Brazilian Journal of Biological Sciences, 2015, v. 2, n. 3, p. 3-12.
ISSN 2358-2731

Role of free-living protozoa in the occurrence and survival of *Vibrio cholerae* O1 in aquatic ecosystem

Ferdous Nawar* and M. Niamul Naser

Advanced Fisheries Research Laboratory, Department of Zoology, University of Dhaka, Dhaka 1000, Bangladesh.
*Corresponding author: Department of Zoology, University of British Columbia, Vancouver, BC, Canada. Email: nawar.ferdous@gmail.com.

Abstract. Cholera disease caused by bacteria *Vibrio cholerae* O1 and O139. Both afflict 3-5 million and cause 100,000-120,000 deaths worldwide each year. Although from the previous study we have information on the association of cholera bacteria with various zooplankton but little is known about what kinds of relationship exists between cholera bacterium with protozoa though they prefer to live more or less at a similar ecological niche. In the present study the role of free-living fresh water protozoa's in the persistence of *V. cholerae* O1 was assessed. The *in situ* association of *V. cholerae* O1 with protozoa showed the association of *V. cholerae* O1 with the fresh water shelled amoeba, *Arcella* but in the case of ciliates and flagellates large numbers of bacteria were detected from food vacuoles. The result also showed that vibrios form biofilms in *Arcella* culturing media and survived up to two months but the bacterial density declined to < 10 cells/mL water within six days in ciliate and flagellate culturing media. Besides in M-PCR results *wbe*, *ctxA*, *rstR*2 genes were positive for *Arcella* culturing microcosm later for up to 55 days but after four days all of the genes were negative for flagellate and ciliate culturing microcosm water. It indicates that tested amoeba, *Arcella* spp help in the survival of *V. cholerae* O1 *in nature* but ciliates and flagellates can graze upon large number of planktonic vibrios and control the abundance of cholera bacteria.

Keywords: Protozoa, Cholera, Ecology, Biofilm, Association.

Received
December 23, 2014

Accepted
May 9, 2015

Released
June 30, 2015

Open Acess
Full Text Article

Introduction

Cholera is a clinical-epidemiologic syndrome which is caused by *Vibrio cholerae* O1 and O139 and is endemic in Bangladesh (Faruque et al., 2004) and different other geographical regions of the world (Colwell, 1996). In south Asia two seasonal peaks of cholera coincide with the dry season and monsoon rain (Emch et al., 2008). In Bangladesh, the freshwater sources become more salty all through dry season and during monsoon the fresh water bodies are inundated by coastal flooding, and this flood water can lead to the contamination of fresh water with brackish water organisms. During this time, the toxigenic strains of cholera bacterium have been isolated from the aquatic ecosystem of Bangladesh in association with diverse groups of arthropods (Alam et al., 2006a, 2006b, 2007; Nahar et al., 2012) as well as unicellular organisms such as protozoan (Abd et al., 2004).

In general, the vegetative stages of free-living protozoa are cosmopolitan in their spatial distribution and have been found in all kinds of waters intimately associated with bacteria (Lamrabet et al., 2012). Moreover, this free-living protozoa plays a vital role in the survival and spread of some pathogenic bacteria in nature (Laskowski-Arce and Orth, 2008). As cholera bacteria need a biological reservoir

in order to grow in high concentration (Sack et al., 2004) and also to protect them from any detrimental conditions (Macarthy, 1996), many of this free-living protozoa help cholera bacteria to survive and grow rapidly in unfavourable environmental condition (Salah and Hadi, 2011). One of this free-living protozoa, *Acanthamoeba* spp secrets a growth promoting factor to promote the continued existence of *V. parahaemolyticus* (Laskowski-Arce and Orth, 2008). *Acanthamoeba* spp is also considered as environmental hosts of several other pathogenic bacteria such as *Franciscella tularensis*, *Chlamydia pneumoniae*, *Legionella pneumophila* and *Mycobacterium* spp (Wagner et al., 2006). This free-living amoeba is the frequent host for different gram negative bacterial endosymbionts (Abd et al., 2004).

Some members of protozoa are also well-organized at gathering microbes as food in aquatic environment and they probably control the abundances of bacteria (Hobbie, 1994; Sherr and Sherr, 1994). Flagellates and ciliates are considered as biological control agent (Sigee, 1999) as they contribute to the decline of microcystis blooms from the natural water body through their site selective grazing (Nishibe et al., 2002). Heterotrophic nanoflagellates can graze upto 90% of picoplankton population as well as viruses, bacteria, cyanobacteria and heterotrophic protists (Pernthanler et al., 1996).

Cholera (*V. cholerae* O1 and O139) is a devastating disease, outbreak of which kills hundreds of thousands of people in the world annually. During coastal flooding, the causative agent of this disease, *V. cholerae* O1 and O139 disperse brackish to fresh water ponds of coastal areas (Colwell, 1996) and survive in association with diverse groups of aquatic organisms. However, we know very little about the relationship of cholera bacteria with freee living protozoa. Aim of our study is to understand the role of protozoa in the survival of cholera bacteria to know how native free living protozoa influence the survivability of cholera bacteria. To address our research question we examined the *in situ* association of *V. cholerae* O1 with diverse types of free living protozoans in Bangladesh. We hypothesize that members of chitinous shell bearing protozoa might help in the survival of cholera bacteria, however, majority of the free living protozoa graze upon vibrios and control the abundances of cholera bacteria.

Materials and methods

Protozoa culture

Protozoa were collected from Ramna Lake and Dhanmondi Lake in Dhaka City. Generally the water samples were collected from the areas having plant growth, organic debris, slimy growth, dead leaves, pieces of stick and surface scum. The samples were then subjected to culture and inverted microscopy. Collected water samples were then transferred into four different beaker (~100 mL) and different types of foods were added such as hay infusion, boiled rice, corn flour and cucumber juice in order to enrich the growth of protozoa. For an example, in order to make hay infusion media, approximately 50 mg of dry hay were boiled for about 10 min, cooled and allowed to settle. The supernatant was used as culture medium and added to the first beaker which was designated as Hay Infusion Microcosm (HIM). Similarly, remaining three set ups for culture were prepared and beakers were named as Boiled Rice Microcosm Water (BRM), Corn Flour Microcosm (CFM) and Cucumber Mash Microcosm (CMM). All the beakers were then sealed and incubated at room temperature (25 °C) in dark condition for about one month. Growth of protozoa at different culture media were routinely measured using the techniques of inverted microscopes. Protozoa were identified using the morphological description of Kudo (1960), Corliss (1979), and Thorap and Covich (2001). Protozoa were counted with a Sedgwick-Rafter cell counting chamber (Graticlues, Ltd, UK) (Wetzel and Likens, 2000) and their growth in culture media were estimated using the Chi-square statistical test.

V. cholerae association assay

V. cholerae O1 biotype El Tor (EM-226) cells isolated from a pond of Mathbaria were grown in Luria–Bertani (LB) broth at 37 °C for 18 h, harvested,

washed with Phosphate Buffer Saline (PBS). The cells were then inoculated into HIMW, BRMW, CFMW and CMMW to a final concentration of 10^7 cfu.mL^{-1}. All the beakers were then sealed with aluminium foil and incubated at room temperature. Periodically, sub-samples were taken from four beakers to conduct plate culture, Direct Flourescent Antibody reactions (DFA) and multiplex Polymerase Chain Reaction (mPCR).

Culturable count of *V. cholerae* O1

Samples were diluted 10-fold serially in PBS and 100 µL of diluted sample was spread on the surface of thiosulfate citrate bile-salts sucrose (TCBS). Inoculated plates were incubated at 37 ºC for 24 h. After incubation, plates were observed and presumptive colonies resembling *V. cholerae* were confirmed as *V. cholerae* O1 by slide agglutination test using polyvalent anti-O1 serum (Nandi et al., 2000). The confirmed colonies represented the total viable and the culturable *V. cholerae* O1 count.

Direct Flourescent Antibody Technique (DFA)

DFA counting was done following a method described by Brayton (1987). For this purpose, samples were stained using fluorescein isothiocyanate-labeled anti-serum specific for *V. cholerae* O1 (New Horizon Diagnostic Corp, Columbia, MD, USA). Stained samples were observed using an epifluorescence microscope (Carl Zeiss; Axioskop 40) connected to a digital camera (AxioCam MRc).

Multiplex Polymerase Chain Reaction (mPCR)

The colonies confirmed as *V. cholerae* O1 by slide agglutination test (antigen-antibody reaction) were subjected to M-PCR for detection of O1 serotype specific *wbe* genes encoding O-antigen biosynthesis pathway, *ctxA* gene encoding subunit A of cholera toxin subunit A and *rstR2* were amplified using M-PCR (Hoshino et al., 1998).

Results

The presence of different food provided in different culture gave rise to diverse types of protozoa with variable concentration. Such as, in the presence of hay infusion, the day 0 count of protozoa under light microscope showed 20 *Arcella*/mL (Shelled amoeba), 10^2 nanoflagellates/mL and 75 ciliates/mL. Three days latter, the abundance of *Arcella* was > 500/mL. But as soon as the number of *Arcella* increased the nano-flagellates and ciliates were decreased by amoeboid grazing. The total count of *Arcella* in this culture showed an increase from 20/mL to 3,400/mL within a period of 15 days (Figure 1.A). The initial cell count of *V. cholerae* in this microcosm in agar plate was 3.7 x 10^7 cfu.mL^{-1} and continued being for up to two months. (Table 1). At day 2, DFA count was 3.3 x 10^8 and epiflourescent micrographs showed the association of bacteria with matured *Arcella* mainly in its surrounding chitinous shell, and inside and outside of shell. Most of the bacteria were found to attach mainly in the middle portion of the shell surrounding its chromadial network; the region where carbohydrate and other metabolic products laid down (Figure 2.A). Bacteria forming a ring surrounding the shell could be visible at day 4 (Figure 2.B), day 8 and in day 15 (Figure 2.C). Bacterial DFA counts remain stable for the first 14 days and after that bacteria form biofilms of coccoid cells (Figure 2.D). DNA templates prepared from HI microcosm *V. cholerae* O1 co-cultured with *Arcella* supported amplification of - *wbe*, *ctxA*, and *rstR2* gens by M-PCR up to 55 days (Table 2).

Supply of boiled rice as the carbon source supported the growth of rhizopods, ciliates and flagellates. The initial cell count showed the abundances of nano-flagellates and cyst however, after 15 days large amoeba and *Paramecium* became the dominant groups. Trophozoites and cysts counts were 3,190 cells/mL and 60 cells/mL, respectively (Figure 1.B). Bacterial count in agar media collected from this microcosm experiment was high

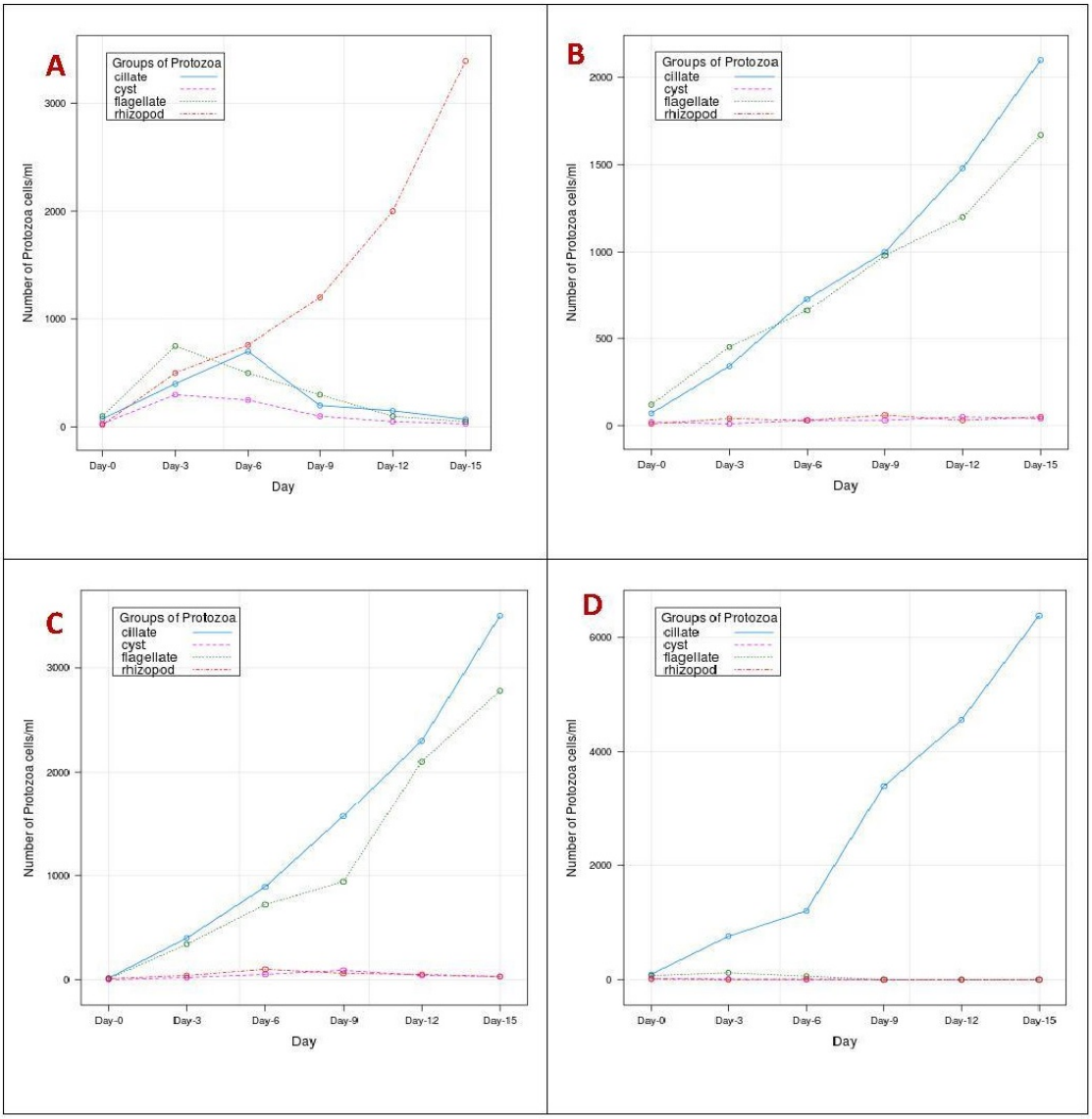

Figure 1. Viable counts of Protozoa in culture media. A) HIW; B) BRW; C) CFW; and D) CMW over a period of 15 days. There exists a statistical association between groups of Protozoa and number of days as the χ^2 test is significant at 5% significance level. A) (χ^2_{15} = 4,848.176, p ≈ 0.000); B) (χ^2_{15} = 232.0472, p ≈ 0.000); C) level (χ^2_{15} = 643.918, p ≈ 0.000); D) (χ^2_{15} = 4,501.134, p ≈ 0.000).

initially which declined within 10 days. Initial count was counts approximately $3x10^7$ colony forming unit/mL^{-1}. The initial DFA count was $3x10^8$, however after 6 days bacteria form biofilm (Table 1), where many bacterial cells were visible inside the food vacuoles rather than in the surrounding water (Figure 2.E). PCR amplification results also follow this pattern where cholera specific genes (*wbe* and *rstR2*) were amplified only in day 4 sample water but no genes were amplified for the DNA prepared from this microcosm water at day 15, 25, 35 (Table 2).

In this experiment we found that corn flour worked best for the mixed culture of ciliates which supported rapid propagation of *Paramecium*, *Oxytricha*, *Coleps*, *Colpoda*, *Holophyra*, *Euglena*, and also some nano-flagellates. Overall, this media had the highest abundances of protozoa. Though the average *V. cholerae* cell count on agar plates was $3x10^7$ cfu.mL^{-1} at day 0 but within 6 days

Table 1. Viable counts (plate count and DFA count) of bacteria in different microcosm water.

Day	Microcosm	Plate count (cfu.mL^{-1})			DFA (cells/mL)
		LA	TTGA	TCBS	
0	HIMW	3.7×10^7	4.1×10^7	2.0×10^7	3.8×10^8
	BRMW	3.4×10^7	4.5×10^7	1.8×10^7	3.6×10^8
	CFMW	5.1×10^7	3.9×10^7	1.5×10^7	4.1×10^8
	CMMW	2.1×10^7	3.2×10^7	2.0×10^6	2.9×10^8
2	HIMW	4.9×10^7	5.5×10^7	2.1×10^7	3.3×10^8
	BRMW	3.4×10^4	4.2×10^4	1.9×10^4	2.5×10^5
	CFMW	6.1×10^3	6.4×10^3	2.8×10^2	3.1×10^5
	CMMW	3.5×10^3	3.7×10^3	2.1×10^2	4.1×10^5
4	HIMW	3.4×10^6	5.3×10^6	3.2×10^6	3.7×10^8
	BRMW	4.1×10^2	5.4×10^2	5.8×10^1	3.2×10^2
	CFMW	2.1×10^2	5.2×10^2	6.1×10^1	2.7×10^2
	CMMW	2.7×10^2	4.3×10^2	5.4×10^1	3.1×10^2
6	HIMW	4.7×10^5	3.4×10^5	5.1×10^5	5.1×10^8
	BRMW	5.6×10^1	4.5×10^1	3.1×10^1	BF
	CFMW	2.2×10^1	2.0×10^1	< 10	BF
	CMMW	1.5×10^1	1.0×10^1	< 10	BF
8	HIMW	3.4×10^4	5.7×10^4	3.2×10^4	6.4×10^8
	BRMW	< 10	< 10	< 10	BF
	CFMW	< 10	< 10	< 10	BF
	CMMW	< 10	< 10	< 10	BF
10	HIMW	5.6×10^3	2.9×10^3	1.6×10^3	3.2×10^8
	BRMW	< 10	< /10	< 10	BF
	CFMW	< 10	< 10	< 10	BF
	CMMW	< 10	< 10	< 10	BF
12	HIMW	4.4×10^2	6.7×10^2	3.8×10^2	4.3×10^8
	BRMW	ND	ND	ND	BF
	CFMW	ND	ND	ND	BF
	CMMW	ND	ND	ND	BF
14	HIMW	5.6×10^1	8.7×10^1	< 10	3.1×10^8
	BRMW	ND	ND	ND	BF
	CFMW	ND	ND	ND	BF
	CMMW	ND	ND	ND	BF
15	HIMW	< 10	< 10	< 10	BF
	BRMW	ND	ND	ND	BF
	CFMW	ND	ND	ND	BF
	CMMW	ND	ND	ND	BF
18	HIMW	< 10	< 10	< 10	BF
	BRMW	ND	ND	ND	BF
	CFMW	ND	ND	ND	BF
	CMMW	ND	ND	ND	BF
25	HIMW	< 10	< 10	< 10	BF
35	HIMW	< 10	< 10	< 10	BF
55	HIMW	< 10	< 10	< 10	BF

HIMW, Hay Infusion Microcsm Water; BRMW, Boiled Rice Microcosm Water; CFMW, Corn Flour Microcosm Water; CMMW, Cucumber Mash Microcosm Water (all of the four microcosm water contained different protozoa inoculated with *Vibrio cholerae* O1). Survivability of bacteria in the presence of different protozoa was monitored over a period of 2 months by plate count (LA, Luria Agar; TTGA, Taurocholate Tellurite Gelatine Agar; TCBS, Thiosulphate Citrate Bile Sucrose Agar); DFA, Direct Flourescent Antibody test. BF, Biofilm; cfu, colony forming unit.

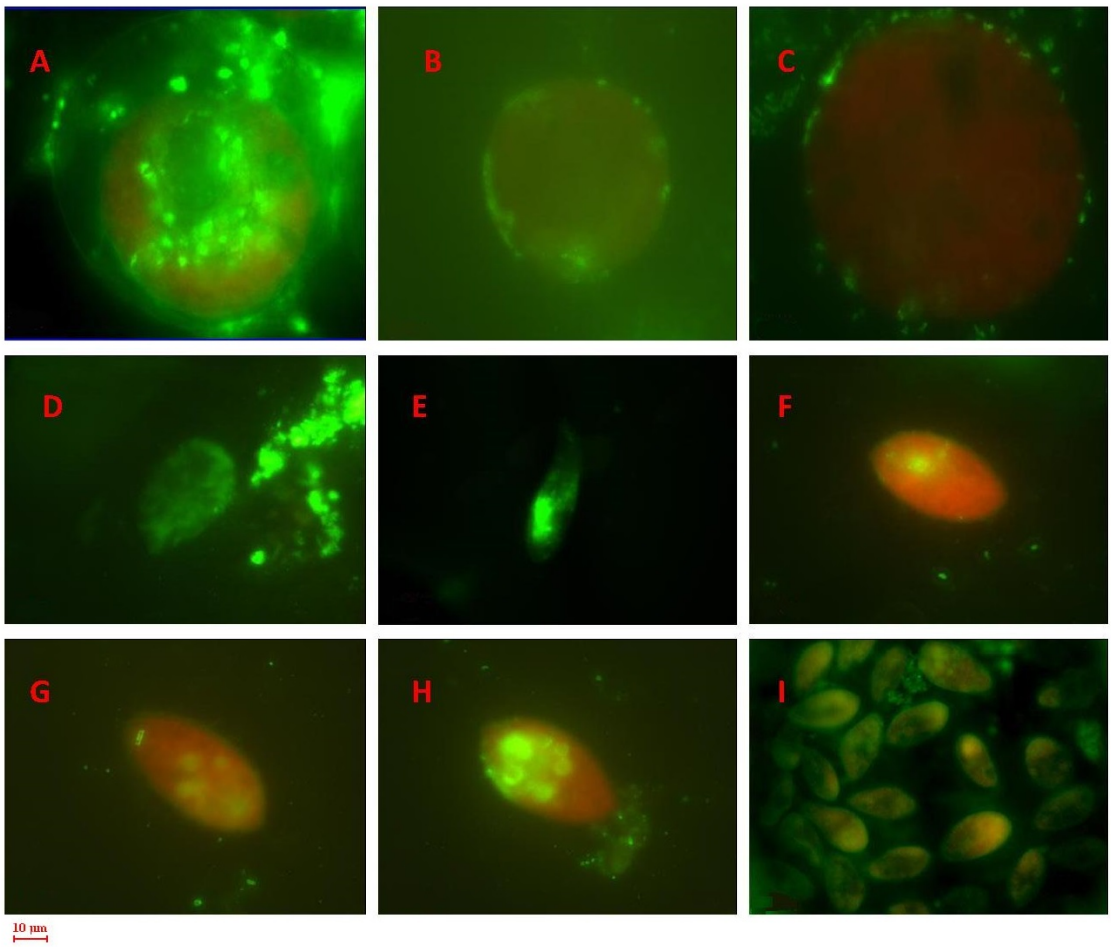

Figure 2. Epiflurescent micrographs of the association of *Vibrio cholerae* O1 with free living fresh water protozoa. Samples were stained with Flourescent Monoclonal Antiboday (DFA) specific for Vibrio cholerae O1 observed under epiflurescence microscope (Carl Zeiss model Axioskop 40). **A)** DFA Day 2 analysis of intracellular localization of *V. cholerae* O1 in *Arcella.* Figure showed that cholera bacteria utilized the chitinous shell and also in the region of chromadial network of an young *Arcella* (magnification 40x)**. B)** DFA Day 4 analysis. Localization of cholera bacteria in the outer shell and sorrounding the space between the inner shell membrane and trophozoite of a mature *Arcella* (magnification 40x). **C**) DFA Day 14 analysis of HIMW. Association of cholera bacteria with an adult *Arcella.* The shell of the *Arcella* is dark chocolate brown color due to the presence of iron oxide. Figure shows the association of large numbers of cholera bacteria with the highly chitinous shell of *Arcella* (magnification 40x). **D**) DFA Day 35 analysis of HIMW. Biofilm formation by cholera bacteria (magnification 10x). **E**) DFA Day 2 analysis of BRMW. Figure showed that cholera bacteria inside the food vacuoles of *Paramecium* (magnification 40x). **F)** DFA Day 4 image of CFMW. Bacteria inside the food vacuoles of ciliate (magnification 40x). **G)** DFA day 2 image of CMMW. Localization of bacteria inside the food vacuoles of ciliate (magnification 40x). **H**) DFA Day 4 image of CMMW. Figure showed that cillite grazes large number of cholera bacteria (magnification 40x). **I)** DFA Day 4 image of CMMW. Ciliates graze most of cholera bacteria and the number of living bacteria in surrounding water is < 100 (magnification x10).

the count was reduced to < 10 cfu.mL^{-1} (Table 1). DFA results showed the presence of *V. cholerae* inside the food vacuoles of ciliates indication ciliates feeds on bacteria (Figure 2.F). Similar to the other microcosm water, genes were amplified from the DNA from this microcosm treatment didn't shows the presence of cholera toxin after 15 days of the initial treatment set up (Table 2).

Cucumber as the food source supported the mono culture of *Paramecium* spp, where over a period of 15 days *Paramecium* had been proliferated

Table 2. Results of M-PCR (multiplex-polymerase chain reaction) analysis.

Day	Microcosm	M-PCR		
		wbe	***ctxA***	***rstR2***
4	HIMW	+	+	+
	BRMW	+	+	+
	CFMW	+	+	+
	CMMW	+	+	+
15	HIMW	+	+	+
	BRMW	-	-	-
	CFMW	-	-	-
	CMMW	-	-	-
25	HIMW	+	+	+
	BRMW	-	-	-
	CFMW	-	-	-
	CMMW	-	-	-
35	HIMW	+	+	+
	BRMW	-	-	-
	CFMW	-	-	-
	CMMW	-	-	-
55	HIMW	+	+	+
75	HIMW	-	-	-

HIMW, Hay Infusion Microcsm Water; BRMW, Boiled Rice Microcosm Water; CFMW, Corn Flour Microcosm Water; CMMW, Cucumber Mash Microcosm Water.

tremendously amounting the approximate count of 4,700 cells/mL. The initial plate count was 2×10^7 on TTGA and LA and 2×10^6 in TCBS, however a week after the count was declined to < 10 (Table 1). DFA analysis showed bacteria inside the food vacuoles of *Paramecium* (Figure 2.G, H, I).

Discussion

Cholera is one of the major public health challenges which occurs epidemically in a specific time of the year in parts of the tropic. This bacteria remains viable throughout the year in association with diverse groups of zooplanktons and phytoplanktons. In the present study, we examined how free living protozoa influence the survivability of cholera bacteria at *in situ* e condition. Our result showed that the pathogenic strains of cholera bacteria can be able to survive and grow *in situ* for a longer period of time only in association with shelled amoeba, however, other groups may have negative effect in survivability of cholera bacteria. We found that when bacteria are co-cultured with *Arcella*, bacteria can capable to survive for more than 60 days in *in situ* condition and latter form biofilm. After 60 days, the growth of cholera bacteria slowly declined following the death of *Arcella* in *in situ*. The better survivability of the bacteria with *Arcella* might be because of its broad, conical, chitinous shell which is transparent and almost colorless when first laid but turn into dark brown color in course of time because of the deposition of iron oxide (Kudo, 1960). This iron oxide is regarded as an important factor in the ecology of cholera bacteria (Patel and Isaacson, 1994). Besides of this chitin and iron oxide, chromadial network might have an advantage for cholera bacteria. According to Jepps (1956), chromadial network is the region of the body of shelled amoebae where carbohydrate and other metabolic products are laid down. In our experiment we detected a huge numbers of bacteria surrounding the outer shell membrane of the shelled amoeba especially in the region of chromadial network.

On the other hand, ciliates and flagellates has food vacuoles which is analogous to the gut of higher organisms. They secretes mucus to feed small organisms and bacteria (Jahn et al., 1961) and when digestion proceeds, the vacuoles decrease in size nearly by 50% and the enclosed bacteria become aggregated (Allen and Fork, 1980). During the cycle of digestion rapid reduction of pH occurs,

dropping to ~3 within 5 min (Fok et al., 1982). By applying this procedure a ciliate or a large flagellate can complete digestion and defecation of 90% of bacterial cells within 40 min (Allen and Fok, 1980; Fenchel, 1986). In our study we found that when bacteria were co-cultured with ciliates and flagellates their survivability declined to 0 within 15 days of inoculation. Our direct flourescent antibody test detected huge numbers of bacteria inside the food vacuoles of ciliates and flagellates, and following the declination of bacteria there were no cholera toxins detected from the sample water, which suggests that ciliates and flagellates graze and completely digest cholera bacteria. The most important grazers of cholera bacteria we found in our study were *Paramecium*, *Holophyra*, *Colpoda*, *Euglena* and *Amoeba*.

The results suggest that *V. cholerae* O1 is able to survive and grow in association with shelled amoeba in natural environments but their occurance and survival might be influenced by another groups of planktonic protozoa as they grazed upon large numbers of planktonic vibrios. Those alimentary chain plays an important role in the epidemiology of cholera because grazing decreases the concentration of vibrios in their natural environmental. The grazing rates of ciliates and flagellates also enable us to predict that in the near future it might be possible to apply ciliates and flagellates to control cholera biologically.

Acknowledgement

This research was supported by the Ministry of National Science, Information and Communication Technology (NSICT) fellowship, Government of the People's Republic of Bangladesh. Technical support of this research was provided by International Centre for Diarrheal Disease Research' Bangladesh (ICDDR'B). The authors thank Dr. Munirul Alam, Marzia Sultana, Rita R. Colwell, A. Huq and B. Sack for their collaboration and support.

Conflict of interest statement

Authors declare that they have no conflict of interests.

References

Abd, H.; Weintraub, A.; Sandstrom, G. Interaction between *Vibrio cholerae* and *Acanthamoeba castellanii*. **Microbial Ecology in Health and Disease**, v. 16, p. 51-57, 2004.

Alam, M.; Hasan, N. A.; Sadique, A.; Bhuiyan, N. A.; Ahmed, K. U.; Nus-rin, S.; Nair, G. B.; Siddique, A. K.; Sack, R. B.; Sack, D. A.; Huq, A.; Colwell, R. R. Seasonal cholera caused by *Vibrio cholerae* serogroups O1 and O139 in the coastal aquatic environment of Bangladesh. **Appl. Environ. Microbiol.**, v. 72, p. 4096-4104, 2006a.

Alam, M.; Sultana, M.; Nair, G. B.; Sack, R. B.; Sack, D. A.; Siddique, A. K.; Ali, A.; Huq, A.; Colwell, R. R. Toxigenic *Vibrio cholerae* in the aquatic environment of Math-Baria, Bangladesh. **Appl. Environ. Microbiol.**, v. 72, p. 2849-2855, 2006b.

Alam, M.; Sultana, M.; Nair, G. B.; Sid-Dique, A. K.; Hasan, N. A.; Sack, R. B., Sack, D. A.; Ahmed, K. U.; Sadique, A.; Watanabe, H.; Grim, C. J.; Huq, A.; Colwell, R. R. Viable but nonculturable *Vibrio cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission. **Proc. Natl. Acad. Sci. USA**, v. 104, p. 17801-17806, 2007.

Allen, R. D.; Fok, A. K. Membrane recycling and endocytosis in *Paramecium* confirmed by horseradish peroxidas pulse-chase studies. **J. Cell. Sci.**, v. 45, p. 131-145, 1980.

Brayton, P. R.; Tamplin, M. L.; Huq, A.; Colwell, R. R. Enumeration of *Vibrio cholerae* O1 in Bangladesh waters by fluorescent - antibody direct viable count. **Appl. Environ. Microbiol.**, v. 53, p. 2862-2865, 1987.

Colwell, R. R. Global climate and infectious disease: the cholera paradigm. **Science**, v. 274, p. 2025-2031, 1996.

Corliss, J. O. **The ciliated protozoa**. 2. ed. Oxford and New York: Pergamon Press, 1979.

Corliss, J. O. The need for the new looks of the taxonomy of protists. **Rev. Soc. Mex. Hist.**, v. 45, p. 27-35, 1995.

Dunkerly, F. Encystations and reserve food formation in *Tinema lineare*. **Trans. Roy. Soc. Edinb.**, v. 53, p. 297, 1923.

Emch, M.; Feldacker, C.; Islam, S. M.; Ali, M. Seasonality of cholera from 1974 to 2005: a review of global patterns. **International Journal of Health Geographics**, v. 7, 13 p., 2008. Available from: <http://www.ij-healthgeographics.com/content/7/1/31>. Accessed in 12 Oct., 2014.

Faruque, S. M.; Naser, I. B.; Islam, M. J.; Faruque, A. S.; Ghosh, A. N. Seasonal

epidemics of cholera inversely correlate with environmental cholera phages. **Proc. Nat. Acad. Sci. USA**, v. 102, p. 1702-1717, 2004.

Fenchel, T. Protozoan filtter feeding. **Prog. Protistol.**, v. 1, p. 65-113, 1986.

Finlay, B. J.; Esteban, G. F. Freshwater protozoa: biodiversity and ecological function. **Biodiver. Conserv.**, v. 7, p. 1163-1186, 1998.

Fok, A. K.; Lee, Y.; Allen, R. D. The correlation of digestive vacuoles pH and size with the digestive cycle in *Paramecium caudatum.* **Journal of Protozoology**, v. 29, n. 3, p. 409-414, 1982.

Hardin, G. Flocculation of bacteria by protozoa. **Nature**, v. 5, p. 94-104, 1943.

Hobbie, J. W. The state of the microbes: a summary of a symposium honoring Lawrence Pomeroy. **Microb. Ecol.**, v. 28, p. 113-116, 1994.

Hoshino, K. Yamasaki, S.; Mukhopadhyay, A. K.; Chakraborty, S.; Basu, A.; Bhattacharya, S. K.; Nair, G. B.; Shimada, T.; Takeda, Y. Development and evaluation of a multiplex PCR assay for rapid detection of toxigenic *Vibrio cholerae* O1 and O139. **FEMS Immunol Med. Microbiol.**, v. 20, n. 3, p. 201-207, 1998.

Islam, M. S., Drasar, B. S.; Sack, R. B. Probable role of blue-green algae in maintaining endemicity and seasonality of cholera in Bangladesh: a hypothesis. **J. Diarrhoeal. Dis. Res.**, v. 12, p. 245-256, 1994.

Jahn, T. L.; Brown, M.; Winet, H. Secretory activity of oral groove of *Paramecium.* **Journal of Protozoology**, v. 8, p. 23, 1961.

Jepps, M. W. **The Protozoa, Sarcodina**. 1. ed. Edinburgh, London: Oliver and Boyd, 1956.

Kudo, R. R. **Protozoology**. 4. ed. Spring-field, Illinios: Charles C. Thomas, 1960.

Lamrabet, O.; Merhej, V.; Pontarotti, P.; Raoult, D.; Drancourt, M. The genealogic tree of mycobacteria reveals a long-standing sympatric life into free-living Protozoa. **PLoS ONE**, v. 7, 8 p., 2012. Available from: <http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0034754>. Acessed in: 12 Oct., 2014.

Laskowski-Arce, M. A.; Orth, K. *Acanthamoeba castellanii* promotes the survival of *Vibrio parahaemolyticus*. **Appl. Environ. Microbiol.**, v. 74, n. 23, p. 7183-7188, 2008.

McCarthy, S. A. Effects of temperature and salinity on survival of toxigenic *Vibrio cholerae* O1 in seawater. **Microb. Ecol.**, v. 31, p. 167-175, 1996.

Michael, A. *Acanthamoeba castellani* promotes the survival of *V. parahaemolyticus.* **Appl. Environ. Microbiol.**, v. 74, p. 7183-7108, 2008.

Miles, H. B. Soil protozoa and earthworm nutrition. **Soil Science**, v. 95, p. 407-409, 1963.

Nahar, S.; Sultana, M.; Naser, N. M.; Nair, B. G.; Watanabe, H. Role of shrimp chitin in the ecology of toxigenic *Vibrio cholerae* and cholera transmission. **Fronters in Microbiology**, v 2, p. 120-125, 2011.

Nandi, B.; Nandy, R. K.; Mukhopadhyay, S.; Nair, G. B.; Shimada, T.; Ghose, A. C. Rapid method for species-specific identification of *V. cholera* using primers targeted to the gene of outer membrane protein OmpW. **J. Clin. Microbiol.**, v. 38, p. 4145-4151, 2000.

Nishibe, Y.; Kawabata, Z.; Nakano, S. Grazing on *Microcystis aeruginosa* by the heterotrophic flagellate *Colodictyon triciliatum* in a hypertrophic pond. **Aquat. Microb. Ecol.**, v. 29, p. 173-179, 2002.

Parry, J. D. Protozoan grazing of fresh water biofilms. **Adv. Appl. Microbiol.**, v. 54, p. 167-196, 2004.

Parry, J. L. **A functional biology of free-living Protozoa**. 3. ed. London: The British Library Publishing Company, 1984.

Patel, M.; Isaacson, M. The effect of iron on the survival of *Vibrio cholerae* O1 in dechlorinated tap water. **Transaction of Royal Society on Tropical Medicine and Hygiene**, v. 88, p. 296-297, 1994.

Perthanler, J.; Šimek, K.; Sattler, B.; Schwarzenbacher, A.; Bobkova, J.; Psenner, R. Short term changes of protozoan control on autotrophic picoplankton in an oligo-mesotrophic lake. **Journal of Plankton Research**, v. 18, p. 443-462, 1996.

Roberts, M. S.; Cohan, F. M. Recombination and migration rates in natural populations of *Bacillus subtilis* and *Bacillus mojavensis*. **Evolution**, v. 49, p. 1081-1094, 1995.

Sack, D. A.; Sack, R. B.; Nair, G. B.; Siddique, A. K. Cholera. **The Lancet**, v. 363, p. 223-233, 2004.

Salah, S.; Abd, H.; Hedenström, I.; Saeed, A.; Sandström, G. Detection of *V. cholerae* and *Acanthamoeba* species from same natural water samples collected from different cholera endemic area in Sudan. **BMC Research Notes**, v. 4, 4 p., 2011. Available from: <http://www.biomedcentral.com/1756-0500/4/109>. Acessed in: 12 Oct., 2014.

Sanchez, J.; Holmgren, J. Virulence factors, pathogenesis and vaccine protection in cholera and ETEC diarrhea. **Curr. Opin. Immunol.**, v. 19, p. 388-398, 2005.

Sherr, E. B.; Sherr, B. F. Bacterivory and herbivory: key roles of phagotrophic protists in

pelagic food webs. **Microb. Ecol.**, v. 28, p. 223-235, 1987.

Sigee, D. C.; Glenn, R.; Andrews, M. J.; Bellinger, E. G.; Butler, R. D.; Epton, H. A. S.; Hendry, R. D. Biological control of Cyanobacteria: principles and possibilities. **Hydrobiologia**, v. 395/396, p. 161-172, 1999.

Thorap, H. J.; Covich, P. A. **Ecology and classification of North American freshwater invertebrates**. 2. ed. New York: Academic Press, 2001.

Wagner, M.; Heinz, E.; Kolarov, I. An *Acanthamoeba* sp. containing two phylogenetically different bacterial endosymbionts. **Environ. Microbiol.**, v. 9, p. 1604-1609, 2006.

Wetzel, R. G.; Likens, G. E. **Limnological analyses**. 3. ed. New York: Springer-Verlag, 2000.