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Biofilm production, adherence and morphological alterations of *Shigella* spp. under salt conditions

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Abstract In this study, we investigated the survival, adhesion and the morphology of *Shigella* after its incubation in various concentrations of salts. Our results showed that, after 48 h of incubation, the rate of cell survival is inversely proportional to the increase of salinity of the medium. In addition to that, the results prove the evidence that the concentration of salt can significantly influence the capacity of *Shigella* to produce biofilm on Congo red agar and on polystyrene microplate wells. Adherence and invasion assays of *Shigella* were performed with KB cells line. Indeed, an increase of the salts concentration enhances the ability of *Shigella* species to attach and to invade the tissue culture cells. The percentage of adherence was increased to 15% and the invasion to 90% at 6% of salt. The atomic force micrographs showed a reduction of the cells' size after stress.

Keywords *Shigella* · Salt · Survival · Adhesion · Invasion · Morphology

Introduction

Shigella spp. is a Gram-negative enteric bacterium which causes bacillary dysentery in human beings, accounting for 20% of the 4.6 million diarrhea-associated deaths among children (Ahmed et al. 2001). *Shigella* was the third most prevalent foodborne pathogen reported in 2004 by the Foodborne Diseases Active Surveillance Network (FoodNet) of the US Centers for Disease Control and Prevention's (CDC) Emerging Infections Program, accounting for 2,231 of the 15,806 total laboratory-diagnosed cases of infections (Centers for Disease Control and Prevention 2005). Food-processing methods have been developed to interfere with bacterial homeostasis, prevent growth, or kill foodborne pathogens (Abee and Wouters 1999). Some common hurdles used to control microbial growth are pH, sodium chloride, and storage temperature. Consumer demand for high-quality fresh or minimally processed products has resulted in many foods using a combination of acid, salt, and cold storage to prevent microbial growth.

Several studies show that changes in temperature and osmolarity have a strong influence on the ability of these pathogens to be the cause of many diseases (Maurelli et al. 1984; Bernardini et al. 1990). Indeed, the characteristics of the suspending medium, such as pH, osmolarity and temperature, are considered to be important factors in altering the physicochemical properties of a bacterial surface (Hamadi et al. 2004). Generally, bacteria have a natural tendency to adhere to surfaces as a survival mechanism and bacterial colonization of solid surfaces has been described as a basic and natural bacterial stratagem in a wide variety of environments (Hunt et al. 2004). The initial step in the colonization is a thermodynamic process mediated by non-specific physicochemical interactions (Donlan and Costerton 2002) which depend on nutrient availability and physical stress caused by environmental factors, growth and nutritional status of the bacterial population. Hydrophobicity of the bacterial cell surface (CSH) is one of the most important factors which govern the mechanism of bacterial adhesion to inanimate and biological surfaces (Vesterlund et al. 2005). Lewis (2001) shows that *Shigella* can be tolerate antimicrobial agents, and can also be extraordinarily resistant to phagocytosis, making biofilms extremely difficult to eradicate from living hosts.

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The aim of this work was to study the survival of four *Shigella* strains at different concentration of sodium chloride. Slime production under salt conditions was realized on Congo red agar. *Shigella* capacity to biofilm formation at various salt levels was tested in polystyrene microtiter plates. Adherence and invasion assays were performed with human oral cavity epidermoid carcinoma (KB) cell line. The morphology of cells was examined by atomic force microscopy.

Materials and methods

Bacterial strains and growth conditions

Four clinical *Shigella* strains were used in this study: to begin with *Shigella sonnei* ATCC 25931 (S1), then *Shigella sonnei* (S2), thirdly *Shigella boydii* (S3), and finally *Shigella flexneri* (*S4*). Cells were maintained at −80°C in Luria-Bertani broth (LB) supplemented with glycerol (15%, vol/vol). For the experiments, the cells were grown at 37°C in Tryptic soy broth (TSB; Difco) for 24 h. TSB broth (100 ml) was prepared with 0, 2, 4, 6 and 8% NaCl and autoclaved (121°C for 20 min) in 100-ml Erlenmeyer flasks. *Shigella* cells were washed three times by centrifugation (13,000 *g* for 10 min at 20°C) with phosphate-buffered saline (PBS) and then suspended in 10 ml of autoclaved TSB. The microcosms (100 ml) were inoculated with these suspensions (approximately 10^9 CFU/ml) and then incubated at 37°C.

Enumeration techniques

Cultures were sampled daily during the first 24 and 48 h. Time zero (inoculation time) and subsequent samples were taken for plate counts. Plate counts of culturable cells were determined by the drop plate method (Hoben and Somasegaran 1982), using Tryptic soy agar (TSA; Difco). The plates were incubated at 37°C, and the number of colonies was counted after 24 h.

Phenotypic characterization of slime-producing bacteria

Qualitative detection of biofilm formation by tested strains was studied by culturing the strains on Congo red agar (CRA) plates as described previously (Freeman et al. 1989). *Shigella* strains were inoculated onto the surface of CRA plates, made by mixing 0.8 g Congo red with 36 g saccharose (Sigma) in 1 l of brain heart infusion agar, and were incubated for 24 h at 30°C under aerobic conditions and followed overnight at room temperature (Chaieb et al. 2007). Slime-producing bacteria appeared as black colonies, whereas non-slime producers remained non-pigmented (Subashkumar et al. 2006).

Adherence assay to measure biofilm production by *Shigella* cells under salt conditions

Biofilm production by *Shigella* strains grown in TSB supplemented with different NaCl concentration was determined using a semi-quantitative adherence assay on 96-well tissue culture plates (Nunc, Roskilde, Denmark) as described previously (Christensen et al. 1985; Mack et al. 2001) with some modifications. An overnight culture grown in TSB (Biorad) at 37°C was diluted to 1:100 in TSB supplement with 2% (wt/vol) glucose and different percent of NaCl (2, 4, 6, and 8%) as reported elsewhere (Rachid et al. 2000). A total of 200 µl of these cell suspensions was transferred in a U-bottomed 96-well microtiter plate. Each strain was tested in triplicate. Wells with sterile TSB alone served as controls. After their incubation for 24 h at 37°C, the culture was removed and the plates were washed three times with 200 µl of phosphate-buffered saline (7 mM Na_2HPO_4, 3 mM NaH_2PO_4 and 130 mM NaCl at pH 7.4) to remove non-adherent cells and dried in an inverted position. Adherent cells were fixed with 95% ethanol and were stained with 100 µl of 1% (wt/vol) crystal violet (Merck) for 5 min. Then, unbound crystal violet was removed and the wells were washed three times with 300 µl of sterile distilled water. The water was then cleared and the microtiter plate was air dried for 2 h. The optical density (OD) of each well was measured at 570 nm using an automated Multiskan reader (Gio De Vitae, Rome, Italy). Biofilm formation was categorized as highly positive ($OD_{570} \geq 1$), low-grade positive ($0.1 \leq OD_{570} < 1$), or negative ($OD_{570} < 0.1$) (Chaieb et al. 2007).

Culture cells adherence and invasion assays

Quantitative adherence assays was performed with human oral cavity epidermoid carcinoma KB cell line as described by Ellafi et al. (2009). KB cells were grown overnight in minimal essential medium (MEM) with Earle's salts and 10% foetal bovine serum in 96-well microtiter plates at 37°C with 5% CO_2. Each *Shigella* strain was grown, at 37° C with shaking, overnight in TSB supplement with 2, 4, 6 and 8% of salt. Bacterial cells were washed three times by centrifugation at 6,000 *g* for 15 min with MEM without serum and put again in the same medium. The number of bacteria in the suspension was adjusted at 10^8 CFU/ml. The monolayers of human cells were inoculated, for each tested strain, with 10^8 CFU/ml, and incubated at 37°C in 5% CO_2 for 60 min. Then, bacterial suspension was removed to exclude the unattached bacteria. The monolayers of KB cells were washed 3 times with Dulbecco's modified Eagle medium (DMEM), and 1 ml Triton X-100 in phosphate-buffered saline (PBS) was added for 5 min at room temperature to release the bacteria from the cells.

Table 1 Effect at various salt levels on the survival of *Shigella* (S1, S2, S3 and S4) incubated at 37°C

Percent of salt	Time (h)	Strains (Log_{10} CFU/ml± standard deviation			
		S1	S2	S3	S4
0	0	9±0.35	9±0.20	9±0.27	9±0.16
	24	8.6±0.75	8.2±0.211	8.72±0.72	8.42±0.42
	48	7.1±0.6	7.1±0.176	7.51±0.51	7.91±0.91
2	0	9±0.1	9±0.14	9±0.17	9±0.2
	24	7.3±0.87	7±0.253	7.32±0.32	7.12±0.65
	48	6.8±0.92	6.5±0.531	6.95±0.95	6.65±0.12
4	0	9±0.265	9±0.247	9±0.24	9±0.22
	24	7.1±0.1	6.71±0.71	6.71±0.71	6.81±0.24
	48	6.3±0.3	6.1±0.145	6.75±0.75	6.25±0.258
6	0	9±0.78	9±0.78	9±0.78	9±0.7
	24	6.6±0.65	6.7±0.751	6.7±0.7	6.42±0.429
	48	5.3±0.3	4.9±0.921	5.9±0.9	5.6±0.682
8	0	9±0.6	9±0.42	9±0.74	9±0.5
	24	5.1±0.325	5.6±0.39	5.3±0.3	4.53±0.536
	48	3.7±0.721	3.5±0.56	4.5±0.52	2.5±0.34

For the study of invasion, following internalization of the bacteria (2 h at 37°C in 5% CO_2), the supernatant containing extracellular bacteria was removed. The cells were then washed with DMEM and cells were then lysed in PBS containing 1% Triton X-100. The number of bacteria was estimated by plating serial dilutions. All experiments were performed in triplicate.

Determination of morphological changes by AFM

In order to visualize any morphological changes in the stressed cells, *Shigella* cells were examined, in triplicate, by Atomic Force Microscopy (Nanoscope IIIA, Digital Instrument, VEECO). For the experiments, the cells were collected, washed three times with PBS, and centrifuged. The final pellet was resuspended in PBS, placed on a round microscope cover slide and was simply dried in air according to the method previously described (Braga and Ricci 1998).

Statistical analysis

Each analysis was performed using the SPSS 13.0 statistics package for Windows. The differences in the degree of biofilm formation were examined by the Friedman test, followed by the Wilcoxon signed ranks test. *P* values of <0.05 were considered significant.

Results

Survival *Shigella* at various salts levels

We investigated the viability of *Shigella* strains in TSB supplemented with various amounts of NaCl (Table 1). After 48 h of incubation in the different media, we have noted a significant decrease of the cell numbers. Indeed, the

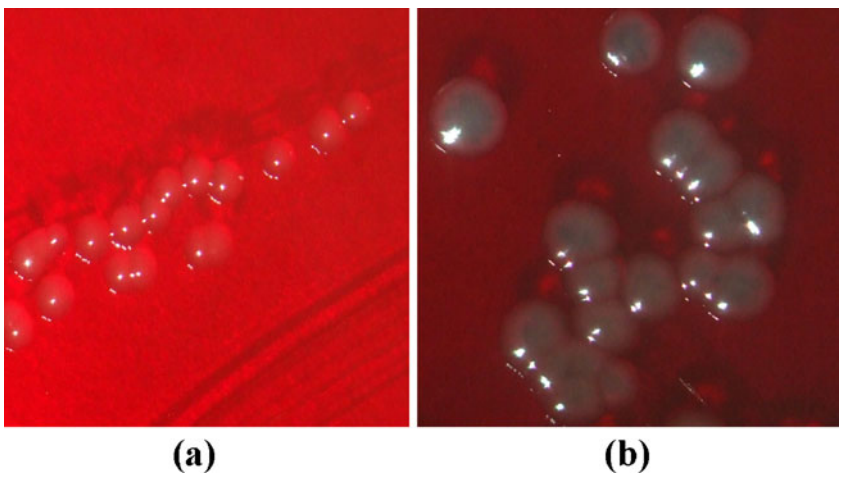

Fig. 1 Detection of slime production by *Shigella* strains before (**a**) and after (**b**) incubation at various salt levels (previously published in World J Microbiol Biotechnol (2009) 25, p 1166)

Table 2 Biofilm formation by *Shigella* at various salt levels

Strains	Percent of NaCl									
	0%		2%		4%		6%		8%	
	$DO_{595\ nm}$	SD	$DO_{595\ nm}$	SD	$DO_{595\ nm}$	SD	$DO_{595\ nm}$	SD	$DO_{595\ nm}$	SD
S1	0.085	0.007	0.225	0.032	0.273	0.034	0.206	0.022	0.112	0.005
S2	0.092	0.003	0.174	0.027	0.219	0.037	0.192	0.063	0.118	0.017
S3	0.098	0.001	0.168	0.088	0.265	0.034	0.186	0.025	0.115	0.015
S4	0.084	0.004	0.143	0.010	0.143	0.016	0.17	0.033	0.113	0.014

SD Standard deviation

survival of *Shigella* strains is inversely proportional to the salt concentration. We also noted that the reduction of the cells viability is significant at 8% of NaCl: the CFU number after 48 h of culture was 3.7, 3.5, 4.5 and 2.5 $\log_{10}$ for S1, S2, S3 and S4, respectively.

Phenotypic determination of slime production of *Shigella* strains

Production of slime by all investigated strains was assessed by culture on CRA plates. Before incubation, *Shigella* cells formed colonies with a red center and a lighter outer zone. After 24 h of their incubation in TSB at different concentrations of salt, all the strains developed colonies with black centers and red contours (Fig. 1).

Biofilm formation by *Shigella* under salt conditions

Biofilm formation of four *Shigella* strains was evaluated in 96-well plates with TSB at different salt concentration (0, 2, 4, 6 and 8%). The results of the OD_{570} presented in the Table 2 showed that all the strains incubated in TSB supplement with salt are able to form biofilm: at 2, 4 and 6% they were considered as highly positive ($OD_{570} \geq 1$) and for 8% ,low-grade positive ($0.1 \leq OD_{570} < 1$). In contrast, the four strains did not show any biofilm formation without NaCl.

Adhesion and invasion assays

Adherence and invasion assays of the four strains of *Shigella* were made with line KB cells. Before their treatment, we have noted that *Shigella* cells are slightly adherent to cells KB (0.8, 1.03, 0.7 and 1.1% for S1, S2, S3 and S4, respectively). Proportionally with the increase of salinity, we have observed a significant increase ($P < 0.05$) in this percentage of adhesion: the high level of adhesion was noted at 6% NaCl and this percentage exceeded the% (Table 3).

For the invasion assays, our results showed a significant increase ($P < 0,05$) in the percentage of membership also for all strains according to salinity (Table 4). We have noted a high level of invasion with 6% NaCl.

Morphological changes of *Shigella* under salt conditions

The cell morphology at different salt conditions was examined by atomic force micrography (Fig. 2). The control *Shigella* cells, whose length is about 3.4 μm, have a normal rod shape with a smooth surface (Fig. 2a.). After 24h of incubation in the different media, we have noted a decrease of the cell size at 4, 6 and 8%. The size of cells is between 3 and 2 μm at 4% NaCl (Fig. 2b), while at 6 and 8%, it is lower at 2 μm. (Fig. 2c).

Table 3 Effect at various salt levels the capacity of *Shigella* strains to adhere to KB cells

Strains	Percent of NaCl									
	0%		2%		4%		6%		8%	
	%Ad	SD	%Ad	SD	%Ad	SD	%Ad	SD	%Ad	SD
S1	0.8	0.053	2.3*	0.275	4.1*	0.87	10.9*	0.8	6.4*	0.35
S2	1.03	0.065	2.98*	0.78	5.76*	0.92	15.3*	0.31	7.86*	0.15
S3	0.07	0.023	1.78*	0.46	3.2*	0.43	8.5*	0.101	8.1*	0.67
S4	1.1	0.01	3.54*	0.58	7.65*	0.13	15.12*	0.87	12.5*	0.38

%Ad % adherence, *SD* standard deviation

*$P<0,05$

Table 4 Effect at various salt levels the capacity of *Shigella* strains to invasion to KB cells

Strains	Percent of NaCl									
	0%		2%		4%		6%		8%	
	%Inv	SD	%Inv	SD	%Inv	SD	%Inv	SD	%Inv	SD
S1	14	0.98	20*	4.2	27.3*	6.2	57*	3.2	34.6*	3.5
S2	16	1.23	21.9*	3.57	29*	3.45	61*	7.36	26.2*	1.5
S3	33	4.56	37.8*	4.73	47.2*	2.65	89.3*	10.59	51.6*	6.7
S4	35.7	2.53	46*	7.2	57.9*	10.1	94.4*	9.35	39.6*	3.8

%Inv % invasion, *SD* standard deviation

*$P<0,05$

Discussion

The present study showed that the survival of *Shigella* is greatly influenced by the salinity. The significant decrease of *Shigella* cells numbers at the beginning of the experiment may be a result of the death bacterial cells caused by the salt. These results are in accordance with those reported by (Zaika and Phillips 2005), who demonstrated that the survival of *Shigella* is affected by the increase of NaCl concentration.

Before their treatement, *Shigella* strains are considered as non-produced slime. After 24 h of incubation at different concentrations of salt, we have noted that the investigated *Shigella* cells passed from colonies with a red center and a lighter outer zone to colonies with black center and red contour. This state is considered a variable phenotype (Touati et al. 2007). This main difference between the normal and stressed strains is that there can be a state of passage toward the biofilm formation in the case of stress

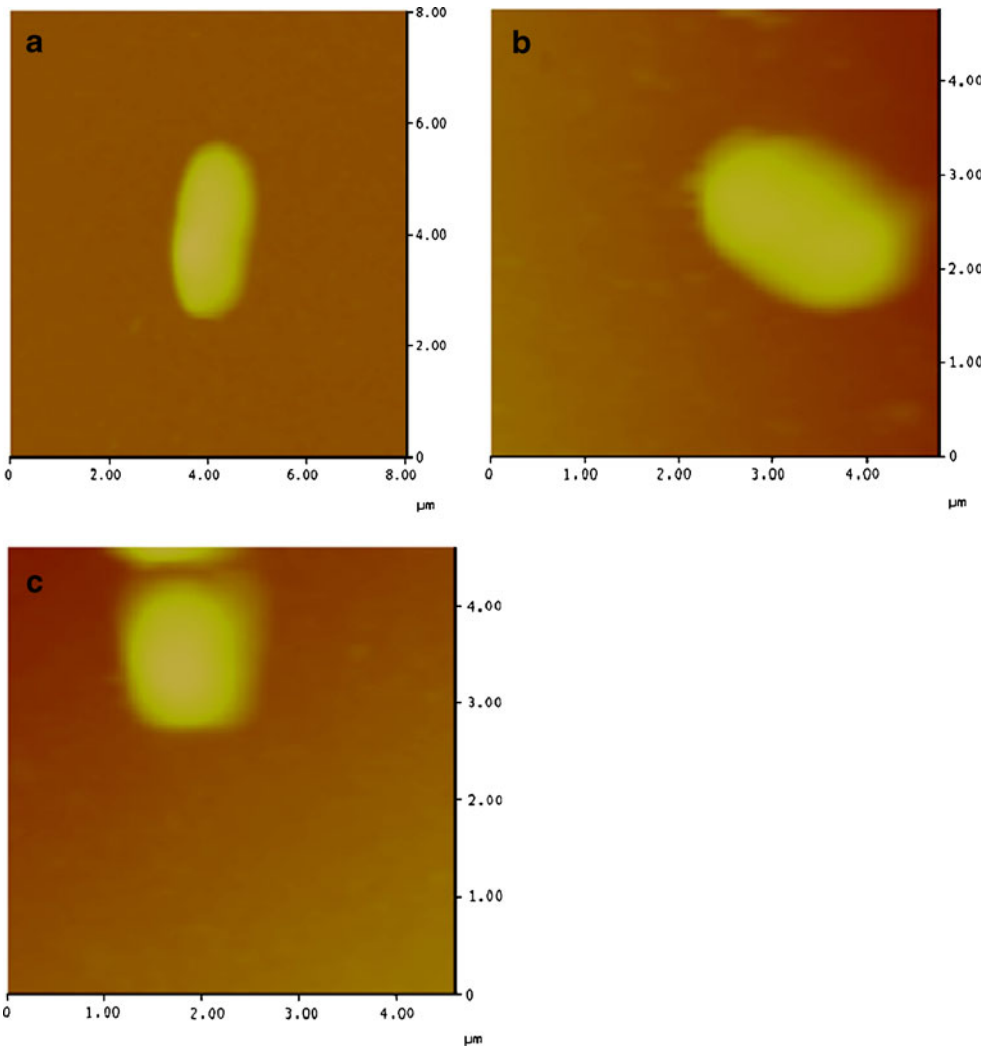

Fig. 2 Atomic force micrographs of stressed *Shigella* spp. cells. **a** Control and cells stressed at 2% NaCl; **b** cells stressed at 4% NaCl; **c** cells stressed at 6 and 8% NaCl. *Bars* 1 µm

elongation. According to Adam et al. (2002), the biofilm is a protective system under environment stress conditions (e.g., physical or chemical treatment).

Bacteria and other microorganisms have a natural tendency to adhere to surfaces as a survival mechanism. Bacterial colonization of solid surfaces has been described as a basic and natural bacterial strategies in a wide variety of environments (Kjelleberg et al. 1983; Jana et al. 2000; Hunt et al. 2004). Our finding showed that salt concentration can significantly influence the capacity of *Shigella* cells to adhere to polystyrene. Similar results have been found with *Escherichia coli, Pseudomonas aeruginosa* and *Listeria monocytogenes* under different environmental conditions. Furthermore, the surface charges and hydrophobicity of bacteria were influenced by the environmental condition (Briandet et al. 1999), explaining, in fact, the variation of bacteria capacity to adhere to substrates.

Adherence and invasion assays of *Shigella* were performed with KB cells line (Tables 2 and 3). Growth at various salts levels enhances the ability of *Shigella* species to attach and to invade the KB cells. Indeed, the increase of invasion capacity, induced by the salts, appears to be due to the increase of adherence. Statistical analysis revealed that the adhesion and invasion were significantly enhanced with the increase of salt percentage. When *Shigella* invades its host, the organism has to cope with a very special environment. Under these conditions, the bacterium is exposed to a variety of stimuli that will most likely affect the expression of genes required for its survival and initiate the development of pathogenic events (Tartera and Metcalf 1993). Previous studies with other enteric pathogens suggested that osmolarity is one of the environmental factors which regulate the interaction of the organisms with eukaryotic cells (Berry et al. 1989; Bernardini et al. 1990; Galin and Curtiss 1991; Ellafi et al. 2009). Understanding the nature of this response to salt will help to elucidate early steps in *Shigella* pathogenesis and may provide insights into why these are such efficient pathogens. Indeed, Leodocia et al. (1995) showed that bile salts influence the capacity of adhesion and invasion of *Shigella*. Other studies have shown that the percentage of *Salmonella typhi* able to penetrate Henle 407 cells and Caco-2 cells was controlled by the osmolarity (Tartera and Metcalf 1993).

The atomic force micrographs revealed reductions in the *Shigella* cells' sizes caused by salt stress. According to (Morita 1993), several bacteria such as *Shigella*, can survive for a long period under stressing environmental conditions owing to gradual changes in cellular physiology and morphology. The reduction of the bacteria size, such as *Shigella*, during the stress is a strategy of survival (Jiang and Chai 1996). This reduction of cell size is the result of the cytoplasmic contraction as well as the reduction of the bacterial periplasm volume (Huisman et al. 1996). The reduction of cell size and the increase of the adhesion property are probably linked. Indeed, bacterial populations under stress react in order to adequate cell metabolism and physiology to stressful conditions. As a consequence, this modification caused a general increase in virulence and resistance against stress (Givskov et al. 1994). These results are in agreement with those reported by Kalchayanand et al. (2004), which showed that hydrostatic pressure and bacteriocin mixture induce change in the morphology of *Escherichia coli* O157:H7 and *Salmonella typhimurium*.

In conclusion, salt dramatically influences the capacity of adhesion and, indirectly, the surface hydrophobicity of *Shigella*. This finding is very important in explaining the pathogenesis of this bacterium.

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