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Influence of Different Physico-Chemical Stresses on Growth and Survivality of *Shigella dysenteriae* and *Shigella flexneri*

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Abstract: This study relates to the survivality of *Shigella dysenteriae* and *S. flexneri* in acid environment and the effect of salt induction on subsequent sensitivity to acid. After 120 min exposure to acidic environment at pH 3, around 30% survivality of both the *Shigella* spp. was recorded. The acid tolerance was found to be dependent on the growth phase and the pH of the growth medium. *Shigella* spp. previously grown in low-salt broth at pH 7.2, produced organisms which were markedly more acid sensitive when subsequently cultured in the same broth supplemented with 200 mM or more salt at 37°C. However The degree of sensitivity varied with the time of exposure in different salts. Almost no viable cells were recorded after 30 min exposure when treated with 500 mM salt at pH 3. Survivality of salt treated *Shigella* spp. was also found to be influenced by different water samples. Viable counts could be observed up to 6 days in normal saline, where as, in pond-, tap- and distilled water *Shigella* spp. were not detected after 96, 72 and 72 h respectively. *Shigella* spp. treated with 500 mM salt went into non-culturable state but remain viable as was detected by acridine orange staining method. No significant changes of the virulence properties of NaCl treated *Shigella* spp. was noted.

Key words: *Shigella* spp., survivality, salt induction, acid tolerance

Introduction

Bacillary dysentery, caused by four species of *Shigella* spp., is an acute gastrointestinal infection causes a great deal of morbidity and mortality in Bangladesh (Khan and Curlin, 1974; Khan *et al.*, 1979). In developing countries, shigellosis, manifested by mild diarrhea, fever, abdominal cramps and severe fluid loss, accounts for at least 500,000 deaths per year in young children (Smith, 1987). Poor understanding of quality control measures and lower sanitation facilities in developing countries are the main causes of shigellosis. By means of human transmission, *Shigella* spp. can contaminate several kinds of foods and aquatic environment (Smith, 1987). Contaminating *Shigella* spp. can be subjected to high concentrations of salt in wide range of situations. They may enter in marine and estuarine waters where the NaCl concentrations can be as high as 500mM, in naturally or artificially salted or brine preserved foods; may also be ingested along with food or water and subjected to salt concentrations of up to 150 mM in animal body (Rowbury *et al.*, 1994).

The low pH of gastric secretions has long been recognized as the first line of defense against food-borne enteric pathogens (Benjamin and Datta, 1995). The striking ability of enteric bacteria to protect themselves against extremely low acidic condition during passage through the stomach increases their survivality in the intestine and help these pathogens to cause an infection. The infective dose (ID) of enteric pathogens thus depends on their relative abilities to resist killing by acid (Benjamin and Datta, 1995). Reduction of gastric acidity has been associated with an increase in survival rates of some common enteric pathogens (Peterson *et al.*, 1989) and with lowering of the ID (Cash *et al.*, 1974; Schlech *et al.*, 1993). It has been reported that, *Shigella* spp. are more acid tolerant (pH 2 to 2.5) than are *Salmonella* and *E.coli* (Gorden and Small, 1993). The ability to survive in such a low pH depended on the growth phase and the pH of the growth medium for *Listeria* spp. and *Salmonella* spp. (Gorden and Small, 1993; Kroll and Patchett, 1993; Foster and Hall, 1990). The prior exposure to a mild dose of a stress may alter the ability to resist the same but more pronounce stress. It has been reported that the pre-exposure to heat, alkylating agents, oxidative stress, ethanol, or starvation leads enhanced tolerance to these stresses (Demple and Halbrook, 1983; Mackey and Derrick, 1987; Michel and Starka, 1986). Rowbury *et al.* (1994) reported that *E. coli* grown in low-salt broth at pH 7.0 was markedly more acid sensitive when subsequently cultured in the same broth with 200-300 mM salt added. Responses to pH stress are of particular interest because organisms can be exposed to extremes of pH in aquatic environments, in foods, and in animals and human bodies (Rowbury *et al.*, 1989), and responses to such stress may influence subsequent ability to survive and cause disease (Foster and Hall, 1990). Survival in acid may have clinical significance, because enteric pathogens must pass through the stomach at pH lower than 3 for up to 2 h before colonizing in intestinal tract. When exposed to environmental stresses, bacteria may enter a viable but non-culturable state. Non-culturable cells have been observed for several gram-negetive bacteria (Roszak *et al.*, 1984; Rollins and Colwell, 1986). Since, non-culturable cells may still retain metabolic activity and, if pathogenic, might maintain their infectiveness (Griems *et al.*, 1986; Oliver, 1993), it is important to determine the viable state of non-culturable cells. Though a number of reports on the survivality of some enteric pathogens to acid and other stresses have been published, however, the response of *Shigella* spp. those stresses is yet to be clarified. Considering the above points, the present study aimed at investigating the acid sensitivity, viability and pathogenesis of *Shigella* spp. upon exposure to different physicochemical stress conditions.

Materials and Methods

Bacterial strains, media and growth conditions: *Shigella dysenteriae* type 1 (strain 6476, 3351R, 24623R, 3351W and PSd-19) and *S. flexneri* (strain H90T and 1095W), obtained from clinical research center of International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), were used throughout the study. The strains were maintained at 4°C in nutrient agar medium, composed of peptone and beef extract in a final concentration of 0.5 and 0.3%, or in MacConkey,s agar. The strains were routinely cultivated in luria broth (LB), pH 7.2 at 37°C.

Acid resistance of *Shigella* spp.: Fresh, over night cultures of *Shigella* spp. in nutrient broth (NB) medium, pH 7.2, diluted 10^{-3} in the same medium at pH 3.0 and were incubated for 2 h at 37°C, unless otherwise stated. Dilutions were plated on NA agar, incubated at 37°C for 24 h, and colony counts were compared with those from plated dilutions of the original culture to determine the percent survival. In order to determine the growth phase dependent acid tolerance of *Shigella* spp., fresh over night cultures were diluted in the same NB medium, and incubation was continued at 37°C with gentle shaking. Samples were withdrawn hourly and centrifuged at 10,000 x g for 5 min. The pellets were suspended in equal volume of NB adjusted to pH levels of 7.2 and 3.0. The pH 7.2 samples were plated immediately on nutrient agar;

the pH 3.0 samples incubated at 37°C for 2h before being plated on nutrient agar. The plates were incubated overnight at 37°C, and CFU were then counted. At each sampling time, the survival percentage was calculated and results were ploted in log graph. Values shown for percentage of survival represent the mean of at least three independent trials from over night cultures from separate colonies. In order to determine the effect of growth medium pH on acid tolerance, the strains were grown to mid exponential phase at 37°C in nutrient broth, pre-adjusted to pH 5.0, 6.0, 7.0 and 8.0. After incubation, the pH of each culture was recorded and the culture viability in pH 3.0 was assayed. Survival percentage was calculated as, "the ratio of viable bacterial cells remained after each treatment to those contained in the initial inoculum multiplied by 100".

Salt induction test: The salt induction test was carried out according to the method described elsewhere (Rowbury *et al.*, 1994).

Survivality in water samples: A number of water samples namely, pond water, tap water, normal saline, distilled water and gram negetive (GN) broth (as a control liquid, composed of tryptone, 2%; dextrose, 0.1%; mannitol 0.2%, Na-citrate, 0.5%, Na-deoxycholate 0.05%, K_2HPO_4, 0.4%, KH_2PO_4, 0.15% and NaCl, 0.5%) were selected randomly to observe their effect on the survivality of *Shigella* spp. treated with 85 and 500 mM NaCl. Cells were grown over night in NB at 37°C, harvested by centrifugation, dissolved in normal saline and were diluted to give a final cell density of approximately 10^6 cfu/ml. Diluted cells were then inoculated into NB medium supplemented with 85 and 500 mM NaCl and incubated for 30 min. After induction, cells were transferred to different aquatic samples and incubated at 4, 25 and 37°C. Viable counts of the cells were estimated according to the method described above.

Viability and morphological variations of salt treated cells: Fresh culture of *Shigella* spp. grown in low-salt broth at 37°C was diluted 100 fold, transferred to nutrient broth medium supplemented with 500 mM NaCl and incubated for 30 min at the same temperature. Cells were harvested by centrifugation, dissolved in yeast extract (0.002%) and nalidixic acid (0.025%) solutions and incubated for 12-18h at the same temperature. Smears of the cells were prepared on the surface of clean slides, fixed with gentle heat and submerged by acridine orange dye according to the procedure described by Roszak and Colwell (1987). Viable but non-culturable cells were observed under a fluorescent microscope equipped with a high mercury lamp (Olympus, Japan). In order to determine the morphological variation of the salt (500 mM, NaCl) treated *Shigella* spp., cells were dissolved in yeast extract (0.002%) and nalidixic acid (0.025%) solutions and incubated at 37°C for 7 days. Morphology of the cells was observed by gram-staining as well as by acridine orange staining methods.

Virulence associated properties of salt treated *Shigella* spp.: Invasiveness of salt treated *Shigella* spp. was determined by the method of Sereny (1955) in guinea pig's eye. The NaCl (85 mM) induced strains were inoculated in screw-cap test tubes containing 3 ml of tryptic soy broth (TSB) and 0.6% yeast extract and incubated for 4 h at 37°C. Cells were then inoculated in blood agar plates and incubated over night at the same temperature. A thick cell suspension was prepared with the freshly grown cells from blood agar plates and 10 µl of this suspension was applied in one eye of guinea pigs and gently messaged to ensure the distribution of the organisms over the conjunctival sac. The animals were observed over a period of 96 h for keratoconjunctivitis. A control experiment also was carried out with cells not induced by NaCl.

Results

Acid resistance of *Shigella* spp.: Effect of acidic condition on the survivality of *S. dysenteriae* and *S. flexneri* was determined by a series of experiments. Both the species of *Shigella* were challenged to pH 3.0 (a pH that is very close to gastric secretion), incubated at different time intervals and percent survivality was recorded. Around 30% survivality of both the species of *Shigella* was noted after 120 min of exposure, that is enough for causing bacillary dysentery when ingested to human intestine (Table 1). Growth phase dependent acid tolerance in *S. dysenteriae* type 1 was determined following the method described above. The results obtained were confirmed by at least three independent trials. Although the individual values varied at times by as much as three fold, the basic shape of the graph remained almost the same. In an experiment (Fig. 1) it was demonstrated that the acid tolerance was highest at late stationary phase (over night cultures), which decreased several logs when cells were at the mid exponential phase. A second peak of high acid tolerance, observed at the early stationary phase, was about 100 fold less than that at the late stationary phase. A similar effect was found for *S. flexneri* (data not shown). These results suggested that some factors in stationary phase cells persist over several generations of log-phase growth. A similar growth phase dependent acid tolerance was also observed in enterohemorrhagic *E. coli* (Benjamin and Datta, 1995). *Shigella* spp. isolated from variety of foods and natural environments that provide a wide range of conditions in terms of nutrients, pH, salinity and temperature. Several genes,

Table 1: Determination of acid resistance at pH 3 of *S. dysenteriae* and *S. flexneriae* grown in nutrient agar

Strain	Survivality (%)[a] at different time (min) interval				
	0	5	30	60	120
S. dysenteriae					
6474	100	85	55	42	30
3351R	100	90	59	40	28
24623R	100	89	56	39	29
3351W	100	89	50	40	27
S. flexneria					
H90T	100	84	47	38	31
1095W	100	91	55	39	25

[a]Fresh overnight cultures in NB, pH 7.2, were diluted 1:1000 in NB adjusted with HCl to pH level of 3.0. The cultures were incubated for 37°C for 2h, and survival percentages were calculated from viable counts.

Table 2: Relationship of growth medium pH and acid tolerance in *S. dysenteriae* and *S. flexneriae*

Growth medium pH		Survival(%) of strtains[a]	
Before growth	After growth	*S. dysenteriae*	*S. flexneriae*
5	4.8	56	63
6	5.9	48	52
7	6.8	25	30
8	7.4	10	14

[a]Survial percentage were calculated from viable counts of the cultures after 2 h of incubation at 37°C in NB acidified to pH 3.0.

responsible for adaptive acid tolerance, have been isolated from *E. coli* O157:H7 and *Salmonella* spp. (Foster, 1991; Benjamin and Datta, 1995). To find out whether the same phenomenon exists in *Shigella* spp. The acid resistance of *S. dysenteriae* has checked and *S. flexneri* grown in various acidic conditions. pH of the growth medium clearly had a significant effect on subsequent survival in acidic pH: higher the growth medium pH, lower the tolerance and vice versa (Table 2). The pattern of this adaptive response was similar to that observed in *Salmonella* spp. (Foster and Hall, 1990).

Induction of acid sensitivity: A number of mono-valent cations including NaCl at different concentrations were employed to

Table 3: Effect of various of salts at different concentrations on survivality of *Shigella dysenteriae* type 1 incubated under conditions of 37°C and at pH 3

	Percent survival											
	NaCl			KCl			$NaNO_3$			Na_2SO_4		
	Time (min)			Time (min)			Time (min)			Time (min)		
Salt (mM)	5	15	30	5	15	30	5	15	30	5	15	30
50	24	17	11	18	15	7	25	17	12	22	16	10
85	32	19	13	28	21	9	30	22	16	31	20	14
100	20	14	8	17	10	5	21	13	10	21	16	7
200	17	9	4	15	5	4	15	11	8	18	11	3
300	9	7	3	7	5	1	7	6	4	5	4	1
400	2	1	1	3	2	1	3	2	0	3	2	0
500	2	0	0	0	0	0	2	0	0	2	0	0

Table 4: Effect of various of salts at different concentrations on survivality of *Shigella flexneriae* incubated under conditions of 37°C and at pH 3

	Percent survival											
	NaCl			KCl			$NaNO_3$			Na_2SO_4		
	Time (min)			Time (min)			Time (min)			Time (min)		
Salt (mM)	5	15	30	5	15	30	5	15	30	5	15	30
50	22	18	7	21	12	8	27	18	9	22	18	8
85	41	21	10	39	19	9	42	20	15	47	21	10
100	18	8	5	11	5	3	19	13	7	22	12	5
200	20	12	5	10	5	2	14	8	5	18	10	4
300	19	7	3	7	2	0	7	6	1	11	6	1
400	1	0	0	1	0	0	3	1	0	1	0	0
500	0	0	0	0	0	0	1	0	0	1	0	0

induce *S. dysenteriae* and *S. flexneri* to determine their subsequent effect on acid sensitivity. Sensitivity towards acid (pH 3.0) was rapidly gained in *Shigella* spp. treated with NaCl and other tested cations at 37°C (Table 3 & 4). Although a slight sensitization was recorded after 5 min, the effect was much more pronounced after 15 min and the effect was almost completed at 30 min. The intensity of the effect was dependent on the concentrations of salts used for experiment. Even after 30 min, NaCl and other salts produced a moderate effect when added at 50, 85 or 100 mM. Sensitization was more prominent at 200 to 300 mM in most cases and almost fully completed at 400 and 500 mM. Experiments were also carried with NH_4Cl and $_{CaCl_2}$ and almost similar effect was recorded (data not shown). A similar type of acid response was reported in *E. coli* (Rowbury *et al.*, 1994).

Survivality in different water samples: Bacteria living in natural environments are subjected to constantly changing and frequently stressful conditions. In view of these criteria, experiments were carried out to find the effect of NaCl treated *Shigella* spp. on the survivality in various water samples. The viable cells of both species of *Shigella* could be observed in normal saline and GN broth (control experiment) for up to 6 days (Fig. 2 & 3). Survivality of *S. dysenteriae* and *S. flexneri* was observed in pond water for more than 96 and 72 h respectively at 37°C. In case of tap water & distilled water viable cells were recorded for upto 72h.

Viability and virulence properties of salt treated *Shigella* spp.: *Shigella* spp. treated with 500 mM salt became non culturable within 30 min. It was reported that non-culturable cells remain viable in a prolonged period of time (Rollins and Colwell, 1986). Acridine orange staining method was employed to determine the viability of salt treated *Shigella* spp. Fluorescent microscopic observation revealed that a huge number of both species of *Shigella* were orange in colour that indicates the viable form of non-culturable cells; on the other hand, non viable cells fluoresce green colour (data not shown). This result correlates with the findings described by Roszak and Colwell (1987). In another experiment, it was found that salt treated *Shigella* spp.

Table 5: Invassiveness of *Shigella* spp. Treated with or without NaCl

Time after inoculation (h)	Keratoconjunctivitis			
	S. dysenteriae		*S. flexneri*	
	Salt induced	Uninduced	Salt induced	Uninduced
0	-	-	-	-
24	+	+	+	+
48	+	+	+	+
72	+	+	+	+
96	+	+	+	+

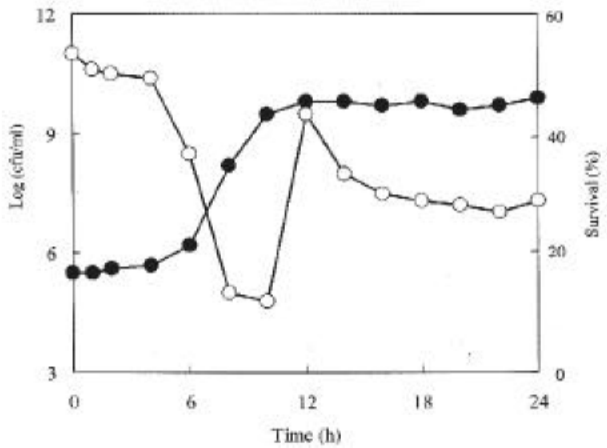

Fig. 1: Growth phase-dependent acid tolerance of *S. dysenteriae* type 1. Cells were grown in nutrient broth, pH 7.2 at 37°C (●). Survival %ages (○) were calculated after incubation of this culture in NB, pH 3.0 for 2h.

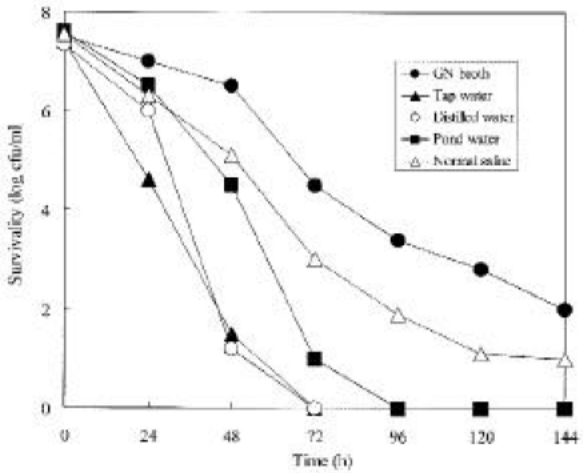

Fig. 2: Survivality of NaCl treated (85 mM) *S. dysenteriae* type 1 in various water samples. Survival percentages were calculated every 24h after incubation in different water samples.

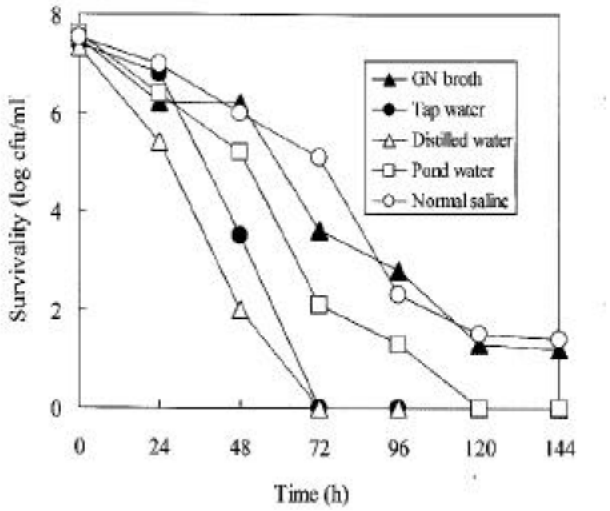

Fig. 3: Survivality of NaCl treated (85 mM) *S. flexneri* in various water samples. Survival percentages were calculated every 24h after incubation in different water samples.

showed no morphological changes, except that a number of elongated cells were observed. This elongation may be due to the effect of yeast extract and nalidixic acid.

A number of assay methods were employed to determine the virulence properties of salt treated *Shigella* spp. Results of sereny test, (Table 5) indicate that both salt treated and non-salt treated *S. dysenteriae* and *S. flexneri* induced the production of keratoconjunctivitis in guinea pigs eye. This result indicates that salt treatment could produce no effective change in invasive properties of *Shigella* spp. No change of virulence properties in salt treated *Shigella* spp. also was observed in haemagglutination test, salt aggregation test and in congo-red binding test (data not shown).

Discussion

Shigella spp. frequently subjected to inhibitory stresses in natural environment including in human stomach after their ingestion through foods or water. Among many stress conditions existing in the natural environment, extreme pH and salinity are more significant as the organisms have to face challenges of salinity in different water bodies and foods and have to pass the stomach acidity to cause shigellosis. This study describes the effect of such stresses on the survivality, viability and virulence properties of *S. dysenteriae* and *S. flexneri*.

In this study, it was found that *Shigella* spp. grown in nutrient broth showed a high level of acid tolerance that may contribute to their relatively low infective dose of 100 to 500 organisms that strongly support the hypothesis of Gorden and Small (1994). In the experiment of growth phase dependent acid tolerance, it was found that stationary phase cells are more acid resistant than log-phase cells. Usually stationary phase cells have to face more stress conditions caused by either nutrient deprivation or other environmental factors such as temperature, salinity and pH. It can be assumed that stress caused by these agents will also lead to a higher level of acid tolerance through some global regulatory factors. Similar growth- phase dependent acid tolerance was also observed in *E. coli* (Benjamin and Datta, 1995). In a separate experiment, it was found that the surviavlity of *Shigella* spp. is dependent on the growth medium pH. The higher the growth medium pH the lower is the tolerance and vice versa. This type of adaptive response was reported earlier in *Salmonella* spp. (Foster and Hall, 1990). It has been reported that the adaptive acid tolerance in *Salmonella* spp. was found to be controlled by several genes whose synthesis during adaptation was crucial for the response in *Salmonella* spp. (Foster, 1991). Foster (1993), showed that the adaptive tolerance involves two distinct responses: pre-challenge adaptation and transient adaptation, which occur during low pH challenge. Such types of phenomena might be involved in *Shigella* spp. The mechanism by which bacteria maintain internal pH in a narrow range (6.5to 8.0) despite the large variations in outside pH is poorly understood (Booth, 1985). However several possibilities have been considered to explain how bacteria achieve pH homeostasis: (i) the buffering capacity of cytoplasm, (ii) low proton permeability, and (iii) the extrusion of protons from the cytoplasm by a membrane-bound pump (Benjamin and Datta, 1995). It has also been proposed that low internal pH may induce specific enzymes that may be involved in pH homeostasis (Booth, 1985). These types of mechanisms might be involved in maintaining high acid tolerance in *Shigella* spp.

Both *S. dysenteriae* and *S. flexneri* were found to be sensitive to acid pH when grown in media supplemented with a series of salt solutions. Induction of acid sensitivity occurred rapidly with high-salt concentration supplemented broth at 37°C. The resistance towards acid challenge of *Shigella* spp. might be attributed by several factors: changes in sodium/proton anti porter system, high osmotic pressure and inactivation of RpoS gene. This type of mechanism also has been reported previously (Ohyama *et al.*, 1992; Panden and Schuldiner, 1987).

The present study demonstrated the effect of various aquatic samples on the survivality of salt treated *Shigella* spp. It was found that *Shigella* spp. treated with 85 mM salt survived in GN broth (control experiment) and normal saline for more than 6 days at 37°C. A similar type of survival pattern was also observed at 25 and 4°C (data not shown). Lower survivality in tap and distilled water might be due to nutrient starvation and or the presence of disinfectants in water samples. On the other hand a significant survival in pond water might be caused by the presence of certain nutrients, though the level of survivality was below the expectation. It has been reported that the presence of detergents, organic materials, humidity, light, turbidity etc may hinder prolong survival of bacterial pathogen (Marsharipov, 1970).

The non-culturable state of *Shigella* spp. and their capacity to persist in aquatic environment are important features in the ecology of *Shigella*. It has been demonstrated in this study that non culturable but viable form of *Shigella* spp. turned to elongated filamentous form due to the presence of yeast extract and nalidixic acid. At the same time, acridine orange stained *Shigella* spp. emitted orange red fluorescence and dead cells were found

to be green in colour. Both of the findings indicate that due to the treatment with high (500 mM) salt, *Shigella* spp. could not be cultured in the conventional media but some of them remained viable. The results detected in this study by acridine orange staining method were consistent with the results observed by Kogure *et al*. (1979) in case of *V. cholerae* 01. The non-culturable but viable *Shigella* spp. reported here have a great significance in understanding the epidemiology of diarrhoea. If those non-culturable cells are ingested by humans, there might be a possibility to revert to culturable state and cause shigellosis, as has been reported for *V. cholerae* 01 in volunteer studies (Colwell *et al*., 1996). In this study it has also been demonstrated that salt treatment could not alter the virulence properties of *Shigella* spp. Finally, it can be concluded that a change in acid tolerance level caused by growth phase variation and growth medium pH underscores the importance of the physicochemical environments of contaminated foods in determining the infective dose. Even acid sensitivity increased after salt induction, the non-culturable forms of *Shigella* spp. that might remain in foods or waters pose a serious question on the judgement of acceptability of these items by conventional methods.

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