (33·3)	16·7 - 50·0 (25·0)
8·0	33·3 - 50·0 (40·0)	11·7 - 35·0 (17·5)	50·0 - 111·0 (100·0)	25·0 - 55·5 (50·0)
16·0	50·0 - 100·0 (66·7)	11·7 - 35·0 (17·5)	125·0 - 166·7 (142·9)	25·0 - 55·5 (50·0)

food extract (*Nahrungsmittelextrakt, extrait alimentaire*)

Food extract. The ranges of minimum inhibitory concentrations of the antiseptic against *Oxford staphylococcus* cultured in Dubos medium containing increasing protein concentrations in the form of meat extract are shown in Table 3. As with the yeast extract, chlorhexidine gluconate, alexidine and cetyl pyridinium chloride were required in higher concentrations to inhibit the growth of *Oxford staphylococcus* with increasing addition of the food extract. Moreover the effect of the food extract in increasing the minimum inhibitory concentrations was greater than that of the yeast extract. For hexetidine, unlike the yeast extract, the food extract increased the minimum inhibitory concentration values. Furthermore, at all concentrations of food extract added, the minimum inhibitory concentrations for hexetidine were higher than

Table 4. Effect of pooled human serum on the minimum inhibitory concentration values for chlorhexidine gluconate, alexidine, hexetidine and cetyl pyridinium chloride against *Oxford staphylococcus* in Dubos medium (μg/ml)

Der Effekt summierten menschlichen Serums auf die, gegenüber dem Oxfordstaphylokokkus im Dubosmedium (μg/ml), minimalen inhibitatorischen Konzentrationswerte für Chlorhexidinglukonat, Alexidin, Hexetidin und Cetyl Pyridinium

Action du mélange de sérum humain sur les valeurs de la concentration minimale inhibitrice du gluconate de chlorhexidine, de l'alexidine, de l'hexétidine et du chlorure de cétylpyridinium à l'égard de Staphylococcus Oxford dans le milieu de Dubos (μg/ml)

Dubos Medium % Pooled Human Serum	Chlorhexidine Gluconate	Alexidine	Hexetidine	Cetyl Pyridinium Chloride
0	0·5 - 1·0 (0·67)	0·35 - 0·44 (0·39)	5·0 - 11·0 (10·0)	0·17 - 0·50 (0·25)
0·5	2·9 - 4·0 (3·33)	0·9 - 1·7 (1·17)	16·7 - 25·0 (20·0)	6·3 - 8·3 (7·14)
1·0	4·0 - 6·7 (5·0)	1·8 - 3·9 (3·5)	33·3 - 100·0 (50·0)	6·3 - 8·3 (7·14)
2·0	10·0 - 22·2 (20·0)	1·8 - 3·9 (3·5)	50·0 - 111·0 (100·0)	10·0 - 16·7 (12·5)
4·0	10·0 - 22·2 (20·0)	1·8 - 3·9 (3·5)	50·0 - 111·0 (100·0)	12·5 - 25·0 (16·7)
8·0	10·0 - 22·2 (20·0)	11·7 - 35·0 (17·5)	50·0 - 111·0 (100·0)	16·7 - 50·0 (25·0)
16·0	40·0 - 66·7 (50·0)	11·7 - 35·0 (17·5)	100·0 - 125·0 (111·1)	16·7 - 50·0 (25·0)
100·0	66·7 - 200·0 (100·0)	17·5 - 38·9 (35·0)	166·7 - 250·0 (200·0)	16·7 - 50·0 (25·0)

pooled human serum (*summiertes menschliches Serum, mélange de sérum humain*)

the other antiseptics. Proportionately, however, the effects of protein addition were greatest on cetyl pyridinium chloride and chlorhexidine with the minimum inhibitory concentration values increased approximately 200 and 100 times respectively at 16% food extract compared with no food extract. For alexidine the increase in the minimum inhibitory concentration values was less than 50 times. For hexetidine the overall effect was the least, with an increase of 14 times. Again, the effects of protein markedly reduced the antibacterial pro-

Table 5. Range of minimum inhibitory concentrations (μg/ml) of chlorhexidine gluconate, alexidine, hexetidine and cetyl pyridinium chloride for the *Oxford staphylococcus* (NCTC 6571) in pooled human saliva

Streuungsbreite minimaler, gegenüber dem Oxford-staphylokokkus (NCTC 6571) inhibitatorischer, Konzentrationen (μg/ml) in summiertem menschlichen Speichel

Fourchette des concentrations minimales inhibitrices (μg/ml) du gluconate de chlorhexidine, de l'alexidine, de l'hexétidine et du chlorure de cétylpyridinium à l'égard du Staphylococcus Oxford (NCTC 6571) dans le mélange de salive humaine

Chlorhexidine gluconate	7.8– 15.6
Alexidine	5.5– 10.9
Hexetidine	62.5–125.0
Cetyl pyridinium chloride	7.8– 15.6

perties of povidone iodine (Table 6), such that at 16% meat extract, the povidone iodine mouthwash was required at a concentration equivalent to a 1 in 2 dilution of the original solution.

Pooled human serum. The range of minimum inhibitory concentration of the antiseptics against *Oxford staphylococcus* cultured in Dubos medium containing increasing protein concentrations in the form of pooled human serum are shown in Table 4. Again, as the concentration of pooled human serum in the medium increased, the minimum inhibitory concentration values for all four antiseptics against *Oxford staphylococcus* similarly increased. The effect of pooled human serum was greatest for cetyl pyridinium chloride and chlorhexidine gluconate with the minimum inhibitory concentration values increased approximately 100 and 75 times respectively at 16% pooled human serum compared with no serum. For alexidine, this increase was 50 times but for hexetidine only 10 times. Nevertheless, again the minimum inhibitory concentration values for hexetidine were always higher than the other cationic antiseptics. Povidone iodine (Table 6) was markedly affected by serum with the MIC values progressively increasing as the concentration of serum increased.

Table 6. The effect of protein addition and saliva on the minimum inhibitory concentration values for povidone iodine against *Oxford Staphylococcus* in Dubos medium (μg/ml)

Der Effekt von Proteinzusatz und Speichel auf die, gegenüber dem Oxfordstaphylokokkus im Dubosmedium (μg/ml), minimal inhibitatorischen Konzentrationswerte des Povidine Iodine

Action de l'addition de protéines (food extract = extrait alimentaire, yeast extract = extrait de levure, sérum) et de salive sur les concentrations minimales inhibitrices de povidone iode à l'égard de Staphylococcus Oxford dans le milieu de Dubos (μg/ml)

Dubos medium % Additive	Yeast Extract	Food Extract	Serum	Saliva
0	33	33		1250
0·5	500	1000	500	
1·0	500	1000	500	
2·0	1000	1111	1000	
4·0	3333	2000	1000	
8·0	>5000	3333	3333	
16·0	>5000	5000	3333	

Table 7. Mean salivary bacterial counts following a single rinse with chlorhexidine gluconate, alexidine, hexetidine, cetyl pyridinium chloride and sterile water $\times 10^6$/ml

Mittlere Anzahl von Bakterien im Speichel nach Mundspülung mit Chlorhexidinglukonat, Alexidin, Hexetidin, Cetyl Pyridinium Chlorid und sterilem Wasser $\times 10^6$ *ml*

Moyenne des numérations des bactéries salivaires après un rinçage unique, avec du gluconate de chlorhexidine, de l'alexidine, de l'hexétidine, du chlorure de cétylpyridinium et de l'eau stérile ($\times 10^6$/*ml*)

Time	Chlorhexidine Gluconate		Alexidine		Hexetidine		Cetyl Pyridinium Chloride		Water	
	Aerobes	Anaerobes	Aerobes	Anaerobes	Aerobes	Anaerobes	Aerobes	Anaerobes	Aerobes	Anaerobes
0 Mins.	43·03	39·97	70·50	158·33	82·17	116·38	45·19	77·78	76·56	174·12
	(25·71)	(24·06)	(58·82)	(138·33)	(62·79)	(88·79)	(25·06)	(65·64)	(100·92)	(211·59)
2 Mins.	2·36	1·35	4·33	13·82	10·88	34·01	3·18	3·98	79·33	156·75
	(3·44)	(1·74)	(4·58)	(20·10)	(8·38)	(36·82)	(5·99)	(7·21)	(92·30)	(161·26)
30 Mins.	10·39	10·67	10·29	21·77	17·97	55·37	13·46	46·27	37·06	72·94
	(13·54)	(14·47)	(11·10)	(33·64)	(13·97)	(72·26)	(12·22)	(53·10)	(20·07)	(84·15)
90 Mins.	7·17	8·36	11·48	31·33	24·21	51·04	9·16	22·97	57·22	81·25
	(10·40)	(12·05)	(8·54)	(44·54)	(10·58)	(58·64)	(5·98)	(23·32)	(64·52)	(117·00)
3 Hrs.	7·32	10·36	19·17	37·90	47·78	143·53	23·24	41·10	59·11	112·25
	(6·74)	(9·46)	(14·32)	(63·56)	(64·36)	(253·21)	(19·49)	(45·55)	(33·90)	(118·22)
5 Hrs.	8·67	11·71	20·57	72·21	50·03	79·45	22·92	39·72	59·17	136·31
	(9·59)	(13·87)	(18·96)	(100·38)	(39·87)	(90·08)	(14·73)	(24·60)	(45·91)	(193·83)
7 Hrs.	12·22	14·67	53·30	120·78	70·22	145·00	40·43	48·67	81·78	130·56
	(9·73)	(15·07)	(83·43)	(208·71)	(39·58)	(140·23)	(29·39)	(46·97)	(53·08)	(102·74)
24 Hrs.	52·34	32·79	32·31	91·66	65·89	84·78	57·38	61·02	113·06	123·78
	(88·47)	(24·38)	(24·45)	(73·40)	(43·60)	(59·19)	(58·77)	(49·97)	(146·40)	(106·64)

Number of Subjects = 10
Standard Deviation ( )

time (*Zeit, moment*), mins (*Minuten, minutes*), hrs (*Stunden, heures*), number of subjects (*Zahl der Probanden, nombre de sujets*), standard deviation (*Standarddeviation, écart-type*)

Tube dilution in pooled human saliva

The ranges of minimum inhibitory concentrations of the antiseptics against *Oxford staphylococcus* cultured in pooled human saliva are shown in Table 5. The values for alexidine, chlorhexidine gluconate and cetyl pyridinium chloride for *Oxford staphylococcus* in saliva were not markedly different whilst hexetidine was required at approximately eight times the concentration of the other antiseptics to inhibit the growth of *Oxford staphylococcus*. The minimum inhibitory concentration of povidone iodine in pooled human saliva, however, was high and represented a 1 in 8 dilution of the original solution.

In vivo antibacterial determinations

The mean and standard deviation of the bacterial counts for the group of subjects obtained over the 24-hour periods after a single rinse with the antiseptic mouthwashes or sterile water, are shown in Table 7. After rinsing with the four cationic antiseptics there was a mean fall in the aerobic and anaerobic salivary bacterial counts which was significant and most marked for chlorhexidine gluconate ($P<0.01$–$P<0.001$). In the case of the sterile water rinse, the 2-min counts were not significantly different from pre-rinse levels ($P<0.05$–$P<0.7$). Furthermore, the fluctuations in the mean counts throughout the rest of the day following rinsing with water were not significantly different from pre-rinse counts. The mean aerobic and anaerobic salivary bacterial counts remained significantly below pre-rinse levels for all antiseptics up to 90 min ($P<0.05$–$P<0.01$). After 90 min

following rinsing with hexetidine, the mean counts returned to levels which were not significantly lower than the pre-rinse counts for both aerobic and anaerobic organisms ($P>0.7$). Aerobic and anaerobic salivary bacterial counts were significantly reduced for cetyl pyridinium chloride 3 hours and for alexidine up to 5 hours. The reduction in both aerobic and anaerobic counts for chlorhexidine gluconate was still highly significant at 7 hours ($P<0.1$ and $P<0.001$, respectively). However, 24 hours following the rinsing counts were not significantly different from pre-rinse levels ($P>0.1$). In percentage terms the immediate mean fall in salivary bacterial counts was similar for alexidine, chlorhexidine gluconate and cetyl pyridinium chloride, ranging from 91 to 95% reduction. The percentage reduction for hexetidine was less, being 84% for aerobes and 70% for anaerobes.

The mean diameter of the zones of inhibition minus the width of the wells (11 mm) for saliva samples against *Oxford staphylococcus* obtained after rinsing with the four antiseptics and sterile water are shown in Table 8. Sterile water produced no antibacterial activity in saliva following rinsing. In the case of the other agents, all subjects showed antibacterial activity at 2 min. For chlorhexidine gluconate all 10 subjects demonstrated antibacterial activity at

Table 8. Mean diameter of zones of inhibition of *Oxford staphylococcus* by saliva following a single rinse with chlorhexidine gluconate, alexidine, hexetidine and cetyl pyridinium chloride (mm) (zone is minus 11 mm well diameter)

Mittlerer Durchmesser der Inhibitationszone des Oxfordstaphylokokkus im Speichel nach einer einzigen Mundspülung mit Chlorhexidinglukonat, Alexidin, Hexetidin und Pyridinium Chlorid (in mm) (abzüglich 11 mm Randzone)

Diamètre moyen des zones d'inhibition de Staphylococcus Oxford par la salive après un rinçage unique avec du gluconate de chlorhexidine, de l'alexidine, de l'hexétidine et du chlorure de cétylpyridinium (mm). Le diamètre de la cupule (11 mm) est retranché du diamètre de la zone

	Chlorhexidine gluconate	Alexidine	Hexetidine	Cetyl pyridinium chloride
No. in Group	10	10	9	9
2 min	8.35	1.65	2.97	2.77
	(0.88)	(0.52)	(1.03)	(0.93)
N	10	10	9	9
30 min	6.30	1.22	1.94	1.11
	(1.54)	(0.59)	(1.01)	(0.92)
N	10	9	8	8
90 min	3.72	0.57	0.72	0.16
	(2.11)	(0.44)	(0.71)	(0.50)
N	10	7	5	1
3 hours	2.25	0.05	0.38	0
	(1.95)	(0.15)	(0.48)	
N	8	1	4	
5 hours	0.25	0	0	0
	(0.63)			
N	2			
7 hours	0	0	0	0

No in group (*Anzahl in der Gruppe, nombre dans le groupe*), N=number of subjects (*N=Zahl der Probanden, N=nombre de sujets*)

both 30 and 90 min. By 3 hours eight subjects showed residual activity and at 5 hours two subjects still demonstrated antibacterial activity. For alexidine a similar diminution in the number of subjects demonstrating antibacterial activity was seen with time so that only one subject demonstrated antibacterial activity at 3 hours. For hexetidine antibacterial activity in the saliva was present in four subjects up to 3 hours. Cetyl pyridinium chloride produced less residual antiseptic activity in the saliva with time since only one subject demonstrated antibacterial activity up to 90 minutes. Statistical analysis to compare the significance of the difference between the zone widths at each time following the original rinse demonstrated that at all time periods chlorhexidine gluconate rinsing produced significantly wider zones of inhibition than all three antiseptics ($P<0.001$). At 2 min the zones of inhibition for cetyl pyridinium chloride were significantly wider than alexidine ($P<0.01$) but not significantly wider than hexetidine ($P>0.6$). Hexetidine produced significantly wider zones than alexidine ($P<0.01$). After 30 min there was no significant difference between alexidine, hexetidine and cetyl pyridinium chloride ($P<0.05$–$P<0.7$). At 90 min alexidine zones were significantly wider than those with cetyl pyridinium chloride ($P<0.05$) but not significantly different from those of hexetidine ($P>0.5$). Hexetidine also showed significantly wider zones than cetyl pyridinium chloride ($P<0.05$).

Discussion

The results of the *in vitro* antibacterial assays demonstrated that alexidine, chlorhexidine and cetyl pyridinium chloride were essentially similar in activity, being effective against all the organisms tested and at low concentrations. Hexetidine also inhibited the growth of the test organisms but at higher concentrations. All antiseptics inhibited the growth of *Candida albicans* and therefore may be suitable in the management of candidal lesions either alone or as an adjunct to other anti-candidal therapy. Chlorhexidine, in particular, has been employed for the treatment of candidal infections (Budtz-Jørgensen & Löe 1972, Olsen 1975 a,b). The similarity in the minimum inhibitory concentration values of alexidine, chlorhexidine and cetyl pyridinium chloride against the test organisms and yet known differences in anti-plaque activity (Gjermo et al. 1970, 1973, Bonesvoll & Gjermo 1978, Roberts & Addy 1980) again emphasizes the lack of correlation between these two parameters of action already established (Gjermo et al. 1970). In fact the use of tube dilution tests may over-estimate the plaque inhibiting properties of antiseptics (Tanzer et al. 1972).

The addition of protein to the media resulted in a reduction in the antibacterial properties of all the antiseptics albeit to a variable degree. By comparison with povidone iodine, an antiseptic known to be adversely affected by the presence of organic matter (Lowbury & Lilly 1974) the extent of the inhibition was less. The effects on povidone iodine were thought to arise from the depression of iodine activity (Gershenfeld & Witlin 1949). Certainly in this study the minimum inhibitory concentration values for povidone iodine were reduced by all forms of protein extract and saliva. Thus at high concentrations, yeast extract, which had little effect on the cationic antiseptics, almost totally inhibited the activity of the povidone iodine preparation. Moreover the reduced activity of povidone iodine in saliva may also in part be responsible for the very limited reduction in salivary flora observed *in vivo* (Addy & Wright 1978).

The experiments employing protein and saliva were again *in vitro* tube dilution experiments which alone do not provide information as to the plaque inhibitory properties of the antiseptics. Nevertheless, consideration of the considerable literature concerned with the activity of the cationic antiseptics enables inferences relevant to the possible mode of action to be drawn. All of the antiseptics used in this study are known to adsorb onto *in vivo* and *in vitro*

surfaces (Rölla et al. 1970, 1971, Bonesvoll et al. 1974, Bonesvoll & Olsen 1974, Bergenholtz & Hanström 1974, Jensen 1977, Jensen & Tustian 1978, Bonesvoll & Gjermo 1978, Addy & Roberts 1979). The variable effect of protein, to which chlorhexidine is known to have an affinity, (Rölla et al. 1970, Hjelford et al. 1973) on the minimum inhibitory values of the respective antiseptics, may therefore be dependent on the amounts adsorbed onto the protein in the media. For alexidine, chlorhexidine and cetyl pyridinium chloride the percentage reduction in antibacterial activity correlates well with the reported differences in adsorption of the antiseptics either *in vitro* or *in vivo*. Thus adsorption to surfaces was greater for cetyl pyridinium chloride than chlorhexidine (Bonesvoll & Gjermo 1978, Jensen & Tustian 1978, Jensen 1978, Addy & Roberts 1979) which in turn was greater than alexidine (Addy & Roberts 1979). The adsorption of hexetidine *in vivo* or *in vitro* has been discussed (Bergenholtz & Hanström 1974) but not quantitatively assessed. Nevertheless, the weakly basic nature of this compound would suggest very low adsorption properties.

Reduced activity of chlorhexidine has been reported following the addition of serum to media (Hennessey 1973) again presumably as a result of adsorption to proteins. Thus the resultant activity of adsorbed antiseptics must also be a consideration in respect of anti-plaque activity, particularly since it is generally accepted that the mechanism of action of the hitherto unrivalled anti-plaque agent chlorhexidine is dependent upon its prolonged retention in the oral cavity as a result of adsorption (Rölla et al. 1970, 1971). Moreover, the differences in binding of food dyes and beverages to hydroxyapatite and polymethyl methacrylate treated with cetyl pyridinium chloride, alexidine and chlorhexidine (Jensen 1977, 1978, Jensen & Tustian 1978, Addy et al. 1979, Addy & Roberts 1979) may be explained by considering both the extent of adsorption and the activity of the adsorbed antiseptics. The observations concerning the monocationic and dicationic nature of cetyl pyridinium chloride and chlorhexidine respectively, as accounting for the differences in chemical activity on surfaces, are therefore worthy of note (Jensen & Tustian 1978, Bonesvoll & Gjermo 1978).

The effect of the different protein extracts on the minimum inhibitory concentration values was consistent with the reported increased binding of cationic antiseptics such as chlorhexidine to denatured protein and albumin (Hjelford et al. 1973) when compared with soluble proteins. The limited effect of a soluble yeast extract on the minimum inhibitory concentration values was therefore not surprising. The effects of saliva may thus have arisen due to adsorption of the antiseptics to protein macromolecules, the total protein content of saliva being approximately 1% (Jenkins 1970). As well as the implications of adsorption to plaque inhibition, excessive quantities of protein may limit the activity of the antiseptic. Thus, although chlorhexidine has been observed to be effective in the management of recurrent oral ulceration of the minor type (Addy et al. 1974, 1976) it was considered largely ineffective in the treatment of major aphthous ulcers and herpetiform ulceration (Addy 1977). The large areas of necrotic slough associated with such ulcers may limit the antibacterial activity and availability of this antiseptic. Similarly, the failure of both chlorhexidine and povidone iodine in the treatment of acute ulcerative gingivitis may be explicable (Addy & Llewelyn 1978).

The duration of the reduction in salivary flora and the time periods of detectable antibacterial activity in saliva following rinsing demonstrated the more prolonged action of chlorhexidine in the mouth. Although the mouthwashes were used at differing concentrations, these duration effects are of clinical relevance since the concentrations are those at which the antiseptics are normally used. Moreover, increasing the concentration of some of the antiseptics may pose problems. Thus alexidine, because of its poor solubility in water, has not been used above a concentration of 0.05%

(Lobene & Soparkar 1973). Hexetidine caused a high incidence of mucosal erosion when used at 0.2% (Bergenholtz & Hanström 1974). The duration effects of chlorhexidine on salivary flora were noted in the initial studies (Schiött et al. 1970). However, these parameters have infrequently been reported for the other oral antiseptics. Thus, although the degree of reduction in salivary flora does not appear relevant to the mechanism of plaque reduction (Stralfors 1961, Davies et al. 1970), from this study it would appear to correlate with the known anti-plaque action of the antiseptics. The very brief reduction in salivary flora observed with povidone iodine (Addy & Wright 1978) similarly would relate to the lack of anti-plaque action of this compound. Furthermore, it is of interest that the anti-plaque action of cetyl pyridinium chloride was increased to that of chlorhexidine by doubling the frequency of rinsing (Bonesvoll & Gjermo 1978). With the exception of hexetidine the period of salivary flora reduction was greater than the duration of the salivary antibacterial action conferred. For cetyl pyridinium chloride and chlorhexidine the difference indicates that the salivary levels determined spectrophotometrically (Jensen & Christensen 1971) and radiochemically (Bonesvoll et al. 1974, Bonesvoll & Gjermo 1978) were at the later time periods not bacteriologically active. However, the maintenance of reduced salivary flora demonstrated the adsorbed antiseptic remaining still exerted a depressant effect. Again, this would support the relevance of differences in activity of adsorbed antiseptics as being particularly important. Desorption of the adsorbed antiseptic may also aid in maintaining a bacteriostatic milieu in the oral cavity. However, the activity of desorbed molecules is unknown. Moreover it is possible from the radiochemical studies (Bonesvoll et al. 1974, Bonesvoll & Gjermo 1978) that the chlorhexidine and cetyl pyridinium chloride measured were attached to the surfaces to which they were initially adsorbed and would include salivary proteins, oral epithelial cells and bacteria. Clinical and laboratory adsorption and staining investigations would suggest that the cationic antiseptics are not truly desorbed (Jensen 1977, 1978, Jensen & Tustian 1978, Addy et al. 1979, Prayitno et al. 1979). Loss from the oral cavity may therefore be the result of the normal turnover of the biological surfaces to which the molecules are adsorbed, as a result of mechanical displacement, or in the case of the weakly basic compounds, absorption through the oral mucosa.

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Zusammenfassung

In vivo und in vitro-Vergleich antibakterieller Eigenschaften einiger antibakterieller Mundspülflüssigkeiten. Relevanz gegenüber der Wirkungsweise

In dieser Studie werden die antibakteriellen Eigenschaften von 4 kationischen Antiseptika, von denen 3 als kommerzielle Mundspülflüssigkeiten auf dem Markt erhältlich sind, miteinander verglichen. Durch Verdünnung im Reagenzglas wurde das Minimum inhibitatorischer Konzentrationen für Alexidin, Cetyl Pyridinium Chlorid, Chlorhexidinglukonat und Hexetidin gegenüber einer Reihe von Standardorganismen ermittelt. Ähnliche Werte wurden für den Oxford Staphylokokkus im Dubos Medium erhalten, zu dem Proteine wie Hefe oder Nahrungsmittelextrakt zugesetzt wurden. Die Verdünnung wurde dadurch auf 16% verdoppelt. Bei 10 Probanden wurde nach einer einzigen Mundspülung mit den Antiseptika oder mit Wasser, einen Tag lang die Speichelbakterien gezählt, sowie die verbleibende antiseptische Aktivität im Speichel gemessen. Alle Antiseptika waren bereits bei geringer Konzentration den getesteten Organismen gegenüber wirksam – die

geringsten inhibitatorisch wirksamen Konzentrationswerte für Hexetidin lagen jedoch im Vergleich am höchsten. Der Zusatz von Nahrungsmittelextrakt zum Serum erhöhte die minimalen Konzentrationswerte aller Antiseptika – doch wurden Alexidin und Hexetidin davon prozentual am geringsten beeindruckt. Die Aktivität einer zum Vergleich herangezogenen 1%-igen Povidoniodinelösung wurde fast völlig ausgeschaltet. Durch die kationischen Antiseptika wurde ein unmittelbar signifikanter Abfall der Speichelbakterienzahlen erreicht. Bei Hexetidin kam es nach 90 Minuten zu der Rückehr zu den vor der Mundspülung vorhandenen bakteriellen Niveaus, bei Cetyl Pyridinium Chlorid nach 3 Stunden, nach Alexidinspülung nach 5 Stunden und bei Mundspülung mit Chlorhexidinglukonat nach 7 Stunden. Antibakterielle Residualaktivität verblieb im Speichel: bei Cetyl Pyridinium Chlorid 90 Minuten, bei Hexetidin und Alexidin 3 Stunden und bei Chlorhexidin 5 Stunden. Die antibakteriellen Eigenschaften, vor allem die Dauer der in vivo Wirkung könnte der Antiplaqueaktivität kationischer Antiseptika entsprechen.

Résumé

Comparaison entre les propriétés antibactériennes in vivo et in vitro de quelques bains de bouche antiseptiques: leur influence sur le mode d'action

Une étude a été effectuée pour comparer les propriétés antibactériennes de 4 antiseptiques cationiques, dont 3 produits du commerce destinés aux bains de bouche. Les concentrations minimales inhibitrices à l'égard d'une série de micro-organismes test standardisés ont été déterminées par dilution en tubes pour l'alexidine, le chlorure de cétylpyridinium, le gluconate de chlorhexidine et l'hexétidine. Les valeurs correspondantes pour le Staphylococcus Oxford ont ensuite été déterminées dans le milieu Dubos auquel était ajoutées des protéines sous forme de levure, ou d'extrait alimentaire, ou de sérum, en doublant les dilutions jusqu'à 16%. Chez 10 sujets, des numérations des bactéries salivaires après un rinçage unique avec les antiseptiques ou avec de l'eau ont été effectuées au cours de la journée, et la durée de la persistance d'une activité antiseptique résiduelle dans la salive a été mesurée. Tous les antiseptiques étaient efficaces à l'égard des organismes testés à basses concentrations, cependant les valeurs de la concentration minimale inhibitrice de l'hexétidine étaient les plus élevées. L'extrait alimentaire et le sérum augmentaient nettement les valeurs de la concentration minimale inhibitrice de tous les antiseptiques, mais, pour l'alexidine et l'hexétidine, l'influence sur les pourcentages était moins marquée. L'activité de 1% de povidone iode, utilisé à titre de comparaison, était presque entièrement annulée. Les antiseptiques cationiques déterminaient une chute immédiate et significative du nombre des bactéries salivaires. Le retour aux valeurs existant avant le rinçage était constaté au bout de 90 minutes pour l'hexétidine, au bout de 3 heures pour le chlorure de cétylpyridinium, au bout de 5 heures pour l'alexidine et au bout de 7 heures pour le gluconate de chlorhexidine. Une activité antibactérienne salivaire résiduelle persistait jusqu'à 90 minutes pour le chlorure de cétylpyridinium, jusqu'à 3 heures pour l'hexétidine et l'alexidine et jusqu'à 5 heures pour le gluconate de chlorhexidine. Les propriétés antibactériennes mesurées, en particulier la durée de l'action in vivo, peuvent représenter un facteur important en ce qui concerne l'action antiplaque des antiseptiques cationiques.

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