PRETREATMENT WITH COLISTIN AND *PROTEUS* SENSITIVITY TO OTHER AGENTS

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The effects of pretreatment with colistin (polymyxin E) on the sensitivity of *Proteus mirabilis, P. vulgaris* and *P. morganii* strains to tris and sodium deoxycholate (DOC) have been studied. Pretreatment of two *P. mirabilis* strains (NCTC 60 and 4199) with low concentrations (0.25~1 μg/ml) of colistin rendered them sensitive to lysis by tris (0.05 M) or DOC (250~1,000 μg/ml) although DOC induced lysis of control (non colistin-treated) suspensions also. In contrast, the other *P. mirabilis* strains, as well as the *P. vulgaris* and *P. morganii* strains were little affected by tris (0.2 M) or DOC (10,000 μg/ml) even after exposure of the cells to high colistin concentrations (up to 500 μg/ml). Colistin-pretreated or control cells of *P. mirabilis* NCTC 60 rapidly lost viability when suspended in water but not when held in 0.16 M sodium chloride solution. Ethylenediamine tetraacetate-pretreated cells of strains 60 and 4199 were fairly sensitive to tris, although the extent of the lysis was less than when colistin was used as pretreating agent. One strain of *P. vulgaris* (NCTC 4175) became sensitive to tris and to DOC following exposure of the cells to ampicillin.

Cells of *Proteus mirabilis, P. vulgaris* and *P. morganii* are resistant to polymyxins although recent reports indicate that these antibiotics have some effect on the outer layers of *P. mirabilis* cells.[1~3] It is likely that, following interaction with the cell wall of sensitive bacteria, the polymyxins act by damaging the cytoplasmic membrane.[4~6] Apart from *Proteus* sp., only *Serratia marcescens* among the Enterobacteriaceae is resistant to polymyxins, and it has been demonstrated with both types of organisms that synergism between a polymyxin and a sulphonamide can occur.[7~9]

In this paper, we have investigated the effects of pretreatment of the cells of various strains of *Proteus* sp. in relation to their subsequent sensitivity to other agents, particularly tris and sodium deoxycholate.

Materials and Methods

Bacterial Strains

The following strains of *Proteus* sp. were obtained from the National Collection of Type Cultures, London: *P. vulgaris* strains 4175, 4635 and 4636; *P. morganii* strains 232, 235 and 1707; and *P. mirabilis* strains 60, 2896 and 4199.

Growth and Pretreatment Methods

Ten ml of an 18-hour culture, grown in nutrient broth (Oxoid) at 37°C was added to 90 ml of broth and incubated in a shaking water-bath at 80 oscillations min^{-1} for 2 hours at 37°C. Sufficient of an antibacterial agent (colistin, chlorhexidine or cetrimide) was added, and incubation continued for 1 hour at 37°C. Washed suspensions were then prepared as described below. In other experiments, cells were pretreated with ampicillin (at 1/2× the minimum inhibitory concentration).

In pretreatment studies involving ethylenediamine tetraacetic acid (EDTA), an overnight culture was washed in borate buffer, pH 9, and suspended for 5 minutes at 37°C in this buffer containing 0.01 M EDTA. Washed suspensions were then prepared as described below.

Preparation of Washed Suspensions

The log-phase culture, pretreated with a drug if necessary, or EDTA-treated suspension was divided into 5 aliquots, centrifuged at 19,000 × *g* for 30 minutes and the cell suspensions triple washed with sterile saline (0.16 M) and then dispersed in tris buffer (0.05 ~ 0.2 M) of various pH values at 37°C or in sodium deoxycholate (DOC) in 0.16 M saline at 37°C. Optical density (O.D.) and viability changes were measured as described below.

Osmotic Shock

P. mirabilis NCTC 60 was grown for 3 hours at 37°C with shaking in broth (with or without 50 μg/ml colistin for the final hour). The suspension was centrifuged at 19,000 × *g* and triple washed with 0.16 M sodium chloride and then resuspended in either 0.16 M sodium chloride, pH 7.4, or glass-distilled water, pH 7.1. During subsequent storage, samples were removed and serially diluted in 0.16 M saline for viable counting by the surface-viable method, colony counts being made after incubation of plates for 24 hours at 37°C.

Culture Media

Control and colistin-pretreated suspensions were streaked on to agar (± DOC), ENDO agar, EMB agar, deoxycholate agar and MACCONKEY agar. Plates were incubated at 37°C for 24 hours and examined.

Optical Density (O.D.) Measurements

These were carried out at 500 nm in a Unicam SP 600 spectrophotometer using 1-cm cells and an appropriate blank.

Viable Counting

Viable counts were made in triplicate on to overdried nutrient agar plates. Colonies were counted after incubation of the plates for 24 hours at 37°C.

Chemicals

These were: ampicillin sodium B.P. (Beecham Research Laboratories, Brentford); benzalkonium chloride (50% w/v solution, B.P.C.: Berk Pharmaceuticals, Godalming); cetrimide, B.P. (Glover's Chemicals, Leeds); chlorhexidine acetate, B.P.C. (I.C.I. Ltd., Macclesfield); colistin sulphomethate sodium (Pharmax, Ltd., Bexley); disodium EDTA and sodium chloride, both 'Analar' grade (B.D.H. Chemicals, Poole); sodium deoxycholate (pure grade, Koch-Light Laboratories, Colnbrook); tris-(hydroxymethyl)-methylamine (referred to as Tris), Triton X-100 (scintillation grade) and Tween (polysorbate) 80 (all from B.D.H. Chemicals, Ltd.).

Minimum Inhibitory Concentration (MIC)

A washed suspension (*ca.* 10^6 viable cells/ml), 0.04 ml was dropped on to previously dried (4 hours, 37°C) nutrient agar plates containing an appropriate drug concentration. The plates were examined for the presence and absence of growth after incubation for 24 hours at 37°C and the MIC (the lowest drug concentration which completely prevented growth) determined.

Results

MIC Values

The MIC values of colistin, tris, DOC and ampicillin against the various strains are depicted in Table 1, which indicates the high resistance of strains to colistin and to DOC. In subsequent experiments, colistin was used in the range 0.125 ~ 500 μg/ml, tris 0.05 ~ 0.2 M and DOC 125 ~ 10,000 μg/ml.

Pretreatment with Colistin

Pretreatment of *P. mirabilis* strains 60 and 4199 with 50 μg/ml of colistin rendered the cells susceptible to 0.05 M tris (Fig. 1 a, c; Table 2); the degree and extent of the lethal effect of tris against the pre-treated cells was pH dependent being slower at pH 8.5, and more so at lower pH values, than at pH 9. Control cells (not pretreated with colistin) were insusceptible to tris at any pH. Decreasing the colistin concentrations to as low as 1 μg/ml (strain 60: Fig. 1b) or 0.25 μg/ml (strain 4199: Fig. 1d)

Table 1. MIC values for colistin and other substances

Organism	Strain (N.C.T.C.)	Colistin (μg/ml)	Tris (M)	DOC (μg/ml)	Ampicillin (μg/ml)
P. mirabilis	60	>1,000	0.25	>4,000	1
	2896	>1,000	0.25	>4,000	16
	4199	>1,000	0.25	>4,000	2
P. vulgaris	4175	>1,000	0.25	>4,000	6
	4635	>1,000	Nd	Nd	8
	4636	>1,000	Nd	Nd	>100
P. morganii	232	>1,000	0.25	>4,000	4
	235	>1,000	Nd	Nd	20
	1707	>1,000	Nd	Nd	12

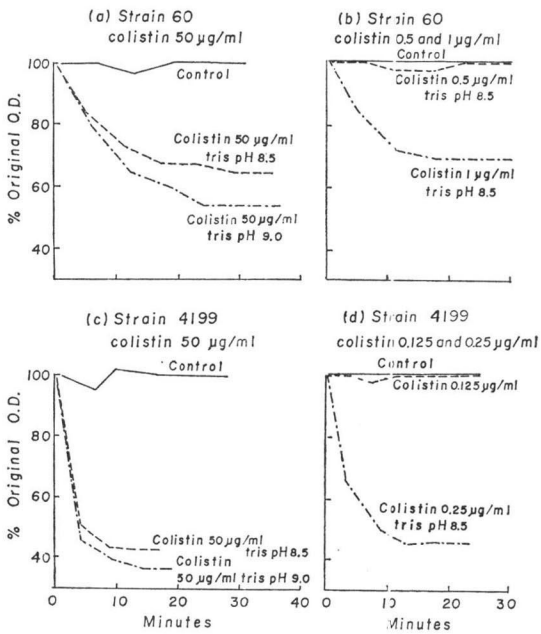
Nd: not done

resulted in lysis on exposure of the treated cells to tris.

In contrast, pretreatment with colistin (50~500 μg/ml) did not render the cells of the other strains markedly sensitive to 0.05~0.2 M tris at any pH; the decrease in O.D. was always <10% and the decrease in viability was correspondingly small and not dissimilar to control cells exposed to tris (Table 2). It is noteworthy that the third strain of *P. mirabilis* tested, 2896, shows a completely different response from strains 60 and 4199. Other strains (not described) of *P. mirabilis* tested resembled strain 2896.

The effects of pretreatment of cell suspensions with colistin on their subsequent sensitivity to DOC in saline are shown in Figs. 2 and 3 and Tables 3 and 4. *P. mirabilis* strains 60 and 4199 were highly sensitive to DOC irrespective of whether or not they had been pretreated with colistin, although the antibiotic did increase the rate of DOC-induced death (Tables 3 and 4). However, the other strains (Table 4) plus additional strains (not described) of the 3 species did not become sensitive to DOC even after the cells had been exposed to colistin.

Fig. 1. Susceptibility to 0.05 M tris of colistin-pretreated cells of *P. mirabilis* 60 and 4199.



Control and colistin-pretreated suspensions were plated on to various media: irrespective of pretreatment or not, colony counts of strains 232, 2896 and 4175 were not inhibited by nutrient agar containing 1,000 μg/ml DOC, or by ENDO, EMB, MACCONKEY or deoxycholate citrate agar media. In contrast, cells of strains 60 and 4199 which had or had not been pre-exposed to colistin grew on all media except EMB agar where colony growth was completely inhibited.

Cell suspensions which had been exposed or unexposed (controls) to colistin were tested for their sensitivity to a range of concentrations of the non-ionic surface-active agents, polysorbate (Tween) 80

Table 2. Effect of tris (pH 9) on *Proteus* strains at 37°C

Colistin pretreatment	Time (min) after exposure to tris	*Proteus* strain*				
		60	4199	2896	4175	232
No	0	100	100	100	100	100
	10	86	82	90	90	100
	20	78	76	83	84	95
	30	74	72	78	78	87
	60	70	70	74	77	82
Yes+	0	100	100	100	100	100
	10	30	29	86	86	90
	20	21	14	81	80	84
	30	15	10	69	72	76
	60	0.5	0.38	61	67	70

Figures are % surviving cells (0 min=100%) after exposure to tris.
* Strains 60,4199 and 2896 are *P. mirabilis*; 4175 is *P. vulgaris*; 232 is *P. morganii*.
\+ 50 μg/ml colistin for strains 60 and 4199; 500 μg/ml colistin for strains 2896, 4175 and 232.

Table 3. Susceptibility of *P. mirabilis* 60 to DOC in 0.16 M saline at 37°C

Colistin pretreatment (50 μg/ml)	Time (min) after exposure to DOC	% Viability* after treatment with DOC (μg/ml)		
		125	250	500
No	0	100	100	100
	10	94	84	14
	20	92	82	10.2
	30	84	70	9.8
	60	79	70	6.2
Yes	0	100	100	100
	10	94	60	5
	20	91	56	1.1
	30	80	50	0.8
	60	78	48	0.5

* 0 min=100%
Cells which were pretreated with colistin (50 μg/ml) and then held in 0.16 M saline (DOC absent) at 37°C showed no decrease in viability.

Table 4. Summary of susceptibility of other *Proteus* strains to DOC in 0.16 M saline at 37°C

Organism	Colistin pretreatment (500 μg/ml)**	% Viability* after exposure for 60 min to DOC (μg/ml)		
		125	250	1,000+
P. mirabilis 2896	No	92	88	72
	Yes	91	86	70
P. vulgaris 4175	No	89	88	68
	Yes	86	82	58
P. morganii 232	No	91	76	62
	Yes	90	75	58
P. mirabilis 4199	No	76	62	4.8
	Yes	70	41	0.34

* Viability at 0 min=100%
\+ With strains 2896, 4175 and 232, concentrations of DOC up to 10 mg/ml were no more effective against colistin-treated cells than against control suspensions.
** 50 μg/ml when *P. mirabilis* 4199 tested.

Fig. 2. Susceptibility to DOC in saline of colistin-pretreated cells of *P. mirabilis* 60 (very similar results were obtained with strain 4199).
(a) control (not pretreated with colistin), (b) cells pretreated with 50 μg/ml colistin.

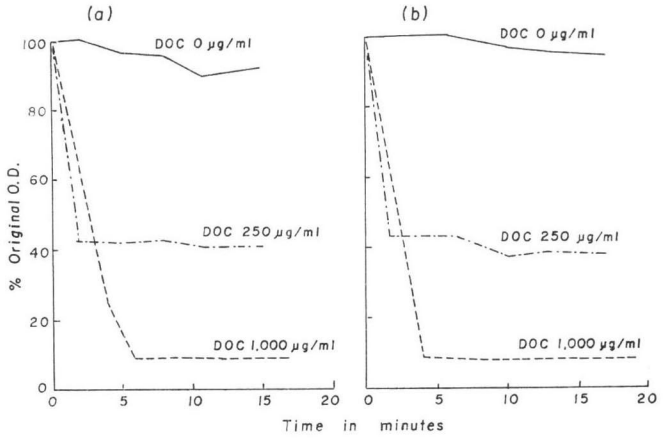


Fig. 3. Susceptibility to DOC in saline of colistin-pretreated cells of *P. morganii* 232.

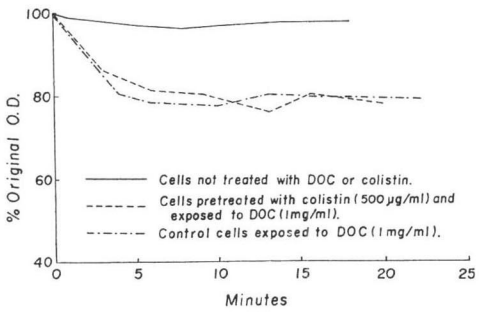
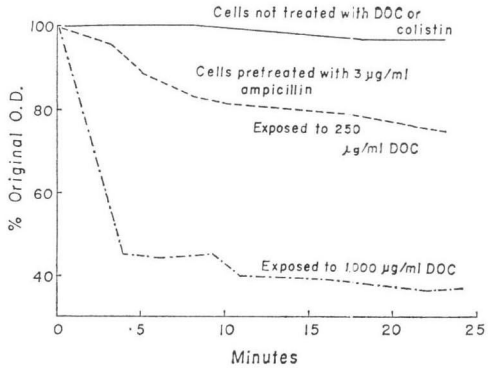


Fig. 4. Susceptibility to DOC in saline of ampicillin-pretreated cells of *P. vulgaris* 4175.
(Untreated cells were not lysed by 1,000 μg/ml DOC).



and Triton X-100, and to EDTA (0.01 M, pH 9). In no instance did cell lysis occur.

Control or colistin-pretreated cells of *P. mirabilis* NCTC 60 rapidly lost viability when suspended in water but not when held in 0.16 M sodium chloride solution.

Pretreated with Other Agents

Pretreatment of cell suspensions with EDTA (0.1 M, pH 9) for 5 minutes at 37°C did not render the cells susceptible to colistin (1,000 μg/ml). However, such pretreated cells of strains 60, 4199 and to a lesser extent, 2896, became rather more sensitive to tris; the extent of this sensitivity was less than with colistin-pretreated cells.

Pretreatment of cell suspensions with 200 μg/ml cetrimide for 60 minutes did not increase the sensitivity of the organisms to tris (pH 9) or DOC. Likewise, chlorhexidine (2.5 μg/ml)-pretreated *P. mirabilis* 60 was not rendered sensitive to tris (0.05 M, pH 9).

Pretreatment of cells with ampicillin (at 1/2 × the respective M.I.C.) and subsequent exposure to tris or DOC gave the following results:

(1) tris: some lysis of *P. mirabilis* strains 60 and 4199 occurred with 0.05 M tris at pH 9 and of

P. mirabilis 2896, *P. vulgaris* 4175 and *P. morganii* 232 with 0.2 M tris at pH 9;

(2) DOC: strains 60 and 4199 were rapidly lysed by DOC concentrations of 250~1,000 μg/ml (*vide* above also). Strains 232 and 2896 showed some lysis after exposure to 1,000 μg/ml DOC (decreases in O.D. over a 25-minute period were, respectively, 18% and 30%), whereas considerably more lysis of *P. vulgaris* 4175 was observed (Fig. 4).

Discussion

Proteus sp. are highly resistant to polymyxins[1,2,10] and this is borne out by the results in Table 1. The reasons for this high resistance are still unclear. SUD and FEINGOLD[1] have proposed that the composition of the envelope lipid is not responsible, but rather the accessibility of the lipids to these drugs, since polymyxins are known to interact with phosphate groups of phospholipids in the cytoplasmic membrane[4,11]. Combinations of polymyxin with a sulphonamide are active against *Proteus* sp.[7~9] and polymyxin B renders the cells of *P. mirabilis* sensitive to tris, DOC and osmotic shock[2]. It is not clear from the work of SUD and FEINGOLD[2], however, whether they found this effect with only one strain; moreover, only one concentration (20 μg/ml) of polymyxin B in liquid medium was apparently tested.

Of the nine strains of *Proteus* sp. tested in the present paper, only two became sensitive to tris; these two were sensitive to DOC irrespective of whether or not they had been pretreated with colistin. Thus, it seems likely that very significant differences must exist in the outer envelope layers between these two strains (60 and 4199) on the one hand and the remaining strains on the other. Studies with EDTA indicate the comparative resistance of all strains to this chelating agent, and thus differences between the strains are unlikely to be in the context of the cation composition of the envelope.

Polymyxins interact with the outer layers of *Pseudomonas aeruginosa*,[4] although damage is believed to result from their effect on the cytoplasmic membrane. This interaction with the outer layers may take place between the antibiotic and wall phospholipid[12] and/or with lipopolysaccharide[13,14]. Preliminary studies in this laboratory suggest that differences in envelope phospholipid may be responsible for the variations in strain response to colistin and that whilst some alternation to this phospholipid may occur in strains 60 and 4199, the cells still remain resistant to the lethal action of the antibiotic because insufficient of the drug is able to reach the cytoplasmic membrane. This alteration to the envelope could, however, be sufficient to render these cells susceptible to tris and osmotic shock. Cells of strains 60 and 4199 are susceptible in liquid suspension to DOC, and rather more so after they have been exposed to colistin (Tables 3 and 4). Lipopolysaccharide is broken into small fragments by DOC[15], but whether the present results indicate this to occur in whole cells is unproven.

SUD and FEINGOLD[2] have been shown that, following removal of the polymyxin, cell sensitivity to normally harmless agents is soon lost, and the results in this paper support this finding.

Pretreatment of cells with cetrimide or chlorhexidine did not increase the sensitivity of the organisms to tris (pH 9) or DOC. In view of the findings of SULING and O'LEARY[16], it is possible that both the surfactant and the other agent must be present at the same time for any synergistic action to occur.

Pretreatment of cells with ampicillin led to extension lysis of *P. vulgaris* 4175 on exposure to DOC (1 mg/ml). BURMAN and colleagues[17] using cholate concentrations of 3~15 mg/ml, generally, have found that ampicillin-treated cells of some Gram-negative strains are lysed by the cholate because of a distortion of the cytoplasmic membrane. Their proposal that the murein sacculus is either a part of the penetration barrier or is responsible for holding together the outer membrane structure could be relevant to this particular strain and to a lesser extent to the other strains.

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