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Chlorhexidine resistance in *Proteus mirabilis*

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SYNOPSIS A total of 104 clinical isolates of *Pr. mirabilis* from three hospitals were screened for their sensitivity to chlorhexidine. The minimum inhibitory concentrations of the antiseptic for these strains ranged from 10 to 800 μg/ml. Two strains sensitive to 20 μg of chlorhexidine/ml were adapted to resistance by growth in subinhibitory concentrations of the antiseptic, their MIC values increasing to 200 and 800 μg/ml. These derived strains exhibited slightly reduced sensitivity to cetrimide and benzalkonium chloride. The chlorhexidine-resistant clinical isolates also exhibited this partially decreased sensitivity to the quaternary ammonium compounds. Both the chlorhexidine-sensitive and -resistant strains were uniformly sensitive to chloroxylenol (Dettol), glutaraldehyde, and 2-phenoxyethanol.

The antiseptic chlorhexidine is active against a wide range of bacteria (Davies, Francis, Martin, Rose, and Swain, 1954) and irritates sensitive mucosal surfaces very little (Beeuwkes and De Vries, 1956; Calman and Murray, 1956). These properties have led to its widespread use in medicine and veterinary practice. In addition it has been claimed that despite its extensive use, there have been no reports to indicate the development of bacterial resistance to chlorhexidine (Longworth, 1971). Gillespie, Lennon, Linton, and Phippen (1967), however, isolated strains of *Proteus mirabilis* from postoperative urinary infections in gynaecological patients undergoing catheterization of the bladder, a procedure which involved the repeated application of chlorhexidine to the urethra, and reported that 10 out of 12 of these strains had minimum inhibitory concentration (MIC) values of 125 to 500 μg chlorhexidine/ml, well above the level of 10 to 50 μg/ml generally required to inhibit Gram-negative bacteria (Davies *et al*, 1954).

Subsequently, during the course of studies on the development of urinary infections in male paraplegic patients undergoing intermittent catheterization of the bladder, Stickler, Wilmot, and O'Flynn (1971) observed that the cleansing of the external genitalia before insertion of the catheter with aqueous solutions containing 600 μg chlorhexidine/ml was seldom effective in eliminating the bacterial flora of the urethral meatus of the patient. Strains of *Pr. mirabilis* that had survived the antiseptic procedure were examined for their sensitivity to chlorhexidine and many of them were found to grow in at least 200 μg of the agent/ml (O'Flynn and Stickler, 1972).

In both these instances strains of *Pr. mirabilis* showing greater resistance to chlorhexidine than would normally be expected were isolated from clinical situations in which they had been repeatedly exposed to the antiseptic. The objectives of the present study were threefold. First to determine how common a phenomenon chlorhexidine resistance is in hospital strains of *Pr. mirabilis*, secondly to establish whether it is possible to develop chlorhexidine resistance in *Pr. mirabilis* in the laboratory, and thirdly to examine the sensitivity of chlorhexidine-resistant strains to other disinfectants and antiseptics.

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Materials and Methods

STRAINS USED

The strains of *Pr. mirabilis* designated 40, 61, 405, 407, and 529 were isolated from the urethral meatuses of paraplegic patients at the National Medical Rehabilitation Centre, Our Lady of Lourdes Hospital, Dublin. They had previously been shown to be able to grow in 200 μg chlorhexidine/ml (O'Flynn and Stickler, 1972). NCIB strain 60 and NCTC strains 2896, 3177, 4199, and 5887 were used as reference organisms. All other strains used in this study were obtained from the routine examination of a range of specimens in the diagnostic laboratories of three Cardiff hospitals. They were

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identified as *Pr. mirabilis* using the methods of Cowan and Steel (1965).

MINIMUM INHIBITORY CONCENTRATION DETERMINATIONS

On agar

The strains to be tested were grown for 24 hr at 37° in broth (Oxoid nutrient broth no. 2). Volumes, each of 0·02 ml (containing approximately 10^4 viable cells) of 1 in 10 000 dilutions of these cultures in broth, were then dropped onto nutrient agar (Oxoid) into which various concentrations of the antibacterial agents had been incorporated (table I). A standard method was adopted for the preparation of the agar plates. The antibacterial agents were added to nutrient agar that had been allowed to cool to 50°. The plates were dried at 37° for 20 minutes and then used directly. After inoculation the plates were incubated at 37° and examined for growth after 48 hours. The lowest concentration of the agent that prevented colony formation was taken as the minimum inhibitory concentration (MIC) for that strain. The tests were performed in duplicate and *Pr. mirabilis* NC1B 60 was included as a control with each batch of strains tested.

Agent	*Source*	*Concentrations Incorporated into Agar for MIC Testing*
Chlorhexidine	A 20% w/v solution of Hibitane (chlorhexidine digluconate) ICI Ltd	10, 20, 50, 100
Cetrimide	Cetavlon, ICI Ltd	200, 400, and 800
Benzalkonium chloride	Winthrop Laboratories Ltd	μg/ml
Glutaraldehyde	Sigma Ltd	0·02, 0·04, 0·08, and 0·16 % w/v
2-Phenoxyethanol	Sigma Ltd	0·1, 0·2, 0·4, and 0·8 % w/v
Chloroxylenol (Dettol)	The commercial product containing 4·8% w/v chloroxylenol Reckitt & Coleman Ltd	

Table I *Antibacterial agents used in the minimum inhibitory concentration tests*

In broth

The MIC values for chlorhexidine and benzalkonium chloride were also determined in liquid media. For these experiments 0·1 ml volumes of 24-hr broth cultures were used to inoculate 5 ml of broth (Oxoid nutrient broth no. 2) containing the same range of concentrations of the two agents as shown in table I. The tubes were incubated at 37° and examined for growth after 48 hours. The lowest concentration of the agent preventing growth was taken as the MIC. These tests were also set up in duplicate.

PHAGE MARKING OF STRAINS

In an attempt to isolate a number of *Pr. mirabilis* bacteriophages the supernatant fluids from overnight broth cultures of a large number of the clinical isolates of this species were dropped onto nutrient agar plates that had been seeded with early log phase cultures of prospective indicator strains. The plates were incubated at 37° and examined after 18 hr for plaque formation. Very few of the strains proved to be lysogenic as determined by this method; phages were, however, isolated which produced plaques on two of the chlorhexidine-sensitive strains (18 and 21). The phage designated α was active specifically on *Pr. mirabilis* 18 and phage β plaqued only on *Pr. mirabilis* 21. No other strain used in this study was sensitive to either of these phages.

Results

SENSITIVITY TO CHLORHEXIDINE OF *Pr. mirabilis* STRAINS

A total of 104 strains isolated from three Cardiff hospitals were screened for their sensitivity to the antiseptic.

The minimum inhibitory concentration determinations were performed on agar and the results are shown in table II. The MIC values for five reference strains of *Pr. mirabilis* are shown in table III.

No. of Strains Tested	*No. of Strains with MIC of Chlorhexidine (μg/ml)*						
	10	*20*	*50*	*100*	*200*	*400*	*800*
104	4	12	41	33	11	2	1

Table II *Sensitivity of some clinical isolates of* Proteus mirabilis *to chlorhexidine*

Strain	*MIC of Chlorhexidine (μg/ml)*
NC1B 60	20
NCTC 2896	50
NCTC 3177	50
NCTC 4199	20
NCTC 5887	50

Table III *Sensitivity of reference strains of* Pr. mirabilis *to chlorhexidine*

Two of the clinical isolates, designated strains 18 and 21 and having minimum inhibitory concentrations of 20 μg chlorhexidine/ml, were chosen for further study as they could be marked by their sensitivity to specific bacteriophages.

ADAPTATION OF *Pr. mirabilis* STRAINS 18 AND 21 TO CHLORHEXIDINE RESISTANCE

The phage-marked, chlorhexidine-sensitive strains

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Strain No.	Source	Minimum Inhibitory Concentration								Viable Count of 24-hour Culture Used as Inocula (cells/ml)
		Chlorhexidine (μg/ml)		Cetrimide (μg/ml)	Benzalkonium Chloride (μg/ml)		Dettol (% w/v)	Glutaralde-hyde (% w/v)	2 Phenoxy-ethanol (% w/v)	
		On Agar	In Broth		On Agar	In Broth				
18	Clinical isolates from a Cardiff	20	20	100	50	50	0·4	0·16	0·8	$3·0 \times 10^9$
21	hospital	20	50	100	100	100	0·4	0·16	0·8	$3·8 \times 10^9$
18^{cr}	Derived from 18	200	200	200	200	200	0·4	0·16	0·8	$5·0 \times 10^9$
21^{cr}	and 21 in the laboratory	800	800	400	200	200	0·4	0·16	0·8	$3·3 \times 10^9$
47	Clinical isolate from a Cardiff hospital	800	800	400	400	200	0·4	0·16	0·8	$5·0 \times 10^9$
40	Clinical isolates	400	400	400	200	200	0·4	0·16	0·8	$4·1 \times 10^9$
61	from National	800	800	800	400	400	0·4	0·16	0·8	$5·8 \times 10^9$
405	Medical	400	400	400	200	200	0·4	0·16	0·8	$4·9 \times 10^9$
407	Rehabilitation	400	400	400	200	200	0·4	0·16	0·8	$4·8 \times 10^9$
529	Centre, Dublin	400	400	100	200	200	0·4	0·16	0·8	$6·0 \times 10^9$
60	NCIB	20	20	50	50	50	0·2	0·16	0·4	$1·8 \times 10^9$

Table IV *Sensitivities of some* Pr. mirabilis *strains to six antibacterial agents*

18 and 21 were grown in nutrient broth (Oxoid nutrient broth no. 2) for 24 hr at 37° and 0·1 ml volumes of these cultures were used to inoculate broths (5 ml) containing various concentrations of chlorhexidine. The tubes were incubated at 37° for 48 hr and examined for growth. Samples (0·1 ml) from the broths containing the highest concentration of chlorhexidine that allowed growth were then inoculated into a further series of broths containing increasing concentrations of this agent. The initial tolerance of the organisms was 10 μg chlorhexidine/ml for strain 18 and 20 μg/ml for strain 21. After four cycles of re-inoculation into broths containing increasing concentrations of the antiseptic, strain 18 acquired the capacity to grow in broth containing 100 μg chlorhexidine/ml and strain 21 grew in 400 μg/ml. These derived strains, designated 18^{cr} and 21^{cr}, were subcultured four times on nutrient agar containing no chlorhexidine. They were then found to have retained their specific sensitivities to phages α and β indicating that they were in fact derivatives of strains 18 and 21. Minimum inhibitory concentration determinations were then carried out on the derived and parent strains and the results are included in table IV.

SENSITIVITY OF SOME *Pr. mirabilis* STRAINS TO OTHER DISINFECTANTS AND ANTISEPTICS

A total of 11 strains, including 18^{cr}, 21^{cr}, their parent strains, and six clinical isolates showing resistance to chlorhexidine, were screened for their sensitivity to cetrimide, benzalkonium chloride, chloroxylenol, glutaraldehyde, and 2-phenoxyethanol. The results are shown in table IV. The viable counts performed on the 24-hr broth cultures used as a source of the inocula indicate that equivalent numbers of cells of each strain were being tested in these experiments.

Discussion

The preliminary survey of the minimum inhibitory concentrations of chlorhexidine for *Pr. mirabilis* strains isolated from three Cardiff hospitals demonstrates the wide variation in the sensitivity of clinical isolates of this organism to the antiseptic, MIC values ranging from 10 to 800 μg chlorhexidine/ml. In a similar survey Martin (1969) tested the sensitivity of 126 *Pr. mirabilis* strains isolated from a single hospital to 25, 50, 100, and 200 μg chlorhexidine/ml and found that most strains were inhibited by 25 or 50 μg/ml, only five strains survived 100 μg/ml, and none could tolerate 200 μg/ml.

Martin (1969) determined the minimum inhibitory concentrations of chlorhexidine for his strains in liquid medium using standard loopfuls of 24-hr broth cultures as the inocula for 1 ml of broth (Oxoid nutrient no. 2) containing the various concentrations of the antiseptic. This method was not used in the preliminary survey of the present study because of the difficulty of ensuring standard inocula in this way. Strains were tested, however, for their MIC of chlorhexidine in broth by a method similar to that of Martin, using 0·1 ml volumes of 24-hr broth cultures to inoculate 5 ml of the broth containing the antiseptic. The MIC values obtained by this method were in good agreement with those derived on solid media (table IV). It is interesting that in the present study the levels of sensitivity of strains of *Pr. mirabilis* from two of the Cardiff

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hospitals were similar to those reported by Martin (1969). The strains shown in table II as growing in 200 and 400 μg chlorhexidine/ml were all isolated from the third hospital. The results of the two surveys indicate therefore that the majority of the strains of *Pr. mirabilis* isolated in hospital are sensitive to chlorhexidine and that it is probably only in special situations where contaminated surfaces are exposed to repeated applications of the antiseptic that resistant strains are generated.

Martin (1969) concluded that the levels of resistance recorded in his survey were not likely to be of practical importance as the normal user strengths of chlorhexidine are 200 to 1000 μg/ml. It seems probable, however, that the strains requiring concentrations of 400 to 800 μg/ml to inhibit their growth under the conditions of MIC testing might well be of clinical significance, particularly as many of them (strains 40, 61, 405, 407, and 529) were isolated from situations in which chlorhexidine had been extensively used (Stickler *et al*, 1971).

The results shown in table IV demonstrate that whereas the 10 clinical strains tested were uniformly sensitive to Dettol, glutaraldehyde, and 2-phenoxyethanol, there appears to be a correlation between chlorhexidine, cetrimide, and benzalkonium chloride resistance. In addition comparison of the MIC values obtained for strains 18^{cr} and 21^{cr} with those of their parent strains 18 and 21 show that the increase in resistance to chlorhexidine is accompanied by a slight (2-4 fold) increase in tolerance to the quaternary ammonium compounds.

These increases were reproducibly demonstrable in both broth and agar with chlorhexidine and benzalkonium chloride. The MIC values for cetrimide in liquid media were not obtained as the turbidity produced by this agent in broth makes interpretation of results difficult.

It is interesting to speculate on the nature of the resistance of the organisms to these cationic antiseptics. Resistance to quaternary ammonium compounds has been reported in a number of Gram-negative species (Chaplin, 1952; MacGregor and Elliker, 1958; Soprey and Maxcy, 1968) and MacGregor and Elliker suggested that in the case of *Pseudomonas aeruginosa* resistance was due to a modification of the cell wall, which obstructed the penetration of the bactericide. Bentley, Davies, Field, and Roberts (1968) reported that a strain of *Pseudomonas* species able to grow in media containing up to 200 μg of chlorhexidine/ml had a lower capacity than normal strains for absorbing the bactericide from the growth medium. Davies and Roberts (1969) investigated the possibility that this might be a reflection of an unusual wall structure and found considerable differences in the wall lipopolysaccharide contents of sensitive and resistant cells. It is possible that some such modification in the chemistry of the cell surface could account for the resistance of the *Pr. mirabilis* strains to chlorhexidine and also for their partial increase in tolerance to quaternary ammonium compounds.

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