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SURVIVAL POTENTIALITY OF *SHIGELLA BOYDII* 15 ATCC12034 IN LABORATORY MICROCOSM USING WATER FROM TWO SOURCES

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Abstract

Shigellosis is a waterborne disease but detection and isolation of *Shigella* in the water cannot be credibly ascertained even during epidemics. The detection, persistence or survival of *Shigella* in water is thus quite imperative to control the disease. The present study attempts to study the survival of *Shigella* in waters of different sources using *Shigella boydii* 15 ATCC12034 as reference culture. Five microcosms were set up; these were pond water (from two locations), tap water, distilled water and Phosphate Buffer Saline (PBS). Relatively longer bacterial persistence was found in microcosms with pond water as compared to that observed in microcosms with PBS and distilled water. With the initial inoculums of 10^5cfu/ml, the strain became nonculturable after 9 and 18 days in PBS and pond water, respectively. But with the inoculum size of 10^6cfu/ml, it survived up to 16 to 22 weeks in pond waters from two different sources. The morphological and biochemical characteristics of the bacteria remained unchanged over this time. Fluorescent microscopy and PCR were carried out to detect the presence of *Shigella boydii* 15 in the waters after it became nonculturable in conventional nutrient media. The survival of *Shigella* in water was possibly inoculum's size dependent; its presence might diminish with time due to lack of nutrients and shifting of physicochemical factors, etc. in water.

Introduction

Diarrhoea continues to be one of the leading causes of morbidity and mortality in the world and is ranked fourth as a cause of death.(1) Even though economic development and progress in health care delivery are expected to catalyze substantial improvements in infectious-disease related morbidity and mortality during the next 30 years, it is predicted that diarrhoea will remain a leading health problem.(2) Shigellosis is an important cause of diarrhoeal deaths. *Shigella* spp. cause an estimated 1 million deaths world-wide and 163 million cases of dysentery annually, predominantly in children younger than 5 years of age in developing countries.(3) Shigellosis is endemic in Bangladesh, and it is estimated that dysentery accounts for 20% of deaths related to diarrhoea among children.(4) The genus *Shigella*, causing shigellosis, is comprised of four species, namely *S. flexneri*, *S. dysenteriae*, *S. boydii* and *S. sonnei*. Clinical infection can

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occur with transmission of as few as 10 *Shigella*.[5] The prevalence of the serotypes of *S. flexneri* in Bangladesh has been described to have a temporal variation in the dominance of different subserotypes. The emergence of some atypical serotypes of *S. flexneri* has also been reported.[6] There are no reports of outbreaks caused by the serotypes of *S. dysenteriae* other than serotype 1 in Bangladesh or any other part of the world.[7] Surprisingly, *S. boydii,* which is thought to be relatively rare, is responsible for nearly one-quarter of shigellosis episodes in Bangladesh lately.[8]

Open waters in the environment play an important role to spread diarrhoeal disease in the community and the presence of *Shigella* in environmental waters has been reported.[9] Although contaminated water is a major causative agent for shigellosis, until now there is little or no credible report on isolation and recovery of *Shigella* from aquatic environment. Probably, the *Shigella*e are fragile or might remain in a state of dormancy or might be starved, injured or stressed. In water, there may be lack of appropriate nutrients or growth factors and the physiological factors may not be favorable for cell resuscitation or repair. There are also reports that cells may undergo "viable but nonculturable" (VBNC) state when exposed longer to some environmental conditions.[10] This phenomenon implies that metabolically active cells are incapable of growth on media normally supporting the growth of those cells. Although other waterborne pathogens like *Vibrio* and *Salmonella* have already been detected by routine culture means in water.[11] *Shigella* in water samples still remains an enigma. Probably they enter into the viable but nonculturable (VBNC) state. Such *Shigella*e can be detected by techniques, which are culture independent, such as PCR (Polymerase Chain Reaction), fluorescent microscopy etc. Therefore, attempts were taken to detect the survival potentiality of *Shigella boydii* 15 ATCC 12034 in water.

Materials and Methods

Shigella boydii 15 ATCC12034 strain was obtained from the Department of Microbiology, University of Dhaka and used for survival study of *Shigella* spp. in laboratory microcosm. The strain was streaked on nutrient agar plate and incubated at 37°C for overnight. Gram staining, biochemical and serological tests and antibiogram were performed according to the standard procedures to reconfirm the strain.[10]

A loopful of freshly cultured *Shigella boydii* 15 strains was suspended in the test tube containing 10 ml of sterile normal saline. It was then vortexed to prepare a homogenous cell suspension. The optical density of the suspension at A_{600} was set at 0.11 and 0.28 with a spectrophotometer to prepare the desired concentration of cell suspension of about 10^5 and 10^6cells/ml, respectively. The number of cells per ml was then assessed by viable counts by the drop plate technique.[12]

Seven 250 m1 conical flasks were taken for microcosm study. The following five different types of microcosms were set in the environmental laboratory of the

Department of Microbiology:

1. Shahidullah Hall pond water with initial inoculum density of 10^5cells/ml.
2. Shahidullah Hall pond water with initial inoculum density of 10^6cells/ml.
3. Jagannath Hall pond water with initial inoculum density of 10^6cells/ml.
4. Phosphate Buffer Saline (PBS) with initial inoculum density of 10^5cells/ml.
5. Distilled water with initial inoculum density of 10^5 cells/ml.

Each water samples and the PBS used for setting the microcosms were autoclaved for sterilization before use. Before inoculation, the load of microorganisms present in all the flasks was checked to confirm that the water was sterile to avoid any contamination.

Microcosm flasks were kept in a shaking incubator each day for several hours for providing aeration and agitation. Samples were taken on day 0, 1, 2, 5, 7, 8, 9, 12, 15, 18 from the flasks containing PBS and Shahidullah Hall pond water (10^5 CFU/ml). In case of Shahidullah Hall pond water (10^6CFU/ml) and Jagannath Hall (10^6CFU/ml), the samples were taken on week 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22. Samples were diluted appropriately and number of CFU of total viable cell counts were assayed on selective MacConkey agar medium and nutrient agar plates following the drop plate method. Finally, a plot of time intervals in days versus log of CFU/ml of water sample was prepared for each type of microcosms.

A number of physicochemical parameters such as conductivity, salinity and total dissolved solid (TDS) were measured in pond waters of Shahidullah Hall and Jagannath Hall by conductivity meter (HACH, USA). The pH was measured using pH–fix 0-14 strip (Fisher, UK). This was done for both sterile and nonsterile pond waters to assess their effects on viability of *Shigella boydii*.

Sample (1.5 ml) was taken from each microcosm in an Eppendorf and centrifuged at 12000 rpm for 10 minutes. The pellet was suspended in 100 µl normal saline and vortexed. Thirty micolitre of the suspension from each eppendorf was taken on (4,6-Diamine 2-Phenyl-Indole (DAPI) membrane and semidried. Ten microlitre of DAPI solution was then added and was incubated at 37°C for 30 minutes. Finally, the viability count was taken under the fluorescent microscope.

PCR was carried out for genes specific for virulent properties of *Shigella,* using chromosomal DNA as template. PCR reactions were carried out with an initial denaturation step at 94°C for 3 min; followed by 35 cycles of 94°C, 1 min; 55°C for 1min 30 sec; 72°C, 1 min 30 sec, with the final extension of 7 min at 72°C.[9] Overnight fresh culture of the isolates at 37°C in nutrient broth was subjected to chromosomal DNA extraction and purification according to the procedure described previously [9]. The PCR products were later electrophoresed in 1% agarose gel.

Results and Discussion

In the present study, *Shigella boydii* 15 ATCC12034 was used as reference culture to observe the survival of *Shigella* in waters of different sources. The purity of the strain was confirmed by microscopic, morphological, cultural, serological and biochemical studies. Survival of *Shigella boydii* on water was considered as their ability to multiply and to form colonies on routine culture media. Five different types of microcosms were set with autoclaved pond water from two different places, autocolaved distilled water and autoclaved PBS and *Shigella boydii* 15 was used with different inoculum size. An initial inoculum of 10^5 CFU/ml was taken to observe the viability pattern of *Shigella boydii* 15 in autoclaved distilled water and no growth was found after 24 hours possibly due to unavailability of nutrients. Culturability of *Shigella boydii* 15 in autoclaved PBS and pond water of Shahidullah Hall was assayed by viable plate count (Fig. 1). Initially the inoculum was 10^5 CFU/ml. There was a sharp decrease from the very beginning of the inoculation in the viable count and no cell was found after 9 days in case of PBS whereas the bacteria survived up to 18 days in autoclaved pond water (Fig. 1).

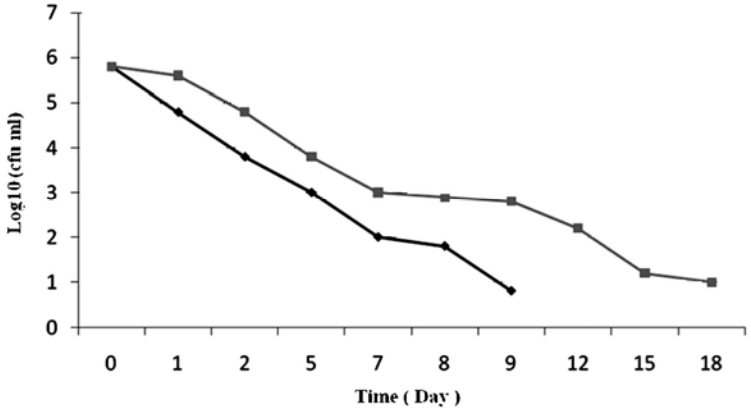

Fig. 1. Survivality of *Shigella boydii* 15 ATCC 12034 in autoclaved pond water of Shahidullah Hall and phosphate buffer saline (PBS). --■-- autoclaved water of Shahidullah hall , --◆-- autoclaved PBS.

Culturability in autoclaved pond water of Jagannath Hall (10^6 CFU/ml) was found to continue for 16 weeks. However, the gradual fall of cell count was observed with time (Fig. 2). The cell count was taken up to 22 weeks and a gradual decrease in cell number was observed in autoclaved pond water of Shahidullah Hall initially containing 10^6 CFU/ml (Fig. 2). The bacterial strain was found to grow in MacConkey plates until 150 days. However, due to time constrain further observation could not be continued.

In a previous study, the viability data concluded that inoculum size was the impact factor for the persistence of the microorganism.[10] The findings of this study elucidate

that at higher density of inoculums, the persistence was longer as compared to lower density. So, in the microcosm with 10^6 CFU/ml inoculums density, the survival was observed for longer days (Fig. 2). The lower cell concentration in the natural water bodies might be a reason for lower survivability coupled with constraint for isolation and recovery of bacteria like *Shigella*. Its persistence in autoclaved water suggests its survival in nature may be limited by biological interactions. One important fact comes out of this experiment was that autoclaving enhances the availability of dissolved organic carbon, perhaps producing a better carbon source for *Shigella boydii* 15 than normal pond water. Other beneficial effects of autoclaving are destruction of bacteriophages and inactivation of thermolabile toxic substances such as antibiotics.

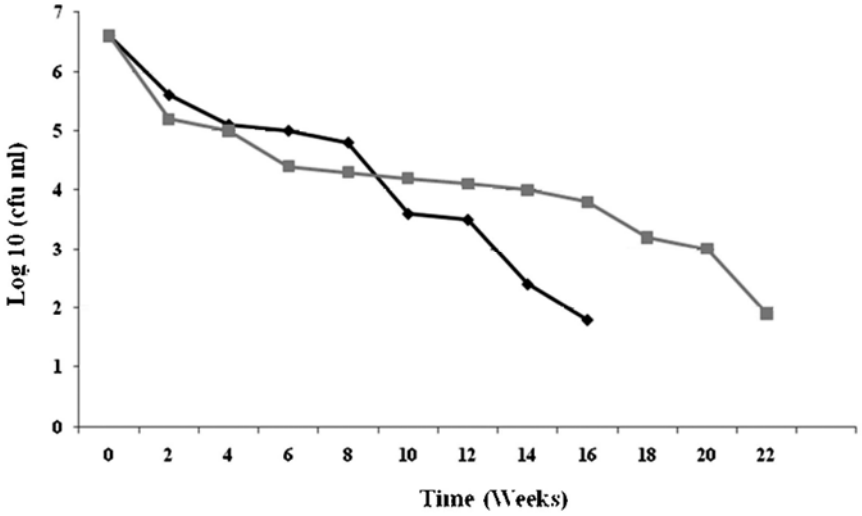

Fig. 2. Survivality of *Shigella boydii* 15 ATCC 12034 in autoclaved pond water of Shahidullah Hall and Jagannath Hall. --■-- Shahidullah Hall , --◆-- Jagannath Hall.

Various physico-chemical parameters (conductivity, salinity, TDS and pH) of the pond waters from Shahhidullah Hall and Jagannath Hall were measured to detect their effects on the survivability of the bacterium (Table 1). These were measured for both sterile and nonsterile water. The parameters were found to be similar for both of the waters but these were relatively lower for pond water of Shahidullah Hall in which longer survivability was observed as compared to Jagannath Hall pond water. Thus the occurrence of *Shigella* spp. in the environment might also be influenced by the same parameters.

When the bacteria became nonculturable on conventional nutrient agar plates, direct detection for viable cells was done by DAPI staining to see whether they were metabolically active or dead. This was done for autoclaved pond water of Shahidullah Hall initially containing 10^5 CFU/ml (Fig. 1), autoclaved pond water of Jagannath Hall

(Fig. 2), autoclaved distilled water and autoclaved PBS (Fig. 1). With no visible growth on to the media the microcosms were kept for another 24 hrs to ensure the absence of even a single cultivable bacterium. Live cells when stained will fluoresce blue under fluorescent microscope and absence of blue cells under microscope indicates the absence of metabolically active cells. No blue fluorescence was observed that indicated all cells were metabolically inactive (Data not shown).

Table 1. Physico-chemical properties of pond waters from Shahidulla Hall and Jagannath Hall before and after autoclaving.

	Parameters	Pond water	
		Shahidullah Hall	Jagannath Hall
Before autoclaving			
	Conductivity (μS/cm)	274	472
	Salinity (%)	0.10	0.20
	TDS (mg/l)	137.1	236
	pH	7.20	7.00
After autoclaving			
	Conductivity (μS/cm)	287	426
	Salinity (%)	0.10	0.20
	TDS (mg/l)	143.70	213
	pH	7.00	7.00

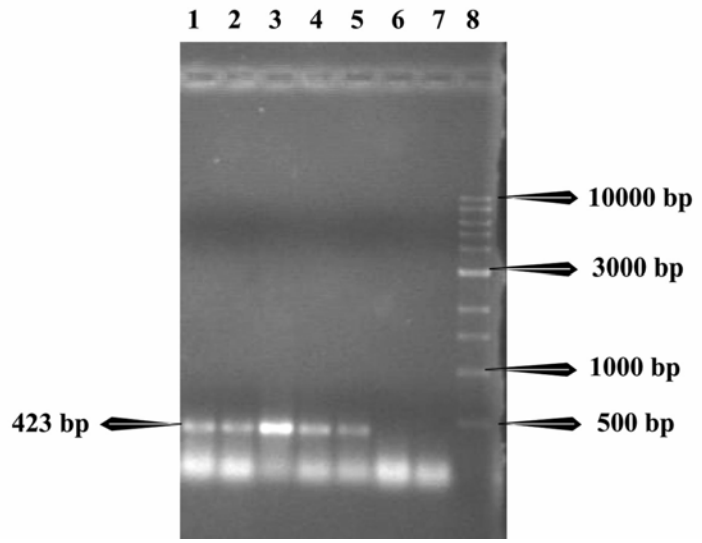

Fig. 3. Amplified product of *ipa*H gene. Lane 1 : DNA extracted from pond water of Shahidullah Hall (10^6CFU/ml), Lane 2: DNA extracted from pond water of Jagannath Hall (10^6CFU/ml), Lane 3: positive control, Lane 4: DNA extracted from pond water of Shahidullah Hall (10^5CFU/ml), Lane 5: DNA extracted from pond water of Jagannath Hall (10^5CFU/ml), Lanes 6 and 7: Negative control, Lane 8: 1 kb marker.

PCR analysis was carried out to detect the presence of virulent gene *ipaH* (invasive plasmid associated H gene in *Shigella* spp.) and *ipaBCD* for autoclaved pond water of Shahidullah Hall and Jagannath Hall initially containing inoculums 10^5 CFU/ml and 10^6 CFU/ml and the bacterial cells could not be recovered by plating method that means bacteria might have undergone to the VBNC state. Both *ipaH* and *ipaBCD* genes are widely used for detection of *Shigella* spp. in water and other environmental sources.[9,13] All four samples gave bands of 423 bp (Fig. 3), an expected size of primers specific to *ipaH* gene which correlate that the strain may be in VBNC state but cannot form colony due to different physico-chemical stresses. All four samples taken from microcosms containing pond waters of Shahidullah Hall (10^6 and 10^5 CFU/ml) and Jagannath Hall (10^6 and 10^5 CFU/ml) gave negative result after PCR for *ipaBCD* gene. Thus it could be assumed that the *ipaBCD* gene might be lost from the strain in course of time of their survival (Data not shown).

Therefore, it could be concluded that the survival potentiality of *Shigella boydii* 15 ATCC 12034 was time and inoculums size dependant. After certain period, the strain became nonculturable on conventional nutrient plate but still their virulent gene was found to be present. So, further detailed study is needed for understanding the survival mechanism of *Shigella* in natural water.

References

1. Murray CJ and AD Lopez 1997a. Mortality by cause for eight regions of the world: Global burden of disease study. Lancet. **349**: 1269-1276.
2. Murray CJ and AD Lopez 1997b. Alternative projections of mortality and disability by cause, 1990-2020: global burden of disease study. Lancet. **349**:1498-1504.
3. Kotloff KL, JP Winickoff, B Ivanoff, JD Clemens, DL Swerdlow, PJ Sansonetti, GK Adak and MM Levine 1999. Global burden of *Shigella* infection: Implications for vaccine development and implementation of control strategies. Bull. WHO **77**: 651-666.
4. Victora CG, SRA Huttly, SC Fuchs, FC Barros, M Garenne and O Leroy 1993. International differences in clinical patterns of diarrhoeal deaths: A comparison of children from Brazil, Senegal, Bangladesh and India. J. Diarr. Dis. Res. **11**: 25-29.
5. DuPont HL, MM Levine, RB Hornick and SB Formal 1989. Inoculum size in shigellosis and implications for expected mode of transmission. J. Infect. Dis. **159**: 1126-1128.
6. Talukder KA, DK Dutta, A Safa, M Ansaruzzaman, M, F Hasan, K Alam, KMN Islam, NIA Carlin, GB Nair and DA Sack. 2001. Altering trends in the dominance of *Shigella flexneri* serotypes and emergence of serologically atypical *S. flexneri* strains in Dhaka, Bangladesh. J. Clin. Microbiol. **39**: 3757-3759.
7. Talukder KA, MA Islam, BK Khajanchi, DK Dutta, Z Islam, A Safa, K Alam, A Hossain, GB Nair and DA Sack. 2003. Temporal shifts in the dominance of serotypes of *Shigella dysenteriae* from 1999 to 2002 in Dhaka, Bangladesh. J. Clin. Microbiol. **41**: 5053-5058.

8. Seidlein LV, DR Kim, M Ali, H Lee, X Wang, VD Thiem, DG Canh, W chaicumpa, MD Agtini, A Hossain, ZA Bhutta, C Mason, O Sethabuta, KA Talukder, GB Nair, JL Deen, Kotloff and J Clemens 2006. A multicentre study of *Shigella* diarrhoea in six asian countries: disease burden, clinical manifestations, and microbiology. PLoS. Med. **3**(9): e353.
9. Faruque SM, R Khan, M Kamruzzaman, S Yamasaki, QS Ahmad,T Azim, GB Nair, Y Takeda and DA Sack 2002. Isolation of *Shigella dysenteriae* type 1 and *S. flexneri* strains from surface waters in Bangladesh : Comparative molecular Analysisis of environmental *Shigella* isolates versus clinical strains. Appl. Env. Microbiol. **68**(8): 3908-3913.
10. Sultana M., MZ Rahman, NK Birkeland and SI Khan 2005. Survival of *Shigella flexneri* cells in Laboratory Microcosms. Bangladesh J. Microbiol. **22**(1): 19-22.
11. Reissbrodt R, I Rienaecker, JM Romanova, PP Freestone, RD Haigh, M Lyte, H Tschape and PH Williams 2002. Resuscitation of *Salmonella enterica* serovar *typhimurium* and enterohemorrhagic *Escherichia coli* from the viable but non- culturable state by heat stable enterobacterial autoinducer. Appl. Env. Microbiol. **68**(10): 4788-4794.
12. Hoben HJ and P Somesagaron 1982. Comparison of the pour, spread and drop plate methods for enumeration of *Rhizobium* spp. in inoculates made from preseterilized peat. Appl Env. Microbiol. **44**:1242-1247.
13. Sharma A, SK Singh and D Bajpai 2010. Phenotypic and genotypic characterization of *Shigella* spp. with reference to its virulence genes and antibiogram. Analysis from river Narmada. Microbiological Res. **165**(1): 39-42.

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