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Note

Physical factors affecting the sporicidal activity of chlorhexidine gluconate

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Abstract

The effects of pH and indirect ultrasonication on the cidal activity of aqueous and alcoholic solutions of chlorhexidine gluconate against *Bacillus subtilis* spores were examined. At moderately elevated temperatures, increasing pH enhanced sporicidal activity. pH markedly enhanced the sporicidal activity between indirect ultrasound and chlorhexidine. 'Alcoholic' chlorhexidine gluconate was more sporicidal than its aqueous counterpart.

Keywords: Chlorhexidine; *Bacillus subtilis*; pH effect; Ultrasonication, indirect

Chlorhexidine is a cationic bisbiguanide which is widely used as a disinfectant, antiseptic and preservative due to its growth inhibitory and cidal activities against both Gram-positive and Gram-negative bacteria (Davies et al., 1954; Gardner and Gray, 1983; Scott and Gorman, 1987). In addition, it is also active against yeasts and fungi, however, the fungistatic action of chlorhexidine is subject to species variation (D'Arcy, 1971; Gardner and Gray, 1983). It has been recognised that low concentrations of chlorhexidine inhibit bacterial spore outgrowth but sporicidal activity is negligible at room temperature (Shaker et al., 1986; Russell, 1990). Sporicidal activity has, however, been observed at higher temperatures. At 98–100°C, after contact times of 26, 15 and 6 min, a reduction in spore viability of 99.999% is obtained with 0.01, 0.1 and 1.0% chlorhexidine gluconate, respectively (Anon, 1981). More recently, we have reported (Gorman et al., 1987) improved sporicidal activity of chlorhexidine gluconate following re-formulation to an alcoholic solution in conjunction with mid-range elevated temperatures (37–70°C). In this study, no viable spores were recovered following 3 h contact with the alcoholic solution of chlorhexidine gluconate (10% v/v) and 4 h contact with the aqueous solution of chlorhexidine gluconate (10% v/v), both at 55°C. In a subsequent study (Gorman et al., 1990), the sporicidal activities of aqueous and alcoholic formulations of chlorhexidine gluconate were improved following exposure to both direct and indirect ultrasound.

Therefore, as an extension of our examination of the sporicidal activity of chlorhexidine, this study reports the effects of pH in the absence and presence of indirect ultrasound on the sporicidal

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Table 1
Effect of pH on the sporicidal activity of chlorhexidine gluconate (aqueous and alcoholic) against spores of *Bacillus subtilis* at 37°C

Time of contact (h)	% surviving spores [a] at 37°C following treatment with							
	10% v/v chlorhexidine gluconate aqueous				10% v/v chlorhexidine gluconate in 50% v/v isopropyl alcohol			
	pH 5.7	pH 7.6	pH 9.0	pH 9.7	pH 5.7	pH 7.6	pH 9.0	pH 9.7
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
1	96.00	91.30	52.10	65.80	100.00	49.70	4.49	5.68
2	85.30	58.90	49.60	20.70	94.60	12.20	3.49	2.09
3	93.30	37.40	32.40	14.90	22.20	9.60	2.69	0.76

[a] Initial viable count approx. 1×10^6 ml^{-1}.

activity of both alcoholic and aqueous formulations of chlorhexidine gluconate.

Bacillus subtilis NCTC 10073 was used as the test organism in this study. Spores were produced as previously described (Gorman et al., 1987). In brief, spores were grown on the medium of Beeby and Whitehouse (1965) by incubation at 30°C for 5 days. Following this, spores were harvested, washed four times with sterile distilled water and incubated in phosphate buffer (0.066 M, pH 7) containing 200 μg ml^{-1} lysozyme for 60 min at 37°C. Spores were washed a further two times before storage as dense suspensions at 4°C.

The germination medium was used as part of the chlorhexidine inactivation procedure (to enhance revival of sublethally injured spores) and has been described by Gorman et al. (1987).

Sporicidal activity of chlorhexidine formulations was determined as follows. An aliquot of standardised spore suspension (0.1 ml, approx. 1×10^8 viable spores ml^{-1}) was added to 9.9 ml of chlorhexidine gluconate, aqueous or alcoholic, solution which had previously been equilibrated to the required temperature. At specified time intervals, 1 ml samples were removed and processed via the inactivation procedure described by us (Gorman et al., 1987). The number of colony forming units ml^{-1} was determined by the drop-plate method of Miles and Misra (1938) on tryptone soya agar (Oxoid). All agar plates were incubated at 32°C for a minimum of 72 h.

Indirect ultrasonic energy was provided by a 150 W ultrasonic bath (Dawe Sonicleaner) to suspensions of spores in either alcoholic or aqueous chlorhexidine gluconate solutions contained in glass, screw-cap, McCartney bottles (wall thickness 2 mm). These were placed on the floor of the ultrasonic bath to produce minimum disturbance of the ultrasonic waves. The volume of water in the bath was maintained at a set level

Table 2
Effect of temperature and indirect ultrasonication [b] on the activity of chlorhexidine gluconate solutions at pH 9.70 (aqueous and alcoholic) against spores of *Bacillus subtilis*

Time of contact (h)	% surviving spores [a] following treatment with								Temperature in ultrasonic bath (°C)
	10% v/v chlorhexidine gluconate aqueous at pH 9.7				10% v/v chlorhexidine gluconate in 50% v/v isopropyl alcohol at pH 9.7				
	20°C	37°C	55°C	I.U.	20°C	37°C	55°C	I.U.	
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	18
1	98.10	65.88	54.50	42.40	100.00	5.68	33.20	8.10	32
2	N.D.	20.70	29.40	13.60	78.10	2.09	17.50	50.00	41
3	80.00	14.90	7.56	0.43	54.50	0.76	0.009	N.D.O.	62

[a] Initial viable count approx. 1×10^6 ml^{-1}.
[b] Indirect ultrasonication of chlorhexidine-immersed spores in an ultrasonic bath. Temperature allowed to rise naturally over a 3 h period of sonication.
I.U., indirect ultrasonication; N.D., not determined; N.D.O., no detectable organisms.

above the level of fluid in the McCartney bottles. At appropriate times, samples of the spore-chlorhexidine mixture were removed and inactivated, prior to incubation and enumeration as described above.

The pH of chlorhexidine formulations was determined potentiometrically and all pH adjustments were performed using either 10% v/v hydrochloric acid (BDH Chemicals, Poole) or 40% w/v sodium hydroxide (BDH Chemicals, Poole).

The effects of pH on the sporicidal activity of aqueous and alcoholic solutions of chlorhexidine gluconate (10% v/v) at 37°C are shown in Table 1. For both aqueous and alcoholic solutions the greatest sporicidal activities were observed at pH 9.7 whereas the lowest activities were observed at pH 5.7, both following 3 h treatment. Increasing the pH of each solution type enhanced the sporicidal activities. At each pH value, alcoholic solutions of chlorhexidine gluconate exhibited greater sporicidal activities in comparison to their aqueous counterparts.

Table 2 lists the effects of temperature and indirect ultrasonication on the sporicidal activities of aqueous and alcoholic solutions of chlorhexidine gluconate (10% v/v, pH 9.7). For both, increasing temperatures correlated with increased spore kill, the extent of which was greatest at 55°C but was optimal using the alcoholic solutions of chlorhexidine gluconate. Indirect ultrasonication was also observed to enhance sporicidal activity of each solution. This enhancement was more marked with the alcoholic (no detectable viability) than the aqueous solution (0.43% surviving spores).

Chlorhexidine is a strong base (pK_a 2.3 and 10.3) which is virtually insoluble in water (Gardner and Gray, 1983). Whilst it has been reported that this agent possesses no sporicidal activity at 20°C, sporicidal activity has been reported when used in conjunction with elevated temperature (Gorman et al., 1987). Both aqueous and alcoholic solutions of chlorhexidine gluconate (10% v/v) exhibit a pH value of approx. 5.7 and, consequently, the results expressed by Gorman et al. (1987) refer to the sporicidal activity at this pH value. In the current study, similar results were obtained using both aqueous and alcoholic chlorhexidine gluconate solutions at pH 5.7. pH enhancement of sporicidal activity is likely to be due to the state of ionisation of chlorhexidine in these solutions. As the pH is increased the percentage ionisation of chlorhexidine will decrease. At pH 5.7, 7.6, 9.0 and 9.7, the percentage ionisations are approx. 0.0001, 0.08%, 0.2 and 1.0%, respectively. Therefore, at higher pH values there will be a greater proportion of unionised chlorhexidine molecules present. It has been reported that the resistance of bacterial spores to chlorhexidine is due, in part, to the nature of the spore coat. Chlorhexidine diacetate was more sporicidal at 20°C to, and showed greater adsorption onto, urea-dithreitol-sodium lauryl sulphate treated spores (Shaker et al., 1988). Therefore, the resistance of bacterial spores to chlorhexidine may be due to the presence of an impermeability barrier to this agent. Chawner and Gilbert (1989) have reported that the preferential site of antibacterial action of chlorhexidine is the cytoplasmic membrane and it appears that, to exert a sporicidal effect, chlorhexidine must diffuse across the spore coats and reach the spore core (Gorman et al., 1990). The enhanced sporicidal activity of chlorhexidine gluconate at pH 9.7 may, therefore, be due to a greater ability to permeate the spore coat in the unionised form. In addition, the alkaline pH of the environment may contribute to the increased sporicidal activity by directly affecting the spore coat integrity. It has been reported that mixtures of 1.5–4.0% sodium hydroxide with sodium hypochlorite are much more rapidly sporicidal than either sodium hydroxide or hypochlorite used singly (Cousins and Allan, 1967).

Indirect ultrasonication was shown to enhance the sporicidal activity of aqueous and alcoholic solutions of chlorhexidine gluconate with no viable spores observed following 3 h contact with the alcoholic solution (Table 2). The activities of both solutions were greatest at pH 9.7 and exceeded that reported by Gorman et al. (1990). This illustrates the apparent synergy between elevated pH and indirect ultrasonication on the sporicidal activity of chlorhexidine.

The difference in sporicidal activities between alcoholic and aqueous solutions of chlorhexidine

gluconate in every condition examined was marked. A softening of the spore coat may arise following contact with alcohol (Gorman et al., 1987), and this may further enhance the diffusion of chlorhexidine in the presence of indirect ultrasonication. Diffusion of unionised molecules of chlorhexidine across the spore coat may be facilitated in a similar manner.

The enhanced sporicidal activity observed at moderately elevated temperatures and the additional benefits of ultrasonic energy on chlorhexidine solutions at high pH may have application in the cold liquid chemosterilisation of thermolabile equipment or materials.

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