Influence of Subinhibitory Concentrations of Disinfectants on Hydrophobicity, Alginate Production, and Motility of *Pseudomonas aeruginosa*

V. MAJTÁN and Ľ. MAJTÁNOVÁ

Institute of Preventive and Clinical Medicine, SK-833 01 Bratislava
e-mail: majtan@upkm.sk

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The ability of subinhibitory concentrations (sub-MICs) of disinfectant substances to interfere with some important aspects of bacterial cell function, such as surface hydrophobicity, production of alginate, and motility was investigated. Hydrophobicity of the tested *P. aeruginosa* strain was evaluated by the method of adherence to hydrocarbon – xylene and by the salt aggregation test of ammonium sulfate. All of the substances tested inhibited the adherence to xylene except for Hexaquart plus. From pure quaternary ammonium compounds (QACs) (Group A) the most effective substances were FD 312, Triquart, and Neoquat S mainly at 1/4 of the MIC. From QACs with other ingredients (Group B) the most effectively reduced adherence Lysoformin 3000, TPH 5225, and ID 212 in the whole concentration range. Sub-MICs of disinfectants had no significant effect on the production of alginate. Divoquat forte (from Group A) and Microbac forte (from Group B) reduced the excretion of this virulence factor at 1/4 of the MIC to 65.3 % and 75.6 %, respectively.

The inhibitory behaviour of both groups of disinfectant substances concerning motility was similar, while Triquart and Benzalkonium chloride (Group A) and Hexaquart S and Diesen forte (Group B) were the most effective at 1/4 of the MIC.

Pseudomonas aeruginosa is one of the most important opportunistic pathogens associated with nosocomial infections and is a leading cause of mortality among patients with immunosuppression, *e.g.* malignancy, cystic fibrosis, burns, and trauma [1]. Quaternary ammonium compounds (QACs) are widely used as disinfectants in hospital practice. They have several advantages over other commonly used disinfectants, such as noncorrosiveness, low toxicity, and high surface activity. However, several reports have described intrinsic and acquired resistance to these compounds, especially among gram-negative species [2—4]. In particular, *Pseudomonas* sp. have been shown to adapt to and grow in high concentrations of QACs [4, 5].

The activity of disinfectants against microorganisms depends on the nature and physiological condition of the organism itself and on the external physical environment. In a previous work we found that commercially manufactured disinfectants on the basis of QACs as active compounds, showed effective antibacterial activity against a clinical isolate of *P. aeruginosa* [6]. There is knowledge that sub-MICs of QACs and disinfectants are not without effect on bacteria, that even though they do not kill bacteria they are still able to modify the production of virulence factors of *P. aeruginosa* [7—9].

The aim of the present study was to investigate the ability of sub-MICs of commercially manufactured disinfectants containing QACs to interfere with hydrophobicity, production of alginate, and motility of *Pseudomonas aeruginosa.* These features are regarded as the important virulence factors of *P. aeruginosa* strains.

EXPERIMENTAL

P. aeruginosa strain was isolated from a patient suffering from nosocomial infection.

Disinfectant Substances

Group A contained QACs: Antibacteric (10 g dodecyldimethylbenzylammonium chloride, Tatrachema, Trnava, Slovak Republic), Antibacteric P (10 g dodecyldimethylbenzylammonium chloride, Tatrachema, Trnava, Slovak Republic), Benzalkonium chloride (alkyldimethylbenzylammonium chloride, Sigma, USA), Divoquat forte (1—8 g alkyl C_{14}—C_{16}, Diversey, Germany), FD 312 (13 g 50 % alkylbenzyldimethylammonium chloride, Bietigheim-Bissingen, Germany), Neoquat S (10 g dialkyldimethylammonium chloride, Weigert, Germany), Sokrena (7 g didodecyldimethylammonium chloride, Bode Chemie, Hamburg, Germany), Triquart (30—35 g alkylbenzyldimethylammonium chloride, Henkel Hygiene, Germany).

Group B contained QACs with other ingredients: Diesen forte (15 g alkyldimethylbenzylammo-

nium chloride, 2 g polyhexamethylenebiguanidinium chloride, 2 g 2-hydroxybiphenyl, Henkel Hygiene, Germany), Hexaquart plus (6.0 g didecyldimethylammonium chloride, 5.5 g dodecyldipropylenetriamine, 4.2 g biguanidinium acetate, B. Braun, Melsungen, Germany), Hexaquart S (3 g didecyldimethylammonium chloride, 7.6 g benzalkonium chloride, B. Braun, Melsungen, Germany), ID 212 (18.8 g 50 % alkylbenzyldimethylammonium chloride, Bietigheim-Bissingen, Germany), Lysoformin 3000 (15 g didecyldimethylammonium chloride, 2 g glutaraldehyde, 2 g glyoxal, Lysoform, Germany), Microbac forte (20 g benzalkonium chloride, 5 g dodecylbispropylidenetriamine, Bode Chemie, Hamburg, Germany), TPH 5225 (20 g benzalkonium chloride, 35 g phenoxypropanol, Schülke & Mayr, Germany).

Determination of Antibacterial Efficacy

The MIC of the disinfectant substances investigated was determined using a macrodilution method. To 9.7 cm^3 of Mueller—Hinton medium (Difco Laboratories, USA) supplemented with 25 mg dm^{-3} of Ca^{2+} and 12.5 mg dm^{-3} of Mg^{2+}, pH 7.2—7.4, in an L-shaped test tube 0.2 cm^3 of bacterial suspension ($A_{600} = 0.5$) and 0.1 cm^3 of the disinfectant were added. At the same time, the L-shaped test tube was used to measure spectrophotometrically the absorbance of the bacterial suspension. The lowest dilution of the disinfectant substance which inhibited bacterial growth was considered as the MIC.

Hydrophobicity Test

Surface hydrophobicity of both cells treated with 1/4, 1/8, and 1/16 MIC of the disinfectants and untreated cells was assessed. Two techniques were used: testing the bacterial adhesion to hydrocarbon (BATH) and the salt aggregation test (SAT).

BATH was performed as originally proposed by *Rosenberg et al.* [10]. The strain was considered hydrophobic when it expressed a percentage of adsorption to xylene of > 35 %.

SAT was performed according to the method of *Blanco et al.* [11] with ammonium sulfate solution (c/(mol dm^{-3}): 0.2, 0.4, 0.6, 0.8, 1.0, 1.4, and 2.0) in 0.2 mol dm^{-3} phosphate buffer at pH 6.8. The strain was considered hydrophobic when it aggregated in ≤ 1.4 mol dm^{-3} ammonium sulphate concentrations.

Alginate Test

The production of alginate was carried out by borate—carbazole method at 55 °C [12, 13]. 3 cm^3 of the H_2SO_4—borate reagent in glass tubes were equilibrated in an ice bath; 0.5 cm^3 of the sample after treatment with 1/4, 1/8, and 1/16 of the MIC of disinfectant was laid on the top and equilibrated again in the ice bath. After agitation with Vortex (4 s) and after equilibration in the ice bath, 0.1 cm^3 of carbazole solution was added to each tube. The samples were heated to 55 °C for 30 min and absorbance at $\lambda = 530$ nm was read.

Motility Test

The bacterial suspensions of both treated with sub-MICs of disinfectants and nontreated bacteria in PUM buffer ($A_{400} = 1.0$) were placed in the volume of 5 mm^3 on the agar surface of the semisolid swarming medium (1 % tryptone, 0.5 % NaCl, 0.25 % agar dissolved in distilled water, pH 7.1). The plates were incubated at 37 °C, viewed against a dark background and the diameters of the swarming zone were measured with a ruler after 18 h.

RESULTS

Changes in the cell surface hydrophobicity of *Pseudomonas aeruginosa* strain after incubation of the cells with disinfectant substances are shown in Table 1. All of the substances tested inhibited the adherence to xylene except for Hexaquart plus, which caused moderate stimulation. From Group A (pure QACs) the most effective substances were FD 312, Triquart and Neoquat S in the whole concentration range. However, their inhibitory activity was significantly higher at 1/4 of the MIC (1.8 %, 3.3 %, and 19.1 %, respectively). From Group B (QACs with other ingredients) the most effectively reduced adherence of *P. aeruginosa* Lysoformin 3000, TPH 5225, and ID 212 in all concentrations tested. Again the 1/4 of the MIC of these substances induced the greatest inhibition of adherence (1.2 %, 3.2 %, and 8.4 %, respectively) against the control value. In addition, the results of influence of adherence to xylene correlated with results of SAT assay.

The effect of disinfectant substances on the production of alginate in *P. aeruginosa* is shown in Table 2. This important virulence factor was not greatly influenced after exposure to their sub-MICs. Inhibition was found with Divoquat forte at 1/4 of the MIC (65.3 %) from Group A and with Microbac forte also at 1/4 of the MIC (75.6 %) from Group B. Antibacteric P, Benzalkonium chloride, Triquart, Hexaquart plus, and TPH 5225 moderately stimulated the alginate production in the all sub-MICs tested. Other substances tested caused only poor inhibition of this virulence factor.

Table 3 reports the findings of the influence of substances studied on motility of *P. aeruginosa*. Migration of *P. aeruginosa* through semisolid agar induced formation of concentric bands which varied in size and rate of growth in dependence on substance and concentration. An expressive inhibitory effect on

Table 1. Effect of Sub-MICs of Disinfectant Substances on the Cell Surface Hydrophobicity of *P. aeruginosa*

Disinfectant	ρ / mg dm^{-3}	Fraction of MIC	Adherence to xylene/% (mean ± SD)	Adherence related to untreated cells/%	SAT $c(NH_4)_2SO_4$ / mol cm^{-3}
Group A					
Antibacteric		0	66.6 ± 2.5	100	0.8
	0.78	1/16	54.9 ± 7.7	82.4	1.0
	1.56	1/8	28.1 ± 2.0	42.2	1.0
	3.12	1/4	23.4 ± 0.6	35.1	1.4
Antibacteric P		0	65.6 ± 3.1	100	0.8
	0.19	1/16	53.3 ± 2.8	81.3	1.0
	0.39	1/8	48.8 ± 0.5	74.3	1.0
	0.78	1/4	12.1 ± 1.7	18.4	0.8
Benzalkonium chloride		0	59.6 ± 0.8	100	0.8
	0.19	1/16	54.0 ± 0.2	90.6	0.8
	0.39	1/8	35.0 ± 1.4	58.7	0.8
	0.78	1/4	29.8 ± 1.1	50.0	1.0
Divoquat forte		0	66.2 ± 2.5	100	0.8
	0.19	1/16	51.3 ± 3.0	77.5	0.8
	0.39	1/8	49.7 ± 0.9	75.1	0.8
	0.78	1/4	46.4 ± 0.8	70.1	1.0
FD 312		0	59.9 ± 2.6	100	0.8
	6.25	1/16	30.9 ± 0.1	51.6	1.0
	12.5	1/8	11.2 ± 0.1	18.7	1.4
	25	1/4	1.1 ± 0.2	1.8	1.4
Neoquat S		0	68.8 ± 1.6	100	0.8
	0.39	1/16	26.1 ± 0.9	38.0	0.8
	0.78	1/8	21.1 ± 2.4	30.7	0.8
	1.56	1/4	13.1 ± 5.5	19.1	1.0
Sokrena		0	68.8 ± 1.2	100	0.8
	0.39	1/16	58.2 ± 0.4	84.6	0.8
	0.78	1/8	35.0 ± 1.1	50.9	0.8
	1.56	1/4	36.4 ± 2.0	52.9	1.0
Triquart		0	64.3 ± 6.6	100	0.8
	1.56	1/16	14.4 ± 1.7	22.3	1.0
	3.12	1/8	11.0 ± 1.8	17.1	1.0
	6.25	1/4	2.1 ± 0.5	3.3	1.4
Group B					
Diesen forte		0	66.6 ± 2.4	100	0.8
	0.78	1/16	54.2 ± 0.6	81.4	0.8
	1.56	1/8	48.1 ± 0.7	72.2	0.8
	3.12	1/4	47.4 ± 0.8	71.2	1.0
Hexaquart plus		0	65.6 ± 1.6	100	0.8
	0.19	1/16	67.8 ± 1.6	103.6	0.8
	0.39	1/8	69.6 ± 3.0	106.1	0.8
	0.78	1/4	67.5 ± 3.5	102.9	1.4
Hexaquart S		0	66.3 ± 0.4	100	0.8
	1.56	1/16	48.7 ± 0.7	73.5	1.0
	3.12	1/8	52.2 ± 0.3	78.7	1.0
	6.25	1/4	1.7 ± 0.4	2.6	1.4
ID 212		0	65.3 ± 1.4	100	0.8
	6.25	1/16	24.4 ± 0.2	37.4	1.0
	12.5	1/8	19.4 ± 0.6	29.7	1.4
	25	1/4	5.5 ± 0.9	8.4	1.4
Lysoformin 3000		0	60.5 ± 0.9	100	0.8
	1.56	1/16	13.4 ± 0.8	22.1	1.4
	3.12	1/8	7.2 ± 1.9	11.9	1.4
	6.25	1/4	0.7 ± 0.5	1.2	1.4
Microbac forte		0	66.3 ± 0.4	100	0.8
	1.56	1/16	33.6 ± 3.5	50.6	1.0
	3.12	1/8	47.2 ± 5.6	71.1	1.4
	6.25	1/4	28.3 ± 1.8	42.7	1.4
TPH 5225		0	64.3 ± 6.6	100	0.8
	0.78	1/16	14.4 ± 1.7	22.4	1.0
	1.56	1/8	11.0 ± 1.8	17.1	1.0
	3.12	1/4	2.1 ± 0.5	3.2	1.0

Table 2. Effect of Sub-MICs of Disinfectant Substances on the Production of Alginate in *P. aeruginosa*

Disinfectant	Fraction of MIC	Production of alginate	
		ρ(mean ± SD) / mg dm^{-3}	%
Group A			
Antibacteric	0	80.5 ± 3.4	100
	1/16	82.1 ± 5.1	102.8
	1/8	81.3 ± 1.8	100.9
	1/4	81.3 ± 3.2	100.9
Antibacteric P	0	79.1 ± 4.8	100
	1/16	73.2 ± 1.2	92.6
	1/8	71.3 ± 4.1	90.2
	1/4	69.6 ± 3.2	88.1
Benzalkonium chloride	0	76.2 ± 4.4	100
	1/16	80.0 ± 1.3	104.9
	1/8	77.5 ± 3.3	101.7
	1/4	78.0 ± 4.0	102.3
Divoquat forte	0	84.2 ± 0.8	100
	1/16	81.5 ± 3.8	96.8
	1/8	69.5 ± 4.5	82.5
	1/4	55.0 ± 8.2	65.3
FD 312	0	80.9 ± 2.8	100
	1/16	87.4 ± 5.1	108.1
	1/8	77.9 ± 4.4	96.3
	1/4	61.7 ± 3.8	76.3
Neoquat S	0	84.6 ± 11.8	100
	1/16	80.4 ± 7.1	95.0
	1/8	76.0 ± 5.8	89.8
	1/4	72.6 ± 4.4	85.8
Sokrena	0	80.6 ± 2.3	100
	1/16	69.9 ± 4.8	86.8
	1/8	77.2 ± 1.2	95.8
	1/4	75.0 ± 2.5	93.1
Triquart	0	83.2 ± 2.4	100
	1/16	84.0 ± 1.1	100.9
	1/8	83.8 ± 4.0	100.7
	1/4	84.1 ± 1.5	101.0
Group B			
Diesen forte	0	81.3 ± 4.2	100
	1/16	69.5 ± 3.3	85.6
	1/8	66.1 ± 2.1	81.4
	1/4	62.9 ± 1.3	77.4
Hexaquart plus	0	82.6 ± 4.2	100
	1/16	82.7 ± 3.1	100.1
	1/8	84.5 ± 1.8	102.3
	1/4	85.0 ± 5.1	102.9
Hexaquart S	0	78.8 ± 1.3	100
	1/16	80.7 ± 1.3	101.5
	1/8	71.9 ± 3.5	91.3
	1/4	70.9 ± 2.0	90.1
ID 212	0	84.0 ± 3.1	100
	1/16	79.1 ± 4.5	94.2
	1/8	73.0 ± 2.2	87.0
	1/4	81.0 ± 5.7	96.5
Lysoformin 3000	0	77.6 ± 3.3	100
	1/16	75.1 ± 1.8	96.8
	1/8	75.7 ± 1.1	97.6
	1/4	61.3 ± 4.0	79.0
Microbac forte	0	79.3 ± 4.1	100
	1/16	81.0 ± 1.9	102.1
	1/8	62.1 ± 3.1	78.4
	1/4	59.9 ± 3.7	75.6
TPH 5225	0	82.8 ± 2.5	100
	1/16	83.0 ± 2.3	100.2
	1/8	84.1 ± 2.1	101.5
	1/4	84.0 ± 2.1	101.4

the motility was observed with Triquart and Benzalkonium chloride from Group A at 1/4 of the MIC. From Group B the inhibition of motility was found with Hexaquart S and Diesen forte also at 1/4 of the MIC. We can state that exposure of the bacterial cells to sub-MICs of all disinfectant substances resulted in inhibition of motility of *P. aeruginosa* compared to the unexposed cells.

DISCUSSION

Disinfectants containing QACs belong to the basic disinfectants [14, 15]. The disinfectants in this study contained either only QACs (8 substances) or QACs with other ingredients (7 substances). Despite the fact that resistance to disinfectants based on QACs is widespread among both clinical gram-positive [16] and gram-negative [17] strains, we found the relatively high antibacterial activity of disinfectants on the clinical isolates of *P. aeruginosa* [6], *S. typhimurium* [18], and *E. cloacae* [19].

Many antimicrobials at levels below their minimal inhibitory concentrations (sub-MICs) may induce changes in the properties of bacteria both *in vitro* and *in vivo* and consequently, may affect bacterial virulence [20—22]. It is known that the bacterial adherence is an important phenomenon contributing to the virulence of bacteria, which is associated with hydrophobicity [23, 24]. Bacterial adhesion increased with increasing bacterial hydrophobicity and *vice versa* [25, 26].

The obtained results showed that disinfectants tested (except Hexaquart plus) reduced surface hydrophobicity of *P. aeruginosa* at sub-MICs. Previous studies have demonstrated that exposure to low concentrations of QACs with carbon chain induced the decrease of the surface hydrophobicity of *Enterobacter cloacae* [27]. On the other hand, nitroxoline at subinhibitory concentrations significantly enhanced cell surface hydrophobicity of *Escherichia coli* [28].

Mucoid *P. aeruginosa* strains producing alginate are associated mainly with cystic fibrosis patients [29]. This acetylated exopolysaccharide is perhaps the most important virulence factor of *P. aeruginosa* strains infecting those patients. The disinfectants studied poorly inhibited the production of alginate at sub-MICs, or some substances such as Antibacteric, Benzalkonium chloride, Triquart, Hexaquart plus, Microbac forte, and TPH 5225 did not affect its production. The disinfectant substances tested in this study are used in hospital environment and therefore this fact, which, however, needs further study, is remarkable because it contributes to pathogenesis of *P. aeruginosa*. In a previous study we found that alginate was leastly inhibited after treatment with sub-MICs of quaternary bisammonium salts compared to another virulence factors of *P. aeruginosa* – elastase, proteinase, phospholipase C [30]. Alginates in

Table 3. Effect of Sub-MICs of Disinfectant Substances on the Motility of *P. aeruginosa*

Disinfectant	Fraction of MIC	Motility after 18 h (mean ± SD)/mm
Group A		
Antibacteric	0	74.0 ± 1.7
	1/16	72.0 ± 3.5
	1/8	63.2 ± 1.3
	1/4	65.5 ± 0.8
Antibacteric P	0	72.2 ± 0.4
	1/16	70.7 ± 1.3
	1/8	70.2 ± 0.4
	1/4	66.0 ± 3.5
Benzalkonium chloride	0	72.7 ± 2.2
	1/16	55.0 ± 1.7
	1/8	55.7 ± 1.3
	1/4	38.2 ± 2.2
Divoquat forte	0	74.2 ± 2.2
	1/16	75.2 ± 2.2
	1/8	66.0 ± 0.8
	1/4	48.2 ± 1.7
FD 312	0	70.7 ± 0.4
	1/16	58.5 ± 2.6
	1/8	52.7 ± 1.3
	1/4	44.5 ± 0.8
Neoquat S	0	76.2 ± 2.2
	1/16	71.0 ± 0.0
	1/8	51.2 ± 2.2
	1/4	46.2 ± 2.2
Sokrena	0	79.2 ± 0.4
	1/16	77.5 ± 4.4
	1/8	52.2 ± 2.2
	1/4	44.2 ± 2.2
Triquart	0	72.2 ± 1.3
	1/16	50.5 ± 2.6
	1/8	44.5 ± 2.6
	1/4	36.2 ± 2.2
Group B		
Diesen forte	0	71.0 ± 0.0
	1/16	76.0 ± 1.7
	1/8	48.7 ± 2.2
	1/4	34.7 ± 1.3
Hexaquart plus	0	72.2 ± 0.4
	1/16	68.2 ± 1.3
	1/8	66.0 ± 0.0
	1/4	61.2 ± 2.2
Hexaquart S	0	71.0 ± 1.7
	1/16	42.7 ± 3.1
	1/8	34.2 ± 2.2
	1/4	25.5 ± 0.8
ID 212	0	74.5 ± 2.6
	1/16	71.0 ± 1.7
	1/8	66.7 ± 2.2
	1/4	51.0 ± 1.7
Lysoformin 3000	0	72.0 ± 1.7
	1/16	63.0 ± 1.7
	1/8	57.2 ± 1.3
	1/4	48.5 ± 0.8
Microbac forte	0	73.2 ± 2.2
	1/16	66.5 ± 2.6
	1/8	61.7 ± 3.1
	1/4	46.5 ± 4.4
TPH 5225	0	73.5 ± 2.6
	1/16	55.0 ± 3.5
	1/8	52.5 ± 0.8
	1/4	47.2 ± 1.3

Results of motility are expressed as the diameter (± SD) of growth zone in the soft agar plate.

the presence of calcium form a gel that surrounds the *P. aeruginosa* cells with a matrix that may shelter large numbers of microcolonies from host defenses and protect them from charge of antimicrobial substances [31]. For determination of MICs of substances studied against *P. aeruginosa* the cultivation medium supplemented with Ca^{2+} was used (see Experimental) and this maybe affected the effect of their sub-MICs on alginate production. In this connection it is interesting that alginate encapsulation improved survival of *P. aeruginosa* in soil treated with disinfectant except formaldehyde [32].

Motility is an important virulence factor which contributes substantially to the invasive capabilities of several organisms. The inhibition of motility reduces the possibility of forming new colonies and spreading the infection away from the first point of contact. Disinfectant substances tested were pure QACs or QACs with other ingredients suppressing the motility of *P. aeruginosa*. Such reduction in motility may be due to the absence of flagella [33] in which the protein (flagellin) synthesis was inhibited during the bacterial growth in the presence of these compounds.

The fact that concentrations of disinfectant substances on the basis of QACs, which are lower than those recommended by the manufacture for practical use, are still able to interfere with bacterial cell functions extends the knowledge about the efficacy of this type of compounds on an important nosocomial pathogen *P. aeruginosa*.

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