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Drug Resistance Studies with Topical Antiseptics

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Abstract □ Species of *Proteus, Serratia,* and *Pseudomonas* became resistant to chlorhexidine after five to eight transfers *in vitro.* Cross-resistance to benzalkonium chloride also was detected. Resistance to povidone-iodine was not encountered. Chlorhexidine resistance was stable after drug-free transfers of *Serratia* and *Pseudomonas* but was transitory for *Proteus.*

Keyphrases □ Chlorhexidine gluconate—resistance by various microorganisms *in vitro* □ Povidone-iodine—resistance by various microorganisms *in vitro* □ Resistance—various microorganisms to chlorhexidine gluconate and povidone-iodine *in vitro* □ Antiseptics, topical—chlorhexidine gluconate and povidone-iodine, resistance by various microorganisms *in vitro*

Chlorhexidine, *N,N″*-bis(4-chlorophenyl)-3,12-diimino-2,4,11,13-tetraazatetradecanediimidamide, first described in 1954 by Davies *et al.* (1), has been used extensively in England and Europe as a preservative, disinfectant, and topical antiseptic. It recently was introduced in the United States for use in hospitals as a topical antimicrobial cleanser.

Resistant strains of *Proteus mirabilis* were isolated from postoperative urinary infections and in paraplegics undergoing catheterization of the bladder following repeated use of chlorhexidine for cleansing the external genitalia (2–4). More recently, Stickler (5) examined 104 clinical isolates of *P. mirabilis* for sensitivity to chlorhexidine and found minimum inhibitory concentrations

Table I—Baseline *In Vitro* Activity of Three Topical Antiseptics against Parent Gram-Negative Rods

Organism	MIC, μg/ml (in Dubos Broth, 48 hr at 35°)		
	Chlorhexidine Gluconate	Benzalkonium Chloride	Available Iodine from Povidone-Iodine
P. mirabilis	8	16	8
Ps. aeruginosa	8	128	8
Ps. cepacia	1	1000	16
Ser. marcescens	8	16	8
Ser. rubidae	32	512	32
Sal. enteritidis	8	32	16

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Table II—Development of Resistance to Chlorhexidine Gluconate [a] by the Serial Passage Technique

Organism	MIC, μg/ml (in Dubos Broth, 48 hr at 35°)																			Geometric Increase at[c]
	1[b]	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	20	
P. mirabilis	8	8	16	32	32	32	64	*128*	*128*	*128*	*128*	*128*	*128*	*256*	*255*	*256*	*256*	*512*	*1024*	128×
Ser. marcescens	8	4	16	64	*128*	*128*	*128*	*256*	*256*	*512*	*1024*	*1024*	*1024*	*2048*	*2048*	*1024*	*2048*	*2048*	*2048*	256×
Ser. rubidae	32	120	256	256	256	256	256	*512*	*1024*	*1024*	*1024*	*1024*	*1024*	*1024*	*1024*	*1024*	*1024*	*1024*	*2048*	128×
Ps. cepacia	1	2	2	4	4	4	8	*16*	*32*	*32*	*64*	*64*	*16*	*16*	*32*	*32*	*32*	*128*	*128*	128×
Ps. aeruginosa	8	4	4	8	8	8	16	32	16	16	16	8	8	16	16	16	8	32	16	2×
Sal. enteritidis	8	8	16	16	16	16	16	8	16	16	32	16	32	16	16	8	16	16	16	2×

[a] Hibiclens. [b] Transfer number. [c] Resistance was considered to have occurred when the MIC value was 10-fold greater than the parent strain (Transfer 1); italicized figure equals resistance.

ranging from 10 to 800 μg/ml with cross-resistance to benzalkonium chloride.

The present study was undertaken to determine the: (*a*) rate and extent to which resistance to chlorhexidine gluconate could be induced in Gram-negative rods as compared to povidone-iodine, (*b*) presence or absence of cross-resistance to topical antiseptics widely used in this country, and (*c*) possible coinduction of other phenotypic changes in strains selected for resistance.

EXPERIMENTAL

Drugs—Chlorhexidine gluconate cleanser[1] as the 4% (w/v) solution, 7.5% povidone-iodine[2], and benzalkonium chloride[3] antiseptic, 1:750 aqueous, were studied.

Procedure—All formulations were diluted in unsupplemented Dubos broth base without glycerin or albumin since this minimally organic medium allowed detection of reproducible values with povidone-iodine. Twofold serial dilutions were prepared in this broth, ranging from 4000 to 0.125 μg of active ingredients/ml. Aliquots of 2 ml of drug were added to 2 ml of broth to achieve the twofold dilution; a fresh pipet was used for each dilution step.

Baseline minimum inhibitory concentrations (MIC values) were obtained by inoculating tubes with 0.1 ml of a 1:1000 dilution of 24-hr trypticase soy broth cultures (~10^4 cells/tube of broth–drug mixture). The inhibitory end-point in micrograms per milliliter was taken as the lowest concentration free of gross turbidity after 48 hr at 35°. The development of drug resistance was then determined by the serial passage technique of Grunberg and Prince (6).

Studies with chlorhexidine gluconate and povidone-iodine were performed concurrently. After 48 hr, the tubes containing growth in the presence of the highest tolerated concentration of drug were diluted 1:1000 and used as the inoculum for the following transfer. A total of 20 passages was performed with each of the following organisms, all initially purified from an isolated colony on MacConkey's agar: *Proteus mirabilis* (ATCC 7002, GBL 15), *Pseudomonas aeruginosa* (ATCC 15442, GBL 67), *Pseudomonas cepacia* (wild strain GBL 110), *Serratia marcescens* (clinical isolate GBL 104), *Serratia rubidae* (wild strain GBL 181), and *Salmonella enteritidis* (clinical isolate GBL 78). The baseline MIC values for the seven organisms are shown in Table I.

Drug resistance was considered to have occurred if the MIC value increased at least 10-fold above the baseline. When resistance was detected, biochemical tests employing freeze-dried miniaturized cupules[4] and intraperitoneal virulence assays in mice (septicemia and death) were performed (7), and the results were compared with the parent strains. These tests included the fermentation of glucose, arabinose, mannitol, sorbitol, rhamnose, sucrose, lactose, and amygdalin; dissimilation of arginine, lysine, ornithine, tryptophan, urea, and citrate; and production of hydrogen sulfide, nitratase, and cytochrome oxidase. When resistant strains emerged, cross-resistance to benzalkonium chloride and povidone-iodine was determined employing the same broth dilution technique in Dubos broth.

[1] Hibitane, Ayerst Laboratories, Montreal, Canada; Hibiclens, Stuart Pharmaceuticals, Wilmington, Del.
[2] Betadine Surgical Scrub, Purdue-Frederick Co., Norwalk, Conn.
[3] Zephiran, Winthrop Laboratories, New York, N.Y.
[4] API System, Analytical Products, South Plainfield, N.J.

RESULTS AND DISCUSSION

The data in Table I show that chlorhexidine gluconate and povidone-iodine were of similar activity in the broth dilution assay and that both were superior to benzalkonium chloride.

The effect of serial transfer on the development of resistance to chlorhexidine gluconate is summarized in Table II. Four of the six organisms studied became resistant to chlorhexidine.

P. mirabilis displayed a 128-fold increase in resistance to chlorhexidine gluconate, with initial resistance seen to emerge by the eighth transfer. Transfer 20 showed MIC values of 512 μg/ml against benzalkonium chloride and 8 μg/ml against povidone-iodine. Thus, resistance to chlorhexidine developed rapidly and extensively with cross-resistance to benzalkonium chloride but not to povidone-iodine. Resistance to chlorhexidine was not permanent since three drug-free transfers produced a strain for which the MIC decreased to 32 μg/ml, a value similar to the value obtained in Transfer 1 with the parent strain. The resistant strains were not altered with respect to biochemical properties or virulence for mice.

Ser. marcescens developed a 256-fold increase in resistance to chlorhexidine gluconate, with initial resistance seen as early as the fifth transfer. Transfer 20 was cross-resistant to benzalkonium chloride (MIC = 512 μg/ml) but not to povidone-iodine (MIC = 8 μg/ml). A similar pattern of resistance and cross-resistance was detected for *Ser. rubidae*. Resistance of these two species of *Serratia* to chlorhexidine was stable, since three drug-free transfers produced strains with MIC values remaining at 512–1024 μg/ml. The resistant strains were not altered with respect to biochemical properties or mouse virulence.

Ps. cepacia developed a 128-fold increased resistance to chlorhexidine. This organism, initially insensitive to benzalkonium chloride (MIC = 1000 μg/ml), did not display baseline cross-resistance to chlorhexidine (Table I). Such resistance had to be induced in a stepwise manner, indicating, perhaps, that chlorhexidine and benzalkonium chloride differ in their mechanism of action against this organism. Resistance of *Ps. cepacia* to chlorhexidine was stable, since three drug-free transfers produced strains with an MIC value remaining at 128 μg/ml. This chlorhexidine-resistant strain also failed to ferment lactose, the only coinduced phenotypic change encountered in this study. Deletion of β-galactosidase was as stable as the acquisition of resistance, since the lac^- condition was maintained after three drug-free transfers.

Neither strain of *Ps. aeruginosa* nor *Sal. enteritidis* became resistant to chlorhexidine gluconate, and additional strains are under test. Similarly, *Staphylococcus aureus* (FDA 209, ATCC 6538) failed to develop resistance to this drug.

In contrast to chlorhexidine, resistance to povidone-iodine was not induced after 20 transfers in any of the six Gram-negative rods studied. The MIC ranges for *P. mirabilis*, *Ser. marcescens*, *Ser. rubidae*, *Ps. cepacia*, *Ps. aeruginosa*, and *Sal. enteritidis* were 8–16, 8–16, 16–64, 8–16,

8–16, and 16–64 μg/ml, respectively, variations normal to the twofold serial dilution technique. These results confirm the work of Houang *et al.* (8), who were unable to detect resistance to this drug.

These studies show that the development of drug resistance can be an important factor in the choice of a skin antiseptic. Pharmaceutical scientists should share equal awareness of this limitation with microbiologists and physicians. There is no evidence from either the literature (8) or the present work that resistance to povidone-iodine is a potential problem in medical practice. However, previous observations of resistance to chlorhexidine and benzalkonium chloride (5) were confirmed and extended. Development of resistance to chlorhexidine in the genus *Serratia* is newly reported here (MIC = 2000 μg/ml). This concentration can be obtained easily in the hospital with only a 20-fold dilution of full-strength surgical scrub.

The practical significance of these findings with respect to nosocomial infections should not be underestimated, especially with increased use of chlorhexidine as a preservative, antiseptic, and oral drug.

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