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Influences of Some Physico-Chimical Stress Conditions on the Survivality and Resistibility of *Shigella flexneri* and *Shigella sonnei*

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Abstract: Impacts of some physico-chemical stress conditions on the survivality and resistibility of *S. flexneri* and *S. sonnei* have been investigated. Acid and salts tolerance and/or resistance of *S. flexneri* and *S. sonnei* are induced during their survivality and pathogenesis with specific mechanisms. Results of the present study revealed that *S. flexneri* and *S. sonnei* could survive longer than 125 min to acidic circumstance at pH 3 and approximately 31% for both the mentioned species of *Shigella* subjected to experiments was reported. The acid challenge for both the species of *Shigella* was found to be dependent on the growth phase and pH of the growth culture media. The data also pointed out that *Shigella* spp. already grown in low-salt Tryptic Soy Broth (TSB) and/ or Nutrient Broth (NB) at pH 7.2, yielded microorganisms which were significantly more acid sensitive when subsequently cultivated in the same broth supported with 200 mM or more salt at 37 °C. Moreover, concerning of the experiments carried out on a number of water specimens, the investigation recorded various survivality and resistibility patterns with salt-treated *S. flexneri* and *S. sonnei.* In addition, regarding response of salt-induction on subsequent acid sensitivity of *Shigella* spp., the obtained results established a different ranges of sensitization. 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. These results assessed by fluorescent microscopic examination, which showed that *Shigella* spp. treated with 500 mM salt went into non-culturable state, but remain viable. In conclusion, salt treatment did not significantly change or interfere with infective characteristics of *Shigella* spp. The importance of these findings in food preservation practice was referred to.

Key words: *Shigella* · Acid Susceptibility · Salt induction · Salt resistance · Viability · VBNC · Virulence.

INTRODUCTION

Shigella is a genus of Gram-negative, facultatively anaerobic, rod-shaped bacteria of the family *Enterobacteriaceae*, made up of nonmotile bacilli that cannot utilize citrate as a sole carbon source and that ferment carbohydrates with acid but no gas production. The genus consists of four species, differentiated by biochemical reactions: *S. dysenteriae* (subgroup A), *S. flexneri* (subgroup B), *S. boydii* (subgroup C) and *S. sonnei* (subgroup D). Their normal habitat is the intestinal tract of humans and higher monkeys; all species cause dysentery. *S. flexneri*, is a species that causes severe dysentery. It has six serotypes and two variants (X and Y); type 2 produces an enterotoxin. *S sonnei*, is also the least pathogenic species, causing a milder but frequently encountered form of bacillary dysentery; organisms are serologically homogeneous, but two antigens, designated I and II, occur in varying proportions [1,2].

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 [3]. Shigellosis is a public health concern in developing countries, particularly for young children who make up 69% of all cases. The majority of shigellosis cases in the Asian, African and Central American regions are caused by *Shigella flexneri* [*S. flexneri*] [4]. Bacteria are transmitted *via* the fecal oral route and require as little as 100 organisms to cause the disease. *S. flexneri* penetrates the epithelial layer of the large intestine, invading and spreading throughout the intestinal epithelial cells.

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A number of proteins are known to be important for these invasion steps, particularly the products of the *mxi-spa* and *ipa* loci located on the virulence plasmid [5].

The low infectious dose [6] allows the disease to be spread effectively by infected food or water and also by person-to-person contact [7]. In Saudi Arabia, the disease is of high incidence and occasionally sporadic in several regions. The isolation rate of *Shigella* in routine surveillance of hospitalized diarrheal cases is not known definitely.

Shigella spp. Can be subjected to a number of stressful conditions including high concentrations of salt (NaCl, KCl. NH4Cl etc.) in a wide range of situations. They may enter in marine and estuarine waters whereas 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 [8]. 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 up to 2h before colonizing the intestinal tract [9]. It has been reported that, *Shigella* spp. are more acid tolerant (pH 2 to 2.5) than are *Salmonella* and *E. coli* [10]. *Salmonella* spp. and *E. coli* can adapt and grow at low pH values if sequential acid adaptation is performed [11-13]. 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. [10,11,14]. The prior exposure to a mild dose of a stress may alter the response to another [15]. Rowbury *et al*. [8] 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 [16] and responses to such stress may influence subsequent ability to survive and cause disease [11]. The capability of pathogenic microorganisms to exist in the viable but non-culturable (VBNC) state has been reported [17]. Therefore, the potential health hazard of *Shigella* spp. existing in the VBNC state may be important, since *Vibrio cholera* 01 could be isolated in the culturable from stools of volunteers after ingestion of VBNC *V. cholera*e 01 [18]. Furthermore, some investigators claim that non-culturable bacteria of selected species can be resuscitated to the culturable state [19]. Since, non-culturable cells may still retain metabolically activity and, if pathogenic, might maintain their infectiveness [20,21], 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 [17]. 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 [4,22,23]. On the other hand, *Shigella flexneri* and *Shigella sonnei* are considered a very pathogenic bacteria for diarrhea in humans, since a several investigations have been carried out on the infections of Shigellosis occurred by the two mentioned bacteria [2,24-26]. Many studies have previously described Shigellosis frequencies caused by different species of *Shigellae* throughout the world, in addition to characterization of *Shigella* spp. [2,26,27].

We have investigated the impacts of some physico-chemical stress conditions on the survivability and resistibility of *Shigella flexneri* and *Shigella sonnei*. The goal of this study was to characterize the acid resistance of *Shigella* spp. and invasiveness of salt treated *Shigella* spp. Subjected to experiments.

MATERIAL AND METHODS

Microorganisms: *Shigella flexneri* and *Shigella sonnei* strains were obtained from the Al-Hada Armed Forces Hospital, Department of Laboratory Medicine, Microbiology Section, in Taif governorate, Kingdom of Saudi Arabia. The Shigella strains were isolated from human tools during an outbreak of dysentery in Taif, K.S.A.

Inoculum: Working cultures were maintained on Xylose Lysine Deoxycholate Agar (XLD) [Scharlau Chemie S.A., Barcelona, Spain, European Union] and on Trypticase Soy Agar (TSA) [TSA, Difco Laboratories, Detroit, Michigan, U.S.A] slants at pH 7.3 and stored at 4°C. Wherever needed, these were activated by transferring loop inocula into 7 ml Tryptic Soy Broth (TSB) (Scharlab, S. L., Barcelona, Spain., European Union) at pH 7.2, grown overnight at 37 °C and then refrigerated. This broth was used to inoculate overnight cultures and was not kept for

longer than 1-week before a new culture was generated from the slant culture. Overnight cultures were started by inoculating into Tryptic Soy Broth (TSB) or/ and Nutrient Broth (NB) [Scharlau Chemie S. A., Barcelona, Spain, European Union] medium containing 1% dextrose (TSB + 1% G) medium using a 0.1 ml of the culture broth and were incubated at 37° C without shaking for at least 18 h.

Acid Tolerance of *Shigella* spp.: Acid tolerance of *Shigella flexneri* and *Shigella sonnei* strains was determined according to the previously reported method [3], and as a technique advised by Al-Bashan [13] for *Escherichia coli* O157:H7 Serotype and *Salmonella typhi* (A Group D Serotype), with some modifications. Briefly, fresh, overnight cultures of *Shigella* spp. in tryptic soy broth (TSB) 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 Trypticase Soy Agar (TSA) and on Nutrient Agar (NA) [CM 0003 Nutrient Agar, Oxoid, Oxoid Ltd, BASIGSTOKE, Hampshire, England], incubated at 37 °C for 24 h and colony counts were compared with those from plated dilutions of the original culture to estimate the percent survival. In order to define the growth phase dependent acid tolerance of *Shigella* spp., fresh over night cultures were diluted in the same TSB or/and NB media and incubation was continued at 37 °C gentle shaking. Samples were withdrawn hourly and centrifuged at 10,000 x g for 5 min. The plates were suspended in equal volume of NB or/ and TSB adjusted to pH levels of 7.2 and 3.0. The pH 7.2 samples were plated immediately on nutrient agar and/or Trypticase Soy Agar; the pH 3.0 samples incubated at 37°C for 2h before being plated on nutrient agar and / or Trypticase Soy 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. As 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 and / or Trypticase Soy 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 investigation was carried out according to the method described elsewhere [3,8] 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 500mM) 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 and/or TSA plates and incubated overnight at 37°C and viable counts were recorded. A mixture of a number of salts at a final concentration of 85 mM 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 and/or TSB 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 and/or TSB media 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%, 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. Subjected to test, cells were grown in low-salt broth at 37°C, diluted 100 fold, transferred to nutrient broth (NB) and /or TSB media supplemented with 500 mM NaCl and incubated for 30 min at the same temperature. A few drops of cells in NB and/or TSB media were plated on NA and/or TSA plates and incubated at 37°C for over night. The rest of the cells from NB and/or TSB 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 [28] and were observed under a fluorescent microscopic equipped with a high mercury lamp (Olympus, Japan). In order to define 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 techniques.

Effect of Salt Treated *Shigella* spp. On Their Subsequent Acid Susceptibility and Viability: The NaCl (85 mM) induced bacterial 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 [29]. The animals were observed over a period of a 96 h for kerato-conjunctivitis. A control experiment also was carried out with cells not induced by NaCl.

RESULTS

Impacts of Acidic Conditions on the Survivality of *Shigella* spp.: Results are recorded in tables 1-4.

The results obtained from this investigation on the relationship of growth medium pH and acid tolerance in *Shigella flexneri* and *Shigella sonnei* are respectively presented in table 1.

Experiments have tested acid resistance of *S. flexneri* and *S. sonnei* 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. Both species of *Shigella* showed marked challenge and resistance to pH 3.0 (a pH that is very close to gastric secretion), incubated at different time intervals and percent survivality was estimated when subjected to several acidic conditions. Also they did not differ significantly in their survival percentages, under these acidic environments with the tests when acid tolerance determinations conducted two hours apart of incubation at 37°C in NB and/or TSB acidified to pH 3. Around 31% survivality of both the species of *Shigella* was recorded after the periods of exposure (125 min) to acidic conditions (data not revealed), that is enough for causing bacillary dysentery when ingested to human intestine. Regarding experiment, carried out on *S. flexneri* at different growth phase, it has been established that

Table 1: Relationship of growth medium pH and acid tolerance in *Shigella flexneri* and *Shigella sonnei*

Growth medium pH		Survival (%) of strains	
Before growth	After growth	*Shigella flexneri*	*Shigella sonnei*
5	4.76	57	60
6	5.97	42	47
7	6.80	25	30
8	7.59	11	14

Survival percentages were calculated from viable count of the cultures after 2 h of incubation at 37°C in NB and/or TSB acidified to pH 3.

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

Percent survival												
	$CaCl_2$			Kcl			NH_4Cl			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	31	19	8	29	15	8	31	15	9	31	17	9
85	40	22	10	39	28	9	37	10	4.5	42	28	13
100	13	9	4	11	5	5	9	5	2.5	13	6.5	3
200	11	5	1.5	5.5	5	1.5	7	4	2	9	5	1
300	10	4	1	5	3	1.5	5	2.5	1	5.5	2.5	1
400	4	1	0	2	1.5	0	3	1	0	3.5	1	0
500	1	0	0	0	0	0	0	0	0	0	0	0

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

	Percent survival											
	$CaCl_2$			Kcl			NH_4Cl			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	32	20	10	30	16	9	34	17	11	32	19	10
85	42	24	12	41	30	10	38	12	8	44	30	15
100	14	8	5	13	6	6	11	6	5	14	7	4
200	12	6	2	10	6	2	8	6	3	11	6	2
300	9	3	1	7	4	1	6	3	1.5	6	3	1.5
400	5	1.5	0	3	2	0	4	1.5	0	4	1.5	0
500	1	0	0	0	0	0	0	0	0	0	0	0

Table 4: Invasiveness of *Shigella flexneri* and *Shigella sonnei* treated with or without NaCl

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

the acid resistance and 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 resistance and tolerance, demonstrated at the early stationary phase, was about 100 fold less than that at the late stationary phase. From table 1, it is clearly evident that the survival (%) strains of *S. flexneri* was somewhat less than of the survival (%) strains of S. sonnei. A similar impact was detected for *S. sonnei* (data not revealed). Results in relation to effects of acidic conditions on the survivality of *Shigella* spp. were confirmed by several independent trials. Although the recorded individual values of these trials varied at times by as much as three fold, the basic shape of the graph remained almost the same There was no a significant difference in survivality (%) between both species of *Shigella* subjected to various acidic conditions. On the other hand, the results of this study suggested that some factors in stationary phase cells persist over several generations of log-phase growth. In each experiment of acidic condition effects on the survivality of *Shigella* spp., a typical growth phase dependent acid tolerance was also observed, and served as indication of this phenomenon exists in *Shigella* spp.

Effect of Salt Treated *Shigella* spp. On Their Subsequent Acid Sensitivity and Survivality: The response of salt-induction on subsequent acid sensitivity and survivality of *Shigella* spp. was manifested in this trial. The observations established in this experiment carried out on *S. flexneri* and *S. sonnei* are tabulated in tables 2 - 4. Both the mentioned species of Shigella treated with NaCl was more sensitive towards acid (pH 3.0) than that was not treated with NaCl and the impact is proportional to time of incubation at 37°C. In each treatment conducted apart on the *S. flexneri* and *S. sonnei* with a few of salts ($CaCl_2$, KCl, NH_4Cl and Na_2SO_4), sensitivity towards acid (pH 3.0) was rapidly gained in both the species of *Shigella* treated at 37°C with the salts pointed out. Although a slight sensitization was recorded after 5 min, the influence was much more pronounced after 15 min and the effect was almost completed at 30 min. In the present experiment, it was found that the concentrations of salts used for trial, play an important role in the increase and/ or decrease of sensitivity and survivality of both the species of *Shigella* treated with these salts. The intensity of the effect was mainly dependent on the concentrations of salts used for induction in the experiment carried out on the above *Shigella* spp. subjected to treatments.

species of *Shigella* separately (Tables 2 and 3) at 50, 85, 100, 200, 300, 400 and 500 mM, but in conjunction with salt concentrations. Acid sensitivity was more evident at 200 to 300 mM in the experiments of effect of various salts at different concentrations on survivality of *Shigella* spp. incubated under condition of 37°C and at pH 3. Sensitization was almost fully completed at 400 and 500 mM. A combination of all the tested salts at a final concentration of 85 mM was examined to find their combined effect on acid sensitivity of *S. flexneri* and *S. sonnei*. The combination of each salt at the above mentioned concentration of 85 mM induced more pronounce impact at pH 3 than that was observed with NaCl only at the concentration of 85 mM. It has been increasingly realized that more fundamental knowledge of acid sensitivity and survivality for *Shigella* spp. is needed to obtain more efficient estimation and control of metabolic chemical reactions in *S. flexneri* and *S. sonnei* microorganisms caused enteric disorders in humans.

Impact of Salt-Induction on Survivality in Different Water Samples: According to the procedures and results of this investigation, *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. Survivality of *S. flexneri* and *S. sonnei* was observed in pond water for more than 125 h at the same temperature. In case of tap water and distilled water, viable cells could not be observed after 65 h. Although the individual values varied up to 2 fold, the same pattern of survivality was observed at 25° and 4° C.

Detection of VBNC state of *S. flexneri* and *S. sonnei*: It was established by plating on NA that *Shigella* spp. treated with 500 mM salt became non culturable within 30 min. On the other hand, non-culturable cells were remained viable in a prolonged period of time. Acridine orange staining technique was employed to determine the viability of salt treated *Shigella* spp. Flourescent microscopic observation revealed that a huge numbers of cells of *Shigella* were orange in colour that indicates the VBNC state of cells. Salt- treated VBNC state of *Shigella*, while treated with yeast extract and nalidixic acid, became elongated as was revealed by fluorescent microscopic observation.

Virulence Properties of NaCl-treated *Shigella* spp.: For determination of virulence properties of salt treated *S. flexneri* and *S. sonnei*, a number of estimation techniques were used. It is evident that the both salt treated and non-salt treated *S. flexneri* and *S. sonnei* caused the induction of keratoconjunctivitis in guinea pigs eye. From the above mentioning result, it can be concluded that salt treatment could induce no effective change in invasive properties of *Shigella* spp. This can observed clearly under the NaCl – treatment of *S. flexneri* and *S. sonnei* separately to establish the virulence properties of the above *Shigella* spp. and also to detect the effect of salt upon the viability of these microorganisms. However, No change of virulence properties in salt treated *Shigella* spp. also was established in a number of tests carried out such as haemagglutination test, salt aggregation test and in congo-red binding test (data not declared).

DISCUSSION

Shigellosis is any condition produced by infection with organisms of the genus *Shigella*, such as bacillary dysentery. Several members of the genus *Shigella* have been examined for differences in extreme acid survival strategies. *S. flexneri*, is one of the *Shigella* species that causes sever dysentery. It has six serotypes and two variants (X and Y); type 2 produces an enterotoxin. Further, the recent investigations pointed out that *S. sonnei* is the least pathogenic species of *Shigella*, causing a milder but frequently encountered form of bacillary dysentery; organisms are serologically homogenous, but two antigens, designated I and II, occur in varying proportions [2].

Impacts of some physic-chemical stress conditions on the survivality and resistanity of *S. flexneri* and *S. sonnei* were investigated. Microorganisms are adapted for optimum functioning in their normal physiological environments. Any extreme change in environmental conditions from the optimum inflicts a stress on an organism. The extent of the change will determine whether the organism is killed, ceases growth, or has an increased lag time and reduced growth rate [30]. Most bacteria are able to tolerate small changes in an environmental parameter and can adapt over the time scale of minutes, hours, or days [31]. Microorganisms do this by both yielding to the stress conditions and making suitable provisions for survival or attempting to resist the stress [32]. For most organisms, this tolerance can be pushed to maximum limits if the cell is provided with sufficient opportunity to sense and adapt to the deteriorating environment. In the present study, it was detected that *Shigella* spp. grown in nutrient broth and /or tryptic soy broth revealed a high level of acid tolerance. Quite a few

of investigations stated that the entire groups of microorganisms such as psychrophiles, acidophiles and halophiles have adapted their lifestyles to prefer these extreme environments[33,34]. Acidophiles are microorganisms that have their growth optimum between about pH 1.0 and 5.5 and halophiles are microorganisms that require high levels of sodium chloride for growth such as 2.8 molal and up to 6.2 molal for extreme halophiles [35]. Changes in environmental conditions away from the optimal value can cause the induction of many elaborate stress responses. These strategies are generally directed at survival rather than growth. It has been demonstrated that the acid tolerance was highest at late stationary phase(overnight cultures), which decreased several logs when were at the mid exponential phase. A second peak of high acid tolerance estimated at the early stationary phase, was about 100 fold less than that at the late stationary phase. A similar growth phase dependent acid tolerance was also observed in enterohemorrhagic *E. coli* [36], *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 gens responsible for adaptive acid tolerance, have been isolated from *E. coli* O157:H7 and *Salmonella* spp. [36,37].To find out whether the same phenomenon exists in *Shigella* spp. the experiments have checked acid resistance of *S. flexneri* and *S. sonnei* 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.[11]. It has been established that in *E. coli*, acid resistance mechanisms include oxidative, glutamate-dependent and arginine-dependent systems which contribute their ability to survive the acidic conditions of the stomach[38]. Although not yet clarified, acid resistance of *S. flexneri* and *S. sonnei* 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 [10].It has been reported that *Shigella* sonnei grown in nutrient broth showed a high level of acid tolerance [3]. *Shigella flexneri* also had increased resistance to extreme acid after pre-exposure to an acidic environment [39]. Therefore, the acidification process of low acidic foods must be performed quickly. The results of this study showed that *S. flexneri* and *S. sonnei* were found to be more acid sensitive (pH 3) when grown in a medium supplemented with a series of salt. These results revealed a close agreement with that reported by Sultana *et al*., [3] on *S. sonnei* and *S. boydii* who stated that *S. sonnei* and *S. boydii* were detected to be more acid sensitive (pH) when grown in a medium completed with a series of salt. Salt-induced acid sensitivity involves a phenotypic change in most or all of the exposed organisms. Rowbury [8], 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 [40,41]. In regard with mode of action of weak acid preservatives against microorganisms, several studies pointed out that the minimum inhibitory concentrations reported to inactivate various microorganisms may vary considerably [42,43]. In principle, growth inhibition can be caused by inactivation of, or interference with, the cell membrane, cell wall, metabolic enzymes, protein synthesis system, or genetic material [42].

The antimicrobial effect of weak acids is both pH-dependent and non-pH dependent, although this has been long established to be more active in an acid than in a neutral environment [44,45]. Acids generally inhibit molecular reactions essential to the microorganisms by increasing the hydrogen ion concentration, which results in a decrease in internal pH (pH_i) [46].This fall in pH_i is a major cause of growth inhibition by weak acids [47].The pH of the environment and the dissociation constant (pKa) of the weak acid determine the proportion of the hydrophobic (undissociated) from in the medium and thus the effectiveness of the weak acid [46,48]. The strength of an acid is defined by its dissociation constant (pKa). This is the pH value when the dissociated and undissociated forms of the acid are in equal amounts. Strong acids such as hydrochloric acid have a much lower pKa value than weak acids. Thus at a pH of between 3 and 6, the pH range for normal foods, strong acids will be dissociated whereas weak acids will be undissoiated. This later form is membrane-permeable and thus allows the weak acid to enter the microbial cell [49].Once inside the cell, weak acids generally encounter a higher pH due to the cell buffers, dissociate and become toxic, which ultimately inhibits cell growth due to the acidification of the cell interior [50,51]. Therefore the lower the pH value, the greater the proportion of the acid in the undissociated form and thus the greater the antimicrobial effect [52,53]. The present investigation has demonstrated that salt-treated *Shigella* can survive *in vitro* when inoculated into a number of aquatic environment. It has been recorded

that the survivality varies with the source of water. Experiments conducted in this field, have been detected that the 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 demonstrated in *S. dysenteriae* and *S. flexneri* [24]. It has been reported that the presence of detergents, organic materials, humidity, light, turbidity etc. may hinder prolong survival of bacterial pathogen [54]. The results of this study has been revealed that salt-treated *S. flexneri* and *S. sonnei*, remain viable as VBNC from that could established by FA technique. It was evident by plating on NA that *Shigella* spp. treated with 500 mM salt became non culturable within 30 min. According to Rollins and Colwell [55], It has been recognized that non-culturable cells remain viable in prolonged period of time. However, a similar type of observation was previously established in *Vibrio cholera* 01 by too many investigators [56]. The non-culturable, but viable Shigella spp. reported here might have a great significance in understanding the epidemiology of diarrhea. If those non-culturable cells are ingested by humans, their might be a possibility to revert to culturable state and cause shigellosis, as has been recorded for *V. cholera*e 01 in volunteer studies [18] and these cells can resume active growth when environmental conditions are restored [57,58]. 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. This result coincides with that of Roszak and Colwell [28]. The present investigation, has also been declared that salt treatment could not alter the virulence properties of *Shigella* spp.

It would be assured that the control of microorganisms, is one of the most important aspects of food preservation. Bacterial destruction ensures food safety, but it also involves the application of more intense treatments that may cause additional food quality losses [59]. In many cases, bacterial destruction is not necessary for food preservation and controlling the environmental factors that affect viability can be sufficient to inhibit bacterial growth. In this case, the microorganisms will not be destroyed, but they will not be able to grow and the preservation techniques used, which are much less intense, will affect food quality to a lesser extent. However, microorganisms will still be metabolically active and viable if transferred to favorable conditions [59-61]. Preservation techniques are designed to prevent the internal environment of the cell such that growth is no longer possible.

It may be concluded that the understanding of the biochemical mechanisms and factors affecting survivality and resistibility of *Shigella* spp. under different physic-chemical conditions, constitutes the main step for resolving the pathogenic disorders caused by these bacteria, a fact which let us consider that the results of this studies were more helpful in estimating the infective dose of the above pathogenic bacteria. Moreover, VBNC state of cells could be a serious information on the judgment of quality of a number of foods. The techniques of this investigation, can be used for determining of some biochemical properties of various pathogenic microorganisms other than *Shigella* spp., in relation with survivality and resistibility in different physic-chemical stress environments.

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