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The effect of disinfectants on *Plesiomonas shigelloides*

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The effects of ten commercially available disinfectants on virulence associated properties of *Plesiomonas shigelloides* were tested. All the disinfectants tested contained quaternary ammonium salts. The majority of the disinfectants when used at subinhibitory concentrations increased surface hydrophobicity as evaluated by bacterial adherence to xylene and decreased bacterial motility in a concentration dependent manner. Disinfectants did not significantly affect lipase activity. However, more than half of the antimicrobials tested increased the resistance of bacteria to hydrogen peroxide. The disinfectants, in a similar manner to antibiotics at concentrations below MIC, interfered with potential virulence factors of *Plesiomonas shigelloides*.

1. Introduction

Plesiomonas shigelloides is a new member of the family of *Enterobacteriaceae* (previously included in the family of *Vibrionaceae*) and belongs to the group of "new" emerging pathogens. These bacteria are commonly isolated from an aquatic environment that is the primary reservoir [1–4], but also they have been isolated from animal and human biological samples. *P. shigelloides* is most commonly associated with gastroenteritis [5–8], but it has been found as the causative agent of various types of extraintestinal infections often in immunosuppressed patients [9–11]. Resistance of this species to antimicrobials has been found mainly with penicillins and aminoglycosides [4, 12, 13].
Though antibiotics at subinhibitory concentrations (sub-MICs) do not kill bacteria they may modulate important bacterial characteristics, including virulence factors. They can potentially affect the course of bacterial infection [14–17]. Less data and information are available in the literature concerning the effect of disinfectants at sub-MIC on bacteria, [18, 19]. Quaternary ammonium salts (QASs) are frequently used as disinfectants. The binding of these membrane active substances to the cytoplasmic membrane interferes with the cell metabolism [20].
The aim of this study was to determine whether sub-MIC concentrations of disinfectants with quaternary ammonium salts as the active components could affect some virulence associated properties of a *P. shigelloides* strain.

2. Investigations and results

The effects of 10 commercially available disinfectants at subinhibitory concentrations on some characteristics of a *P. shigelloides* strain were tested. Table 1 summarises the main composition characteristics and MIC values of the disinfectants. The MICs of the disinfectants showed a wide range of values between 12.5 mg/l and 800 mg/l.

Eight disinfectants, among them Almyrol®, the most effective at all concentrations tested, increased surface hydrophobicity of bacterial cells (Table 2). The bacterial surface hydrophobicities after treatment with disinfectants were in the range of 108.4%–118.6% (1/4 of MICs), 103.9%–136.0% (1/8 of MICs) and 108.2%–135.4% (1/16 of MICs) in comparison to nontreated bacteria. On the other hand, the hydrophobicity expressed as bacterial adherence to xylene was effectively decreased by Divoquard® forte at all concentrations tested (to 38.2%–69.3% of the control value) and by Hexaquart® L at two concentrations, to 67.7% (1/4 of MIC) and 87.2% (1/16 of MIC) of the control value.
The disinfectants Areades® B, Hexaquart L and Salvanos® plus, and also Almyrol, Desam® GK, and Lysoformin® 3000 at two concentrations and Divoquard forte at one concentration respectively, decreased bacterial cell motility. The most significant reduction of motility was observed with Almyrol at a concentration of 1/16 of MIC (to 65.2% of the control value), and also with Divoquard forte at 1/4 of MIC (to 67.6%), Hexaquart L at 1/4 of

Table 1: Composition of disinfectants and their MICs values for *P. shigelloides*

Disinfectants	Active substances	MIC (mg/l)
Divoquard® forte	QASs	25
Hexaquart® L	QASs	100
Salvanos® plus	QASs	50
Sokrena®	QASs	50
Desam® GK	QASs, aldehyde	800
Fordesin®	QASs, aldehyde	25
Lysoformin® 3000	QASs, aldehyde	12.5
Almyrol®	QASs, biquanide	25
Areades® B	QASs, biquanide	50
Loritodes® GA 303	QASs, alcohol	50

Table 2: The effects of the disinfectants on some properties of *P. shigelloides*

Disinfectant	Fraction of MIC	BATH*	Motility (mm)	Lipase (U/ml)	H_2O_2*** sensitivity
Divoquard® forte	0	49.5 ± 0.4 (100)**	20.7 ± 0.6 (100)	34.5 ± 0.8 (100)	38.7 ± 2.9 (100)
	1/16	34.3 ± 0.3 (69.3)	22.3 ± 0.6 (107.7)	36.5 ± 0.3 (105.8)	34.0 ± 1.2 (87.8)
	1/8	28.6 ± 0.4 (57.8)	27.7 ± 1.2 (133.8)	34.8 ± 0.4 (100.9)	31.7 ± 2.9 (81.9)
	1/4	18.9 ± 0.4 (38.2)	14.0 ± 1.2 (67.6)	36.4 ± 0.4 (105.5)	36.3 ± 0.6 (93.8)
Hexaquart® L	0	50.8 ± 0.3 (100)	18.3 ± 0.6 (100)	34.8 ± 0.7 (100)	42.0 ± 1.2 (100)
	1/16	44.3 ± 0.4 (87.2)	17.7 ± 0.6 (96.7)	35.8 ± 0.5 (102.9)	41.7 ± 0.6 (99.3)
	1/8	64.3 ± 0.2 (126.6)	15.3 ± 0.6 (83.6)	35.1 ± 0.6 (100.9)	42.0 ± 1.2 (100)
	1/4	34.4 ± 0.4 (67.7)	13.7 ± 0.6 (74.9)	35.8 ± 0.6 (102.9)	40.0 ± 0 (95.2)
Salvanos® plus	0	52.0 ± 0.5 (100)	23.0 ± 0 (100)	32.8 ± 0.6 (100)	36.7 ± 0.6 (100)
	1/16	75.7 ± 0,3 (145.6)	22.7 ± 0.6 (98.7)	33.2 ± 0.7 (101.2)	37.0 ± 1.2 (100.8)
	1/8	65.5 ± 0.4 (126.0)	20.3 ± 0.6 (88.3)	31.7 ± 0.4 (96.6)	37.3 ± 0.6 (101.6)
	1/4	60.2 ± 0.2 (115.8)	20.7 ± 0.6 (90.0)	32.9 ± 0.9 (100.3)	37.0 ± 0 (100.8)
Sokrena®	0	50.9 ± 0.3 (100)	20.0 ± 1.2 (100)	32.4 ± 0.7 (100)	35.7 ± 1.2 (100)
	1/16	63.4 ± 0.4 (124.9)	20.3 ± 0.6 (101.5)	31.2 ± 0.8 (96.3)	36.0 ± 1.2 (100.8)
	1/8	61.4 ± 0.3 (120.6)	19.7 ± 0.6 (98.5)	31.6 ± 0.8 (97.5)	37.7 ± 1.2 (105.6)
	1/4	56.8 ± 0.4 (111.6)	19.7 ± 0.6 (98.5)	32.1 ± 0.6 (99.1)	37.0 ± 0 (103.6)
Desam® GK	0	51.9 ± 0.3 (100)	22.3 ± 0.6 (100)	34.4 ± 1.2 (100)	27.0 ± 0 (100)
	1/16	65.8 ± 0.3 (126.8)	22.0 ± 1.2 (98.6)	35.5 ± 0.6 (103.2)	22.7 ± 0.6 (84.1)
	1/8	64.8 ± 0.5 (124.8)	25.7 ± 1.2 (115.2)	34.2 ± 0.5 (99.4)	24.7 ± 1.8 (91.5)
	1/4	59.1 ± 0.1 (113.9)	21.3 ± 0.6 (95.5)	35.8 ± 0.5 (104.1)	21.0 ± 1.2 (77.8)
Fordesin®	0	51.6 ± 0.4 (100)	25.7 ± 0.6 (100)	32.3 ± 0.8 (100)	28.0 ± 1.2 (100)
	1/16	65.2 ± 0.4 (126.3)	25.7 ± 0.6 (100)	30.4 ± 0.6 (94.1)	25.7 ± 1.2 (91.8)
	1/8	64.3 ± 0.4 (124.6)	25.3 ± 0.6 (98.4)	29.0 ± 0.5 (89.8)	26.0 ± 1.8 (92.8)
	1/4	59.7 ± 0.3 (115.7)	22.3 ± 0.6 (86.8)	30.7 ± 0.8 (95.0)	26.7 ± 1.2 (95.3)
Lysoformin® 3000	0	51.0 ± 0.2 (100)	26.3 ± 1.8 (100)	36.6 ± 0.8 (100)	37.7 ± 0.6 (100)
	1/16	55.2 ± 0.3 (108.2)	26.3 ± 0.6 (100)	36.2 ± 0.6 (98.9)	35.7 ± 0.6 (94.7)
	1/8	53.0 ± 0.3 (103.9)	21.0 ± 1.2 (79.8)	36.3 ± 0.6 (99.2)	36.3 ± 1.2 (96.3)
	1/4	55.3 ± 0.5 (108.4)	19.0 ± 0 (72.2)	34.5 ± 0.3 (94.3)	35.7 ± 0.6 (94.7)
Almyrol®	0	50.5 ± 0.3 (100)	25.0 ± 1.2 (100)	33.3 ± 0.6 (100)	38.0 ± 1.8 (100)
	1/16	68.4 ± 0.3 (135.4)	16.3 ± 0.6 (65.2)	35.3 ± 0.6 (106.0)	36.0 ± 1.2 (94.7)
	1/8	68.7 ± 0.4 (136.0)	20.3 ± 0.6 (81.2)	34.1 ± 0.3 (102.4)	37.7 ± 1.2 (99.2)
	1/4	59.9 ± 0.3 (118.6)	25.0 ± 0 (100)	35.7 ± 0.6 (107.2)	34.7 ± 0.6 (91.3)
Areades® B	0	51.9 ± 0.1 (100)	22.0 ± 1.2 (100)	33.8 ± 0.7 (100)	35.3 ± 0.6 (100)
	1/16	65.1 ± 0.4 (125.4)	21.0 ± 0 (95.4)	34.8 ± 0.6 (103.0)	36.3 ± 1.8 (102.8)
	1/8	61.8 ± 0.4 (119.1)	21.7 ± 0.6 (98.6)	34.6 ± 0.7 (102.4)	35.7 ± 0.6 (101.1)
	1/4	59.4 ± 0.4 (114.4)	21.0 ± 1.2 (95.4)	35.6 ± 0.6 (105.3)	36.3 ± 1.8 (102.8)
Loritodes®GA, 303	0	51.3 ± 0.4 (100)	28.7 ± 1.2 (100)	32.5 ± 0.7 (100)	41.3 ± 0.6 (100)
	1/16	67.9 ± 0.3 (132.3)	28.3 ± 0.6 (98.6)	30.9 ± 0.6 (95.1)	44.0 ± 1.2 (106.5)
	1/8	67.0 ± 0.4 (130.6)	29.3 ± 1.8 (102.1)	29.5 ± 0.6 (90.8)	47.0 ± 0.6 (113.8)
	1/4	59.4 ± 0.4 (115.8)	27.7 ± 1.8 (96.5)	30.0 ± 0.6 (92.3)	48.7 ± 0.6 (117.9)

* Percentage decrease in absorbance of the lower aqueous phase compared with that of the original suspension
** Percentage BATH, motility, lipase and H_2O_2 sensitivity in parentheses
*** The diameter of the zone of inhibited growth

MIC (to 74.9%) and Lysoformin 3000 at 1/4 of MIC (to 72.2%). A marked increase in motility was found only after treatment with Divoquard forte at 1/8 of MIC (to 133.8% of that of nontreated bacteria). Motility of bacterial cells after using other concentrations of disinfectants were in the ranges of 86.8%–100% (1/4 of MICs), 78.8%–115.2% (1/8 of MICs) and 95.4%–107.7% (1/16 of MICs) of the control values.

With regard to lipase activity, Almyrol, Areades B, Divoquard forte and Hexaquart L at three concentrations tested, and Desam GK and Salvanos plus at two concentrations only slightly enhanced the activity of this enzyme. The maximum increase was found after treatment with Almyrol at 1/4 of the MIC to 107.2% of the control value. Lipase activity after application of other concentrations of disinfectants was in the range of 100.3%–106% in comparison to nontreated bacteria. Similarly, reduction of the enzyme activity of treated bacteria with Lysoformin 3000, Fordesin®, Sokrena®, Loritodes® GA 303 at all concentrations and with Desam GK at one concentration was weak. Fordesin at 1/8 of the MIC decreased lipase activity to 89.8% of the control value.

More than half of the disinfectants (Almyrol, Divoquard forte, Lysoformin 3000, Desam GK and Fordesin at three concentrations and Hexaquart L in one concentration) decreased the sensitivity of bacterial cells to hydrogen peroxide, i.e. treated cells were more resistant to the effect of reactive oxygen species. The most effective decrease of sensitivity to hydrogen peroxide as compared with control was observed after use of Desam GK at 1/4 and 1/16 of MIC (to 77.7% and 84.1% respectively) as well as with Divoquard forte at 1/8 and 1/16 of MIC (to 81.9% and 87.8%). Reduction of sensitivity of bacterial cells to hydrogen peroxide after treatment with the other concentrations of the disinfectants was less pronounced, ranging from 91.3% to 99.3% of control values. On the other hand, Sokrena, Loritodes GA 303, Salvanos plus and Areades B at all concentrations slightly enhanced sensitivity, i.e. they de-

creased the resistance of bacterial cells to hydrogen peroxide. The total increase of sensitivity was in the range of 100.8%–117.9% as compared with control values.
The *P. shigelloides* strain did not produce short chain unsubstituted homoserine lactones and none of the disinfectants tested initiated their production.

3. Discussion

As a potential etiological agent, mainly of gastroenteritis, *P. shigelloides* might produce several pathogenic and virulence factors, including enterotoxins, endotoxin, hemolysin and invasive factors. They could participate in the pathogenesis of both gastrointestinal and extraintestinal infections [21–23]. Additional factors as motility, surface hydrophobicity, response to oxidative stress and enzyme lipase might also play a role in the process of interaction with the host.
The majority of disinfectants tested in our study at sub-MICs increased the surface hydrophobicity of bacterial cells. Only two (Divoquard forte and Hexaquart L) out of the 10 tested reduced bacterial adherence to xylene. There is a divergence among the literature data concerning the hydrophobicity of bacteria treated with antimicrobials. In these studies, hydrophobicity, an important parameter in the nonspecific adhesion of bacteria to interfaces, was affected after treatment with antimicrobial agents. However the effect of the agents dependend on bacterial species, antimicrobial agent and concentration. Some authors have reported reduced surface hydrophobicity of antimicrobial-treated bacteria [15, 17, 18, 24, 25], while others have found enhanced bacterial adherence [26, 27]. In some cases, the fall in bacterial surface hydrophobicity was associated with changes in bacterial structures such as outer membrane proteins or lipopolysaccharide [16]. It is evident that different antimicrobials may selectively modulate the bacterial surface hydrophobicity.
Swimming motility, as one form of flagella-dependent bacterial translocation, may play an role in the pathogenicity and virulence of bacteria. Flagella contribute to bacterial movement and may therefore promote progression from local to systematic infections [28]. The majority of the disinfectants tested in our experiments reduced the motility of *P. shigelloides*. The extent of the decrease dependend on the disinfectant and its concentration. Almyrol, Divoquard forte, Hexaquart L and Lysoformin 3000 at certain concentrations were most effective in reducing motility. Similarly, inhibition of motility after treatment with antibiotics has also been found in other bacteria [17, 29].
No appreciable changes were found in the lipolytic activity of *P. shigelloides* treated with the disinfectants. Enzyme activity was slightly increased or weakly suppressed. The data of Molinari et al. [29] who found that macrolide antibiotics did not significantly change lipase activity in *Pseudomonas aeruginosa* are in agreement with our results. Also the lipase activity of *Acinetobacter baumannii* was only weakly modified after treatment with imipenem [30]. On the other hand, some *Klebsiella* strains treated with imipenem have shown increased enzyme activity [31]. Though the lipolytic activity of some bacteria may participate in the pathogenesis of diseases [32], a possible role of *P. shigelloides* lipase as a virulence factor is not yet clear.
Aerobic bacteria produce several endogenous harmful by-products, including hydrogen peroxide, which at certain intracellular concentrations can be detoxified by protective substances [33, 34]. When these harmful agents exceed an acceptable concentration, oxidative stress leading to bacterial viability loss will occur [35]. Six disinfectants out of the ten tested increased the resistance of bacteria to hydrogen peroxide to different extents in comparison with the untreated bacteria. Desam GK and Divoquard forte were the most efficient in this respect. On the other hand, bacterial cells treated with the other disinfectants showed slightly increased sensitivity on exposure to hydrogen peroxide.
Sofer et al. [36] showed that erythromycin at sub-MICs simultaneously suppressed the production of *Pseudomonas aeruginosa* hemagglutinins, protease, hemolysin and homoserine lactone autoinducers. In our case, *P. shigelloides* did not exhibit production of short chained unsubstituted AHLs and the cultivation of bacteria with disinfectants did not evoke their production. However, it is possible that *P. shigelloides* may produce other types of signaling autoinducers which can control the studied activities of *P. shigelloides*.
In conclusion, the majority of multicomponent disinfectants with QASs as active substances increased surface hydrophobicity and decreased bacterial motility at sub-MICs. Only slight changes were shown in lipolytic activity. *P. shigelloides* treated with six disinfectants showed a higher resistance to hydrogen peroxide. No disinfectants provoked production of AHLs. Even at sub-MICs disinfectants modified some bacterial properties, similarly to antibiotics.

4. Experimental

4.1. Materials

Bacterial strain: *P. shigelloides* O7 H40 was isolated from a patient suffering from diarrhoea. Serotyping of the isolate was performed according to the International Antigenic Scheme using specific anti-*Plesiomonas* sera [37].
Disinfectants used in the study: Almyrol®, Fordesin®, Lysoformin® 3000, (Lysoform, Berlin, Germany), Sokrena® (Bode Chemie, Hamburg, Germany), Loritodes® GA 303 (Otto Oehme GmbH, Allesberg, Germany), Areades® B (Arcana Hygienesysteme GmbH, Vienna, Austria), Hexaquart® L (B/Braun Medical AG, Switzerland), Salvanos® plus (I.N.D.I.A. Industrie Chimiche S.p.A. Padova, Italy), Divoquard® forte (Unilever Magyarország, Rakospalota, Hungary), Desam® GK (Bochemie a.s., Bohumín, Czech Republic).
Medium: Mueller-Hinton broth supplemented with (25 mg/l Ca^{2+} and 12.5 mg/l Mg^{2+}-MHB) was used in all experiments.

4.2. Methods

4.2.1. Minimal inhibitory concentration (MIC)

The macrodilution broth method with two-fold serial dilutions of disinfectants was used. The lowest dilution of the disinfectants allowing no visible growth after a 24 h incubation at 37 °C was taken as the MIC.

4.2.2. Cultivation of bacterial strain with disinfectants

Bacterial suspension (0.2 ml, $A_{600} = 0.5$) in MHB (9.7 ml) after addition of 0.1 ml of disinfectants at concentrations of 1/4, 1/8 and 1/16 of the MICs were incubated for 24 h at 37 °C. The cells obtained after centrifugation of the bacterial suspensions were washed and used for determination of acylated homoserine lactones and after adjusting for an absorbance of 1.0 for hydrophobicity and motility assays. The culture filtrates obtained after centrifugation of the bacterial suspensions were sterilized (0.22 μm, Millipore) and used for lipase assay.

4.2.3. Cell surface hydrophobicity

The surface hydrophobicity of bacteria was evaluated by their adherence to the hydrocarbon xylene (BATH) [38]. In the assay, bacterial cultures (4 ml) were vortexed with xylene (1 ml) for 60 s and then incubated for 30 min at 37 °C. After phase separation, hydrophobicity was determined as a percentage decrease in the absorbance of the lower aqueous phase as compared with the absorbance of the initial cell suspension. Strains were considered hydrophobic when they expressed a percentage of adherence to hydrocarbon of ≥35% [39].

4.2.4. Motility

Bacterial suspensions (5 µl, $A_{400} = 1$) were inoculated on the surface of semi-solid agar (1% tryptone, 0.5% NaCl, 0.35% agar dissolved in distilled water, pH 7.1). The diameter of rings was measured against a dark background after incubation of the assay plates at 37 °C for six h [17].

4.2.5. Lipolytic activity

Enzyme activity was determined with Tween 20 as substrate [40, 41]. Culture filtrate (0.5 ml) was incubated with 0.1 ml of 10% Tween 20 in 0.05 M Tris-HCl pH 7.6 (buffer), 0.1 ml $CaCl_2$ in buffer and 2.3 ml of buffer at 37 °C in a water bath for 2 h. Tween was cleaved and the liberated fatty acids reacted with calcium to form insoluble salts. One unit of lipase activity was defined as the amount of enzyme which under these conditions increased absorbance by 0.01.

4.2.6. Sensitivity to oxidative stress

The method of Hassett et al. [42] was used. A control bacterial suspension (100 µl) as well as the test bacterial suspensions after 24 h treatment with disinfectants were spread uniformly on tryptic soya agar plates. Sterile filter paper disks (7 mm diameter) soaked with 10 µl of 30% H_2O_2 were placed in triplicate on each plate. The sensitivity of the bacterial suspensions to H_2O_2 was determined as the zone of clearing surrounding each disk and was scored after 24 h of incubation at 37 °C.

4.2.7. Acylated homoserine lactones – AHLs (C_4–C_8)

A control *P. shigelloides* bacterial suspension as well as the test cell suspensions after incubation with disinfectants at sub-MICs were streaked on an LB agar plate in parallel to the monitor strain – *Chromobacterium violaceum* CV 026 (the strain was kindly provided by Prof. P. Williams, UK). *P. aeruginosa* 550 was used as a positive control for induction of *C. violaceum* CV 026. The strain tested that produced AHLs (C_4–C_8) induced CV 026 to produce of a purple pigment – violacein [43].

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