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Survivality and Virulence of *Shigella sonnei* and *Shigella boydii* in Different Physico-Chemical Stress Conditions

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Abstract: Acids and salts are important environmental conditions encountered by *Shigella* spp. during its survivality and pathogenesis. These studies have shown that *S. sonnei* and *S. boydii* could survive longer than 120 min to acidic environment at pH 3 and approximately 32% survivality for both the species was recorded. The acid tolerance was found to be dependent on the growth phase and 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. A differential survival pattern was recorded with salt-treated *Shigella* spp. in a number of aquatic samples. Fluorescent microscopic examination revealed that *Shigella* spp. treated with 500 mM salt went into non-culturable state but remain viable. However, salt treatment could produce no significant changes to the infective properties of *Shigella* spp.

Key words: *Shigella*, acid tolerance, salt induction, survivality, VBNC, virulence

Introduction

Shigella, a well-known pathogen that causes gastrointestinal infection in human, is prevalent in less developed countries where conditions of poor sanitation and personal hygiene increase the incidence of disease. The low infectious dose (DuPont *et al.*, 1989) allows the disease to be spread effectively by infected food or water, and also by person-to-person contact (Smith, 1987). In Bangladesh, the disease is hyper endemic and occasionally flares into epidemics. The isolation rate of *Shigella* in routine surveillance of hospitalized diarrheal cases is approximately 11-12% (Hossain *et al.*, 1990). On the other hand, the annual number of Shigella-episodes throughout the world was estimated to be 164.7 million, of which 163.2 million were in developing countries (with 1.1 million deaths) and 1.5 million industrialized countries (Kotloff *et al.*, 1999). It has also been reported that the median percentages of isolates of *S. flexneri*, *S. sonnei*, *S. boydii* and *S. dysenteriae* were, respectively, 60, 15, 6 and 6% in developing countries and 16, 77, 2 and 1% in industrialized countries (Kotloff *et al.*, 1999).

Shigella spp. can be subjected to a number of stressful conditions including high concentrations of salt (NaCl, KCl, NH_4Cl etc.) in a wide range of situations. They may enter in marine and estuarine waters where the NaCl concentrations can be as high as 500 mM, in naturally or artificially salted or brine preserved foods; may also be ingested along with food or water and subjected to salt concentrations up to 150 mM in animal body (Rowbury *et al.*, 1994). Contaminating *Shigella,* when ingested with food, has to face the low pH of gastric secretion. Survival in acid may have clinical significance, because enteric pathogens must pass through the stomach pH < 3 for upto 2h before colonizing the intestinal tract (Giannella *et al.*, 1972). 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 is depended on the growth phase and the pH of the growth medium for *Listeria* spp. and *Salmonella* spp. (Gorden and Small, 1993; Kroll and Pachett, 1993; Foster and Hall, 1990). The prior exposure to a mild dose of a stress may alter the response to another (Jenkins *et al.*, 1990). 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). The capability of pathogenic microorganisms to exist in the viable but non- culturable (VBNC) state has been reported (Islam *et al.*, 1993). Therefore, the potential health hazard of *Shigella* spp. existing in the VBNC state may be important, since *Vibrio cholerae* 01 could be isolated in the culturable form from stools of volunteers after ingestion of VBNC *V. cholerae* 01 (Colwell *et al.*, 1996). Furthermore, some investigators claim that non-culturable bacteria of selected species can be resuscitated to the culturable state (Roszak *et al.*, 1984). Since, non-culturable cells may still retain metabolically 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. A significant problem in elucidating the potential hazard of non-culturable pathogenic bacteria is the inability to detect such cells in the natural environment by routine culture methods. The fluorescent antibody (FA) technique, a highly selective and sensitive method, can detect VBNC shigellae in laboratory microcosm (Islam *et al.*, 1993). Like two other species of *Shigella* (*S. flexneri* and *S. dysenteriae*) both *S. sonnei* and *S. boydii* are almost equally important as diarrheal pathogens, since a number of reports have been published on the outbreak of shigellosis caused by these two species (Alamanos *et al.*, 2000; McCall *et al.*, 2000; Kotloff *et al.*, 1999). We have reported the effect of a number of physicochemical stress conditions on the survivality of *S. flexneri* and *S. dysenteriae* (Sultana *et al.*, 2002). However, the response of S. *sonnei* and S. *boydii* to those stresses is yet to be clarified. This report describes response of the two strains of *Shigella* upon exposure to different physicochemical stress conditions. This is the first report on such type of study.

Materials and Methods

Bacterial strains, media and growth conditions: *Shigella sonnei* and *S. boydii*, obtained from clinical research center of International Centre for Diarrhoeal Disease Research, Bangladesh (ICDDR,B), were used throughout the study (1998- 1999). 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.: Acid resistance of both the *Shigella* spp. was determined according to the previously reported method (Sultana *et al.*, 2002). Briefly, 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 nutrient agar (NA), 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 colony-forming units (cfu) were then counted. At each sampling time, the survival percentage was calculated and results were plotted 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: This study was carried out according to the method described elsewhere (Rowbury *et al.*, 1994) with some modifications. Cells of *Shigella* spp. were grown overnight at 37°C in a low-salt broth (LSB) medium that is composed of 10 g of Oxoid L37 Peptone and 10 g of Oxoid L-29 Lab Lemco powder per liter. Over night grown cells were diluted 20-50 fold in fresh LSB medium and reincubated at 37°C for few hours with shaking. Cells were harvested by centrifugation and diluted in normal saline to give the final cell count of approximately 10^9 cfu/ml. Freshly prepared bacterial suspension (approx. 10^6 cfu/ml) was inoculated to a new nutrient broth medium supplemented with a series of salt solutions including NaCl (50, 85, 100, 200, 300, 400 and 500 mM) at pH 3.0 and incubated at 37°C for 5, 15 and 30 min. After incubation, cells were further diluted in normal saline, plated on NA plate and incubated overnight at 37°C and viable counts were recorded. A mixture of a number of salts at a final concentration of 85mM was tested to determine their combined effect on the survivality of *Shigella* spp. following the same method described above.

Effect of aquatic samples on the survivality of salt treated *Shigella* spp.: Cells of both the species of *Shigella* 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 inoculated to a number of water samples namely, pond water, tap water, normal saline, distilled water and gram negative (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%) and incubated at 4, 25 and 37°C. Viable counts of the cells were estimated according to the method described above.

Viable but non-culturable cells of *Shigella* spp.: To determine the VBNC state of NaCl-treated *Shigella* spp., cells were grown in low-salt broth at 37°C, diluted 100 fold, transferred to nutrient broth (NB) medium supplemented with 500 mM NaCl and incubated for 30 min at the same temperature. A few drops of cells in NB medium was plated on NA plate and incubated at 37°C for over night. The rest of the cells from NB were harvested by centrifugation at 10,000 x g for 10 min. 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) and were observed under a fluorescent microscope equipped with a high mercury lamp (Olympus, Japan). In order to determine the morphological variation of the salt- treated (500 mM, NaCl) *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.

Invassiveness of salt treated *Shigella* spp.: 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 massaged to ensure the distribution of the organisms over the conjunctival sac following the method of Sereny (1955). 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

Effects of acidic conditions on the survivality of *Shigella* spp.: Both 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 32% survivality of both the species of *Shigella* was noted after 120 min of exposure (data not shown), that is enough for causing bacillary dysentery when ingested to human intestine. In an experiment, conducted with *S.sonnei* at different growth phase (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. boydii* (data not shown). 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. 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, 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. we have checked acid resistance of *S. sonnei* and *S. boydiii* grown in various acidic conditions. pH of the growth medium clearly had a significant effect on subsequent survival in acidic pH: the higher the growth medium pH, the lower the tolerance and vice versa (Table 1). The pattern of this adaptive response was similar to that observed in *Salmonella* spp. (Foster and Hall, 1990).

Response of salt-induction on subsequent acid sensitivity: Effect of salt treated *Shigella* spp. on their subsequent survivality on acid was monitored in this experiment. Both the species of *Shigella*, treated with NaCl was more sensitive towards acid (pH 3.0) than that was not treated with NaCl and the effect is proportional to time of incubation at 37°C (Fig 2 and 3). In a separate study, carried out with a number of salts ($CaCl_2$, KCl, NH_4Cl and Na_2SO_4), sensitivity towards acid (pH 3.0) was rapidly gained in *S. sonnei* at 37°C (Table 2). Although a slight sensitisation 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. Sensitization was more prominent at 200 to 300 mM in most cases and almost fully completed at 400 and 500 mM. A similar type of acid response was reported in *E. coli* (Rowbury *et al.*, 1994) and *S. dysenteriae* and *S. flexneri* (Sultana *et al.*, 2002). A combination of all the tested salts at a final concentration of 85 mM was examined to find their combined

Table 1: Relationship of growth medium pH and acid tolerance in *Shigella sonnei* and S. *Boydii*

Growth medium pH		Survival (%) of strains[a]	
Before growth	After growth	S. *sonnei*	S. *Boydii*
5	4.72	58	55
6	5.93	45	40
7	6.77	28	23
8	7.56	12	9

Survival pereantages were calculated from viable count of the cultures after 2 h of incubation at 37%°Cin NB acidified to pH 3.

Table 2: Effect of various salts at different concentration on survivality of *Shgella sonnei* incubated under condition of 37 °C and at pH 3

Salt (mM)	Percent survival											
	$CaCl_2$			KCl			NH_4Cl			Na_2SO_4		
	Time (min)			Time (min)			Time (min)			Time (min)		
	5	15	30	5	15	30	5	15	30	5	15	30
50	30	18	8	28	14	7	32	16	8	30	18	8
85	40	22	11	40	29	8	39	8	5	42	29	14
100	12	7	3	11	5	4	7	5	3	12	6	3
200	10	4	1	9	5	1	6	3	2	10	5	1
300	7	2	0.5	6	3	1	4	2	1	5	2	1
400	3	0	0	1	2	0	2	1	0	3	1	0
500	1	0	0	0	0	0	0	0	0	0	0	0

Table 3: Invassiveness of *Shigella sonnei* and S. *boydii* treated with or without NaCl

Time after Inoculation (h)	Keratoconjunvtivitis			
	S. sonnei		*S.boydii*	
	Salt induced	Uninduced	Salt induced	Uninduced
0	-	-	-	-
24	+	+	+	+
48	+	+	+	+
72	+	+	+	+
96	-	-	+	+

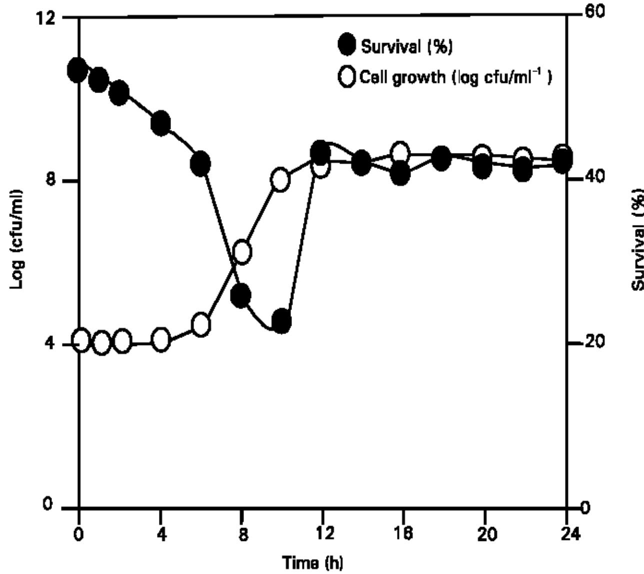

Fig. 1: Growth phase-dependent acid tolerance of *S. sonnei*. Cells were grown in nutrient broth, pH 7.2 at 37°C. Survival percentages were calculated after incubation of this culture in NB, pH 3.0 for 3 h.

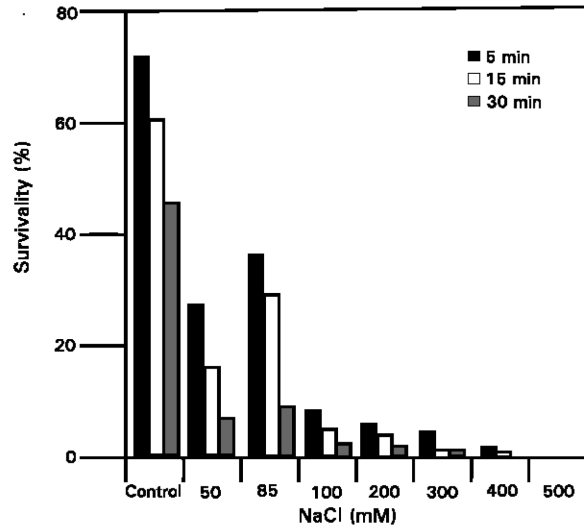

Fig. 2: Effect of NaCl concentrations on the survivality of *S. sonnei* incubated at 37°C, pH 3.0.

effect on acid sensitivity of *S. sonnei*. The combination of each salt at the above mentioned concentration produced more pronounce effect at pH 3 than that was obtained with NaCl only at 85 mM (Fig. 4)

Effect of salt-induction on survivality in different water samples: *Shigella* spp. treated with 85 mM NaCl were inoculated to a number of water-based samples and incubated at different temperatures. Viable cells of both species of *Shigella* could be observed in normal saline and GN broth (control experiment) for up to 7 days at 37°C (Figs. 5 and 6). Survivality of *S. sonnei* and *S. boydii* was observed in pond water for more than 120 h at the same temperature. In case of tap water and distilled water viable cells could not be observed after 60h. Although the individual values varied upto 2 fold, the same pattern of survivality was observed at 25 and 4°C.

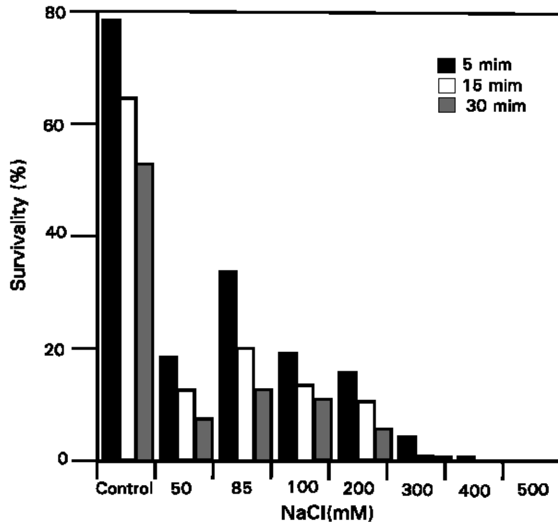

Fig. 3: Effect of NaCl concentrations on the survivality of *S. boydii* incubated at 37°C, pH 3.0.

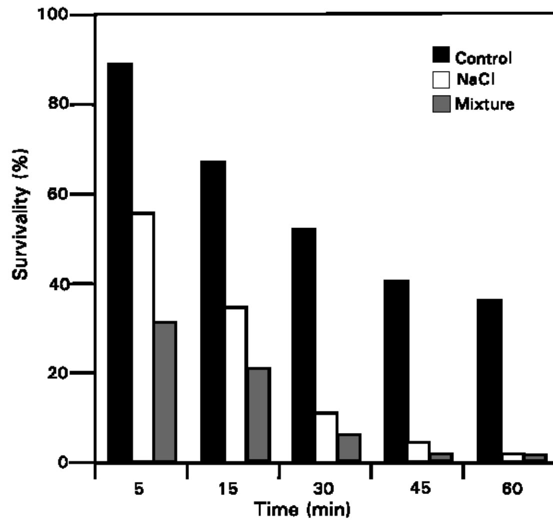

Fig. 4: Effect of a combination of salts at a final concentration of 85mM on the survivality of *S. sonnei* at 37°C, pH 3.0.

Detection of VBNC state of *S. sonnei*: It was revealed by plating on NA that *Shigella* spp. treated with 500 mM salt became non culturable within 30 min. However, non-culturable cells remain viable in a prolonged period of time as was reported by 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 cells of *Shigella* were orange in colour that indicates the VBNC state of cells (Fig 7A). This result correlates with the findings described by Roszak and Colwell (1987). Salt-treated VBNC state of *Shigella*, while treated with yeast extract and nalidixic acid, became elongated as was revealed by fluorescent microscopic observation (Fig. 7B).

Virulence properties of NaCl-treated *Shigella* spp.: A number of assay methods were employed to determine the virulence properties of salt treated *Shigella* spp. Results of Sereny test, (Table 3), indicate that both salt treated and non-salt treated *S.*

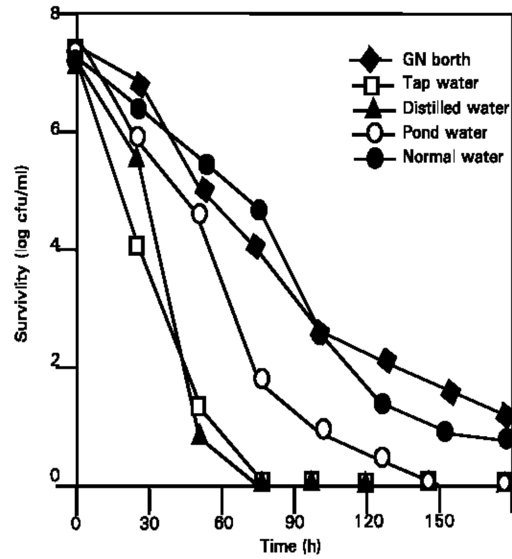

Fig. 5: Survivality of NaCl-treated (85 mM) *S. sonnei* in various water samples. survival percentages were calculated every 24h after incubation in different water samples.

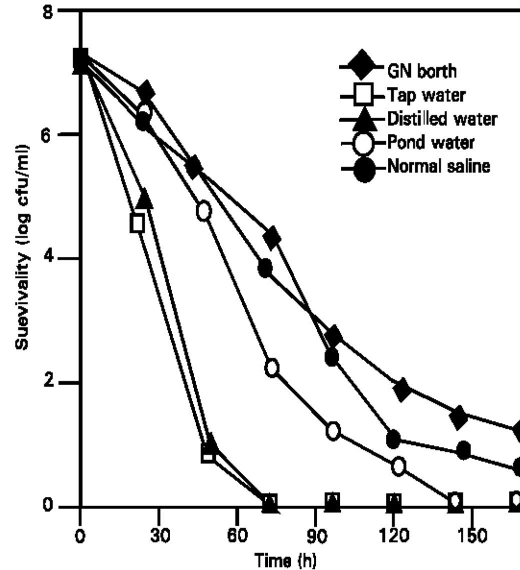

Fig. 6: Survivality of NaCl treated (85 mM) S. *boydii* in various water samples. Survival percentages were calculated every 24h after incubation in different water samples.

sonnei and *S. boydii* 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

The effect of salt-induction on *S. sonnei* and *S. boydii* and their subsequent survivality towards acid and virulence properties of the salt-treated cells were studied. It was found that *Shigella* spp. grown in nutrient broth showed a high level of acid tolerance. It has been reported that in *E. coli* acid resistance mechanisms include oxidative, glutamate-dependent and arginine-dependent

Fig. 7: Detection of viable but non-culturable form of *Shigella* spp. (A) VBNC of *S. sonnei*, determined by acridine orange staining method under fluorescent microscope; (B) yeast extract and nalidixic acid treated elongated VBNC of *S. sonnei* detected under fluorescent microscope.

systems which contribute their ability to survive the acidic conditions of the stomach (Lin *et al.*, 1996). Although not yet clarified, acid resistance of *S. sonnei* and *S. boydii* may also dependent on such types of mechanisms that may contribute to their relatively low infective dose that strongly support the hypothesis of Gorden and Small (1993). In the experiment of growth phase dependent acid tolerance, it was found that stationary phase cells are more acid resistant than log-phase cells. This finding suggests that log-phase cells of *Shigella* spp. are sensitive to adverse environmental conditions such as pH, heat, antibiotic, radiation etc. The lower sensitivity of stationary-phase bacterial cultures may be attributed to several physiological properties not present in exponential growth. These include increased proteolysis (Matin *et al.*, 1989), induction of several starvation-related proteins (Groat and Matin, 1986) etc. 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 survivality 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. is controlled genetically through the synthesis of several genes that involves two distinct responses: pre-challenge adaptation and transient adaptation (Foster, 1991, 1993). *S. sonnei* and S. *boydii* were found to be more acid sensitive (pH 3) when grown in a medium supplemented with a series of salt. Salt-induced acid sensitivity involves a phenotypic change in most or all of the exposed organisms. Rowbury (1994), reported that salt induction is independent of DNA and protein synthesis. The range of salts which sensitize suggests that it is the rise in osmotic pressure which is the trigger. Induction of acid sensitivity also might be attributed by several other factors including changes in sodium/proton anti porter system and inactivation of RpoS gene. This type of mechanism also has been reported previously (Ohyama, 1992; Pandan and Schuldiner, 1987). In this study we have demonstrated that salt-treated *Shigella* can survive *in vitro* when inoculated into a number of aquatic environment. However, the survivality varies with the source of water. 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. Almost similar pattern of survivality was recorded in *S. dysenteriae* and *S. flexneri* (Sultana *et al.*, 2002). It has been reported that the presence of detergents, organic materials, humidity, light, turbidity etc. may hinder prolong survival of bacterial pathogen (Marsharipov, 1970). In this study it has been demonstrated that salt-treated *Shigella* spp. remain viable as VBNC form that could be detected by FA technique. A similar type of observation was detected in *Vibrio cholerae* 01 (Kogure *et al.*, 1979). The non-culturable but viable *Shigella* spp. reported here might 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) and these cells can resume active growth when environmental conditions are restored (Colwell, 1993; Whitesides and Oliver, 1997). In this study, it has also been demonstrated that salt treatment could not alter the virulence properties of *Shigella* spp. In summary, theses studies may resolve the understanding on the survivality of the *Shigella* spp. in a number of physico-chemical stress conditions that may be helpful in determining the infective dose of these pathogenic bacteria. On the other hand, VBNC state of cells could be a serious information on the judgement of quality of a number of foods.

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