Global Journal of Medical research
Volume 12 Issue 3 Version 1.0 May 2012
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals Inc. (USA)
Online ISSN: 2249-4618 & Print ISSN : 0975-5888

A Study on Virulence and Signaling Mechanism Related to Biofilm Formation and Exopolysaccharide Expression in Certain *Vibrio cholerae* Environmental Isolates

By Smritikana Biswas, Swati Sen, Parimal Dua, Prithwiraj Mukherjee, Sougata Bhunia & Chandradipa Ghosh

Vidyasagar University, Medinipur-West, West Bengal, India

Abstract - *Vibrio cholerae*, causative agent of profuse watery diarrhea forms biofilm structure which is adaptive advantage for the *Vibrio cholerae*. This study was carried out to characterize certain *Vibrio cholerae* environmental strains collected from the water reservoirs surrounding Midnapore town, West Bengal, India in respect of serogroups, antibiotic susceptibility, protease activity, haemolytic activity, pathogenecity and determination of biofilm forming ability and also signaling system involved in the regulation of biofilm. Multiplex PCR assay revealed that all were from non O1/non O139 serogroups and *ctxA, tcpA* negative. All of them were sensitive to streptomycin, tetracycline, nalidixic acid but resistant to ampicillin, norfloxacin and chloramphenical and 70% were polymyxin B resistant. Beside this these *Vibrio cholerae* isolates were strongly hemolytic, moderate to high biofilm forming. In this study we found that these strains did not follow the biofilm controlling previously reported flagella-mediated, hapR-mediated as well as quorum sensing autoinducer(s)-mediated signal transduction pathway.

Keywords : *Vibrio cholerae environmental isolates, serogrouping, antibiogram, pathogenecity, biofilm.*

GJMR-A Classification : *NLMC Code: QW 730, QW 90, QW 55*

Strictly as per the compliance and regulations of:

A Study on Virulence and Signaling Mechanism Related to Biofilm Formation and Exopolysaccharide Expression in Certain *Vibrio cholerae* Environmental Isolates

Smritikana Biswas [α], Swati Sen [σ], Parimal Dua [ρ], Prithwiraj Mukherjee [ω], Sougata Bhunia [¥] & Chandradipa Ghosh [§]

Abstract - *Vibrio cholerae,* causative agent of profuse watery diarrhea forms biofilm structure which is adaptive advantage for the *Vibrio cholerae.* This study was carried out to characterize certain *Vibrio cholerae* environmental strains collected from the water reservoirs surrounding Midnapore town, West Bengal, India in respect of serogroups, antibiotic susceptibility, protease activity, haemolytic activity, pathogenecity and determination of biofilm forming ability and also signaling system involved in the regulation of biofilm. Multiplex PCR assay revealed that all were from "non- O1/non O139" serogroups and *ctxA, tcpA* negative. All of them were sensitive to streptomycin, tetracycline, nalidixic acid but resistant to ampicillin, norfloxacin and chloramphenical and 70% were polymyxin B resistant. Beside this these *Vibrio cholerae* isolates were strongly hemolytic, moderate to high biofilm forming. In this study we found that these strains did not follow the biofilm controlling previously reported flagella-mediated, hapR-mediated as well as quorum sensing autoinducer(s)-mediated signal transduction pathway.

Keywords : Vibrio cholerae environmental isolates, serogrouping, antibiogram, pathogenecity, biofilm.

I. Introduction

Cholera is still considered as major public health concern in developing countries as *Vibrio cholerae* has the potential to be transported internationally and has the ability to occur in explosive endemic form ignoring temporal or spatial gap (McCarthy et al., 1994). Indian subcontinent has come out as an important epicenter of cholera in most of the cholera pandemics (Kumar et al., 2009). Epidemiological data reveals that the *Vibrio cholerae* organisms belonging to O1 and O139 serogroups are etiological agent of epidemic cholera. On the other hand, *Vibrio cholerae* strains from non-O1/non-O139 serogroups are associated with sporadic cases of moderate to severe gastroenteritis and extra-intestinal infections in humans (Chen et al., 2007; Dziejman et al., 2005; Zoelsson et al., 2006, Kovacikova and Skorupski, 2002; Kaper et al., 1995). In developed countries *Vibrio cholerae* non-O1/non-O139 predominates over the enteric infections caused by *Vibrio cholerae* O1/ O139. *Vibrio cholerae* non-O1/non-O139 usually causes a less severe diarrhea than *Vibrio cholerae* O1/ O139, although certain strains, especially those that produce cholera toxin, can cause severe cholera-like disease (Datta et al., 1986). Some non O1 *Vibrio cholerae* strains despite being negative for ctxA and tcpA, the major virulence markers, were reported to cause significant mortality and morbidity in Bangladesh (Islam et al., 1988). *Vibrio cholerae* non-O1/non-O139 was again reported to be associated with cholera like disease in 1996 in Kolkata, India (Sharma et al., 1998). Earlier reports showed that epidemic strains of *Vibrio cholerae* exclusively possess ctxA and tcpA those are the most important virulence factors for cholera pathogenesis (Nair et al., 1988). But later *Vibrio cholerae* non-O1/non-O139 strains were also found to possess ctxA, tcpA (both classical and El Tor types) (Ghosh et al., 1997, Chakraborty et al., 2000).

Vibrio cholerae organisms frequently change their characteristics regarding antibiotic susceptibility producing serious problem in health management and this is due to presence of drug resistant gene which is solely responsible for the transmission and spread of the multidrug resistant properties among the pathogen (Mukhopadhyay et al., 1996). *Vibrio cholerae* O1 strains

Author α : Research scholar, Department of Human Physiology with Community Health, Vidyasagar University, Medinipur-West, West Bengal, India, Pin 721102. E-mail : physio.smritikana@gmail.com
Author σ : Research scholar, Department of Human Physiology with Community Health, Vidyasagar University, Medinipur-West, West Bengal, India, Pin 721102. E-mail : swaati.senn@gmail.com
Author ρ : Research scholar,Department of Human Physiology with Community Health, Vidyasagar University, Medinipur-West, West Bengal, India, Pin 721102. E-mail : parimal.dua@gmail.com
Author ω : Research scholar, Department of Human Physiology with Community Health, Vidyasagar University, Medinipur-West, West Bengal, India, Pin 721102. E-mail : muprithwiraj@gmail.com
Author ¥ : Research scholar, Department of Human Physiology with Community Health, Vidyasagar University, Medinipur-West, West Bengal, India, Pin 721102. E-mail : sougata.physiology@gmail.com
About § : Associate Professor, Department of Human Physiology with Community Health, Vidyasagar University, Medinipur-West, West Bengal, India, Pin 721102. E-mail : ch_ghosh@mail.vidyasagar.ac.in

isolated from cholera patients in Dhaka during January 1986 were found to be sensitive to tetracycline, streptomycin, chloramphenicol, amoxicillin or nalidixic acid (Nakasone et al., 1987). Furthermore all *Vibrio cholera* El Tor biotype isolates during 1988 and 1989 in Bangladesh were sensitive to tetracycline (Siddique et al., 1989) while El Tor strains re-emerged during the 1991 epidemic in Bangladesh were tetracycline resistant (Siddique et al., 1992). In addition certain isolates from non-O1/non-O139 serogroups of *Vibrio cholerae* were found to be resistant to polymyxin B (Kiiykya et al., 1992). Polymixin B was mainly used for differentiating El Tor biotype strains from classical biotype strains as the El Tor strains were found to be resistant to polymixin B (Matson et al., 2010). Reports reveal that *Vibrio cholerae* non-O1/non-O139 strains cause pathogenesis in a complex manner and possess various virulence factors in different combinations. No single virulence factor can predict about the pathogenicity of the strain (Honda et al., 1985; Takao et al., 1985; Arita et al., 1986; Yoshimura et al., 1986). A large number of *Vibrio cholerae* non-O1/non-O139 strains are able to produce cholera like toxins, i.e., heat-stable enterotoxin, thermostable direct haemolysin, hemaagglutinin/ protease, shiga-like toxin those play significant role in the enteropathogenecity (Yamamoto et al., 1984; Ogawa et al., 1990; Kaper et al., 1994). Protease produced by *Vibrio cholerae* non-O1/non-O139 strains serves as attachment factor (s) that has important link with virulence properties as it enhances toxicity by nicking cholera toxin (Booth et al., 1984) or degrading intestinal mucin (Crowther et al., 1987). The majority of non-O1/non-O139 *Vibrio cholerae* strains are hemolytic and can lyse red blood cells (RBC) from a variety of animals by forming small pores in the cytoplasmic membrane and they show cytolytic activity against cultured cell lines (Yoshio et al., 1987; Shinoda et al., 1985; Yamamoto et al., 1990). It was previously described that highly haemolytic strains are highly enterotoxic suggesting a putative relation between haemolytic activity and enterotoxicity. Hemolysin produced by non-O1/non-O139 *Vibrio cholerae* was speculated to be an enterotoxic factor that is responsible for gastroenteritis (Yoshio et al., 1987).

Vibrio cholerae, the human pathogen is a natural inhabitant of aquatic environment. For successful survival in aquatic ecosystem in response to stresses they form association with the surface structures of crustaceans, mollusks etc. and develop the diarrheogenic disorder when human host is encountered (Yildiz et al., 2009). Non-O1/non-O139 strains are autochthonous inhabitant of the brackish water and estuarine environment in association with zooplankton and are reported to be important in evolutionary scale as they can change their serogroup through the horizontal gene transfer which is relevant mechanism for the emergence of newer variants of *Vibrio cholerae* (Rammurthy et al., 1993). Chitin, a polymer of N-acetylglucosamine and component of crustacean crab shell not only induces competence for natural transformation but also mediates the acquisition of genes conferring antibiotic resistance during the growth of a *Vibrio cholerae* strain on a crab shell fragment immersed in seawater (Meibom et al., 2005).

Biofilm is the adaptive surface growth through formation of a three dimentional multicellular-conformation. Biofilm formation is highly regulated and involves several steps. Expression of a matrix material termed exopolysaccharide (EPS) by individual cell in a biofilm is critical for the development of mature biofilm (Costerton et al., 1995; Kolter et al., 1998). This specialized structure enhances growth and survival of these organisms for their long persistence in the environmental reservoirs by providing nutrients and protection from predators and antimicrobial compounds (Donlan et al., 2002). A HapR (HA protease regulator)-dependent and a flagellum-dependent signaling pathways controlling EPS expression have been described in *Vibrio cholerae* (Zhu et al., 2002; Watnick et al., 2001). In a subset of epidemic causing *Vibrio cholerae* the LuxO-HapR regulatory loop plays leading role in EPS regulation in response to cell population density. In this subset EPS expression and biofilm formation are negatively regulated by HapR, and LuxO acts via HapR by repressing it (Zhu and Mekalanos, 2003; Vance et al., 2003). On the other hand, in another subset of *Vibrio cholerae* absence of flagella leads to excess EPS expression involving participation of sodium-driven flagellar motor and VpsR (Lauriano et al., 2004). On further investigation this subset revealed existence of quorum sensing autoinducers- and flagellum-dependent parallel but converging EPS signaling circuits independent of input of LuxO-HapR (Biswas et al., 2012, unpublished)

So, on the basis of this we conducted present investigation to characterize environmental *Vibrio cholerae* strains collected from the water reservoirs surrounding Midnapore town, in the state of West Bengal of India in respect of their serogroups, antibiotic susceptibility, protease activity including pathogenecity and also determining the potential for survival in nature through identifying biofilm forming ability of the isolated *Vibrio cholerae* strains. Besides we were also interested to find out whether HapR-mediated quorum sensing pathway or quorum sensing autoinducer(s) and flagellum-mediated parallel signaling pathways predominate in the regulation of biofilm formation in these *Vibrio cholerae* environmental isolates.

Manuscript received "Date here here"

II. Methods and Materials

a) Collection of Samples

Samples were collected during the period of January to March, 2009 from fresh water bodies

surrounding Midnapore town, southern part of state of West Bengal, India and processed by the method mentioned by Nair et al., 1988.

b) Bacterial strains and culture methods

The surface water collected from the fresh water bodies of different water reserviors was passed through sterile membrane filter (pore size 0.22 μm, Millipore) and filtered sample water was allowed to grow in alkaline peptone water (APW) containing of 1% (wt/vol) peptone and 1% (wt/vol) NaCl at pH 8.5. After 12 h incubation at 30°C the surface growth was streaked onto thiosulphate citrate bile salt (TCBS) agar plate. After screening *Vibrio cholerae* isolates by TCBS agar plate pure single colony was transferred onto Gelatin agar plate after an APW passage. Other bacterial strains used in this study were allowed to grow in Luria-Bertani (LB) broth, supplemented with specific antibiotics. LB broth without NaCl and with 10% sucrose was used for counter selection with sacB-containing plasmids. The strains of bacteria used are listed in Table 2 along with their source.

c) Multiplex PCR assay for virulence genes

Two sets of multiplex PCR assays were conducted using specific primer pairs listed in Table 1. First set of multiplex PCR assay was performed to detect serogroup using specific primer pairs for genes encoding O1 somatic antigen (rfbO1) and O139 somatic antigen (rfbO139). Second multiplex PCR was conducted to detect ctxA and tcpA (both classical and El Tor) using specific primer pairs. PCR reaction mixture and the thermal cycling conditions for PCR were carried out by the method mentioned by Kumar et al., 2009. All PCRs mentioned earlier were carried out using chromosomal DNA from these *Vibrio cholerae* environmental strains as template and isolated by the method of Sambrook et al., 2001.

d) Detection of Motility

Motility of the isolated *Vibrio cholerae* strains were tested by the method mentioned by Rasid et al., 2003 using swarm plate containing 0.3 % LB agar.

e) Detection of protease activity

Protease activity of the isolated *Vibrio cholerae* strains were detected by using a single-diffusion technique in agar gel containing skim milk as a substrate (Finkelstein et al., 1983).

f) Antibiotic susceptibility test

Antibiotic susceptibility test was done against the *Vibrio cholerae* environmental isolates by the method mentioned by Okoh and Igbinosa, 2010. Antibiotics used for this study were ampicillin (50μg/ml), streptomycin (100μg/ml), kanaycin (40μg/ml), tetracycline (50μg/ml), nalidixic acid (30μg/ml), norfloxacin (40μg/ml), chloramphenical (20μg/ml) and polymyxin B (300 IU).

g) Detection of haemolysis

Haemolytic ability of the bacterial isolates was examined on Blood agar media using sheep blood. *Vibrio cholerae* non O1 strains inoculated in Brain Heart Infusion Broth (BHB) were spread on to sheep blood (5% vol/vol) agar and incubated at 37◦ C for 24 hours. Blood agar plates were examined for haemolysis around the colonies (Singh et al., 1996).

h) Toxicity to adult mice

Crude toxin was prepared from the each of the bacterial isolates by the method mentioned by Kiiyukia et al., 1992. Toxicity to adult mice was determined through the intra-peritoneal injection of 0.5-ml of crude toxin into 4-week-old male mice. Four animals were used per strain. Epidemic strain MO10 lac- O139 Bengal was used as a positive control. The number of dead mice was recorded after 48 h.

i) Biofilm Formation Assay

Biofilm assay was performed by the method originally mentioned by Watnick et al., 2001with further modifications made by Lauriano et al., 2004. The strains were grown in LB broth overnight and after that normalised to identical densities using OD600. Then 5 μl of culture was inoculated into 500 μl of LB broth in 10-ml sterile borosilicate test tubes and kept statically at 30◦C for 22 hours. The tubes were rinsed thoroughly with distilled water to remove all non-adherent cells. Further the tubes were incubated for 30 minutes after addition of 600 μl of 0.1% (w/v) crystal violet and were rinsed again with distilled water. Lastly 1 ml of dimethyl sulfoxide was added, the test tubes were vortexed and kept standing for 10 minutes, and the OD570 was measured.

Table1 : Oligonucleotide primer sequence.

Target gene or encoding region	Primer sequence (5'-3')
O1*rfb1* P-A	GTTTCACTGAACAGATGGG
O1*rfb1* P-B	GGTCATCTGTAAGTACAAC
O139*rfb1*P-A	AGCCTCTTTATTACGGGTGG
O139 *rfb1*P-B	GTCAAACCCGATCGTAAAGG
CtxA P-A	CTCAGACGGGATTTGTTAGGCACG
CtxA P-B	TCTATCTCTGTAGCCCCTATTACG
tcpA, cl P-A	CACGATAAGAAAACCGGTCAAGAG
tcpA, cl P-B	ACCAAATGCAACGCCGAATGGAGC
tcpA, ET P-A	GAAGAAGTTTGTAAAAGAAGAACAC
tcpA, ET P-B	GAAAGGACCTTCTTTCACGTTG
HapR P-A	GCTCTAGAGCGACCTCTTGCTCAGAAATC
HapR P-B	GCGGATCCGCGTTTTTCGATTGATGCGTC
HapR P-C	GCGGATCCCAAGTCTCCGTTGCAACAGTG
HapR P-D	GCGCGTCGACGCTGGCCATGTTATCGACATC
FlaA P-A	GCTCTAGACTACTGCAATAACGAGATTGC
FlaA P-B	GCGGATCCGTCACAGCATCAGTAACCTGC
FlaA P-C	GCGGATCCCATCCAAACCACGAGATTGCG
FlaA P-D	GCGGTCGACATGACCATTAACGTAAATAC
CqsA P-A	GCTCTAGAATGCATTTAACGAAAATA
CqsA P-B	GCGGATCCAACAGGAGATGAACGAAATAC

CqsA P-C	GCGGATCCTGTTGTTCTTCCAGTAATGAC
CqsA P-D	GCGGTCGACATGAACAAGCCTCAACTTCC
LuxS P-A	GCTCTAGAATGCCATTATTAGACAGTTT
LuxS P-B	GCGGATCCATCTTTGTTTGGCATAGTAA
LuxS P-C	GCGGATCCTGCGGCACTGCGGCGATGCA
LuxS P-D	GCGGTCGACTTAGTGAACCTTCAGCTCAT
VpsLPr-A	GCGAATTCATATTGTTCTGTTTTTCCTTTC
VpsLPr-B	GCGGATCCCGAGTATTCTGCTTTTTTCCTTCATC GC

for 10 minutes, and the OD_{570} was measured.

j) Plasmid construction

All plasmid constructs and strains used in this study are displayed in Table 2. All PCRs were performed using MO10 lac- chromosomal DNA as template and specific primer pairs which were designed depending on the specific gene sequence from complete *Vibrio cholerae* genome sequence (Kwok et al., 1990; Heidelberg et al., 2000). SM10 λpir was used for the propagation of the pir-dependent plasmid and conjugation in *Vibrio cholerae*. The mutations were performed in the choromosome of the bacterial isolates via homologous recombination method (Lauriano et al., 2004).

ΔhapR, ΔflaA, ΔcqsA and *ΔluxS,* plasmid constructs had been constructed by the method described by Lauriano et al., 2004 with further modification in our laboratory. Around 160-500 bp fragment 5' of the gene deletion was generated through PCR using specific primer A and B presented in Table1. This PCR generated fragment of gene deletion after digestion with XbaI and BamHI was ligated into similarly digested suicide vector pKEK229, pir dependent derivative of CVD442 (Correa et al., 2000) to produce plasmid constructs containing deletion. Similarly around 160-500 bp fragment 3' of the gene deletion was PCR generated using the corresponding primer C and D listed in Table 1. The PCR generated 3' fragment of the gene deletion digested with BamHI and SalI was ligated into similarly digested pKEK229 containing 5' of the gene deletion mentioned earlier.

The promoter-lacZ fusion containing transcriptional reporter plasmid of vpsL was prepared by PCR amplification of the respective promoter using primer pairs Promoter A and B for corresponding gene (Table 1). PCR generated fragment was digested with EcoRI and BamHI, and ligated into the corresponding sites of plasmid vector pRS551 (Simons et al., 1987).

k) β - Galactosidase assays

Vibrio cholerae environmental strains were transformed with plasmid pSH117, the transcriptional reporter construct (Table 1). Bacterial cells grown in LB broth were harvested at OD600 of ~ 0.2 to 0.4, permeabilized with chloroform and sodium dodecyl sulfate and assayed for β-galactosidase activity following the method mentioned by Miller 1992.

III. Result and Discussuion

The Gram-negative bacterium *Vibrio cholerae* is inhabitant of aquatic ecosystems as well as a human pathogen. *Vibrio cholerae* causes the profuse watery diarrhea called cholera by colonizing the small intestine and subsequently producing a potent enterotoxin, cholera toxin (CT) (Norris et al., 1974; Rabbani et al., 1990; WHO 1980). Cholera is endemic in southern Asia, parts of Africa and Latin America, where seasonal outbreaks occur widely and sanitation is rudimentary (Kaper et al., 1995). In the areas of endemic infection it appears in regular seasonal pattern and also as explosive outbreaks (Glass et al., 1982; Kaper et al., 1995), indicating a possible role of environmental factors in triggering the epidemic process. Majority of *Vibrio cholerae* strains in the environmental reservoir have been observed to belong to the non-O1/non-O139 serogroups. Although the *Vibrio cholerae* non-O1/non-O139 isolates usually gives rise to the sporadic diarrheal disorders and limited outbreaks certain non-O1/non-O139 strains are also reported to be involved in cholera-like epidemics (McCormack et al., 1969). In this context we developed an interest to study the endemic environmental isolates in relation to their virulence properties including serogroups. In addition we made an attempt to understand their persistence ability in the environmental reservoir and also the signaling system confirming the environmental persistence in these organisms. With this aim we collected water samples from different fresh water bodies recognized as water reservoirs and used for the domestic purposes in the surroundings of Midnapore town, West Bengal, India during the period of January to March, 2009 and finally isolated twelve *Vibrio cholerae* strains (Table 2).

Table 2 : Plasmid and strains used in this study.

Strains	Description	Source
Vibrio cholerae strains		
SB100 to SB 117	Environmental isolates	Collected from fresh water bodies surrounding Midnapore town, West Bengal, India
***Escherichia coli* strains**		
SM10λ *pir*	*Thi thr leu tonA lacY supE* RP4-2-Tc:: Mu λ *pirR6K* Km^r	Laboratory collection
pKEK229	R6K *ori sacB mob* Amp^r	Laboratory collection
pSH101	*Δ hapR* in pKEK229	This study
pSH102	*Δ flaA* in pKEK229	This study
pSH103	*Δ cqsA* in pKEK229	This study
pSH104	*Δ luxS* in pKEK229	This study
pSH117	*vpsL-lacZ* in pRS551	This study

a) Serogrouping of the Vibrio cholerae isolates

We performed multiplex PCR assay with the primer pairs specific for serogroups O1 and O139 (Table 1). All the *Vibrio cholerae* isolates were found to be from non-O1/non-O139 serogroups (Table 3a). In another multiplex PCR we found that these non-O1/non-O139 strains were negative for ctxA and tcpA those are major virulence markers (Table 3a). Previous report also suggested that *Vibrio cholerae* strains isolated from environment are mostly non-O1/non-O139 than O1 and O139 in riverine and estuarine areas and are also CT (cholera toxin) and TCP (toxin co regulated pilus) negative (Karaolis et al., 1998). However report shows that some members of *Vibrio cholerae* non-O1/non-O139 predominate over O1 and O139 strains in producing gastroenteritis and also produce cholera-like toxin (Janada et al., 1988).

b) Antibiotic susceptibility among Vibrio cholerae environmental isolates

Multi-drug-resistant property is found to get continuously increased among the different pathogenic *Vibrio cholerae* strains from time to time and in different geographic locations producing serious treatment crisis and this is due to presence of self-transmissible transposon-like element (SXT element) encoding resistance to sulfamethoxazole, trimethoprim and streptomycin in *Vibrio cholerae* O139 and O1 (Waldor et al., 1996). Depending upon the several case reports on the increased changes in the pattern of drug resistance we performed antibiotic susceptibility test against different antibiotics. Our study showed that all the isolated *Vibrio cholerae* strains (100%) were sensitive to streptomycin (100µg/ml), tetracycline (50µg/ml), nalidixic acid (30µg/ml) but 100 % were resistant to ampicillin (50µg/ml), norfloxacin (40µg/ml) and chloramphenical (20µg/ml) and 70% were resistant to polymyxin B (300IU), but few strains were found to be sensitive (58%) as well as resistant (41.7%) to kanamycin (40µg/ml) (Table 3a). These results revealed that multiple antibiotic resistant properties were present among these bacterial strains. Previous studies had shown that *Vibrio cholerae* is resistant to streptomycin, tetracycline and sensitive to ampicillin but our findings in the present study differed from those reports. We also observed that most of our *Vibrio cholerae* isolates belonging to non-O1/non-O139 serogroups were polymixin B resistant. Polymyxin B is an antimicrobial peptide which is mainly used to differentiate the classical and El Tor biotypes of *Vibrio cholerae* as El Tor biotypes are so long identified as polymyxin B resistant (Chatterjee et al., 2003). However Kiiyekya et al., 1992 reported that some non-O1/non-O139 strains were also becoming resistant to polymyxin B. We predict that due to changing environment in aquatic reservoir they have evolved either acyltransferase like enzyme which confers resistance to antimicrobial peptide drug polymixin B or may have evolved some other strategy for lipid acylation for their survival. Beside this antimicrobial peptide resistance was reported to have a close association with virulence and also to confer greater fitness within the host for their survival (Jyl et al., 2010 Poyart et al., 2003; Somerville et al., 1999). Our observation of newer pattern of antibiotic susceptibility in these non-O1/non-O139 environmental strains supports the concept of substantial mobility in genetic elements encoding antibiotic resistance in these *Vibrio cholerae* strains.

c) Pathogenecity

Pathogenesis of *Vibrio cholerae* non-O1/non-O139 is very complex involving combination of several virulence factors and suggestion is there that a single factor can not be responsible for the enteropathogenecity (Honda et al., 1985; Takao et al., 1985; Arita et al., 1986; Yoshimura et al., 1986). We examined protease activity, which is also considered as virulence factor in *Vibrio cholerae* in these isolated non-O1/non-O139 environmental strains those were already identified to be negative for ctxA and tcpA. We observed that all of them were able to hydrolyze protein (Table-3b). According to previous report protease activity has an important role in adhesion to surface including virulence expression in *Vibrio cholerae* (Booth et al., 1984).

Earlier report also suggested that *Vibrio cholerae* hemolysin/cytolysin acts as a virulence factor for contributing to the pathogenesis in case of C. elegans infection via a CT- and TCP-independent process (Vaitkevicius et al., 2006) and non-O1/non-O139 strains are found to produce El Tor like hemolysin. Non-O1/non-O139 strains were reported to be involved in the fluid accumulation in rabbit ileal loop and infant mice as well as in adult rabbit (Yoshio et al., 1987). So, depending on these reports we studied the haemolytic activity among the isolated non-O1/non-O139 *Vibrio cholerae* environmental strains and surprisingly we found that all isolates caused complete haemolysis of erythrocytes of sheep blood (Table 3b). We further examined the toxicity of crude toxin prepared from the isolated non-O1/non-O139 environmental strains to adult mice and 75% of the mice died after 48 hours (Table 3c). In present investigation we identified that crude toxin was lethal to adult mice and we predict that death of each mice was caused probably due to presence of enterogenic haemolysin in these non-O1/non-O139 envoironmental strains. Yamanoi et al. (1980) demonstrated that mice were killed by intraperitoneal injection of live cells of *Vibrio cholerae* non-O1/non-O139 isolates. Another report by Yoshio et al. (1987) also described non-O1/non-O139 haemolysin as enterogenic.

d) Biofilm formation and related signaling systems

Vibrio cholerae organisms from the non-O1/non-O139 serogroups are frequently found in

aquatic environment. In aquatic ecosystems they exist both as free-living organism and also in association with zooplanktons (Rivera et al., 2001). During their life cycle both in aquatic environment and eukaryotic host they face a number of stresses i.e., chlorine water, antibiotics, bactericidal agents etc. and to combat stresses they have evolved an adaptive feature, known to be formation of biofilm on biotic and abiotic surfaces. Biofilm is a three-dimentional conformation of surface-attached bacterial cells that confers the bacterial cells in it fitness for survival by overcoming the adversities and thus ensures their long persistence in nature. The stability of biofilm structure is critically determined by expression of exopolysaccharide (EPS) (Yildiz et al., 1999; Wai et al., 1998). In this study we were interested to understand the biofilm forming ability of the newly isolated non-O1/non-O139 environmental strains. We observed that all of these *Vibrio cholerae* non-O1/non-O139 strains were able to produce moderate to high biofim (Fig.1). Then to draw correlation between biofilm formation and EPS expression in these isolates we studied the expression of *vpsL*, the first gene from the *vpsL-Q* operon in the vps biosynthetic gene cluster as a marker for EPS expression using promoter *vpsL-lacZ* fusion transcriptional reporter construct. In each isolate expression of *vpsL* was well correlated with the biofilm formation (Fig. 2). This strongly suggests that the biofilm formation in the present isolates was dependent on expression of exopolysaccharide. On the basis of these observations we suggest that these non-O1/non-O139 environmental strains of *Vibrio cholerae* have the capability to persist in the environment for long period through the formation of biofilm. Different environmental factors determine the expression of virulence associated genes and also other appropriate sets of genes required for their growth in each niche. Earlier reports have suggested the existence of a hapR-dependent quorum sensing pathway and a flagellum-dependent signal transduction cascade, respectively to control the exopolysaccharide (EPS) expression, biofilm formation and expression of virulence genes like cholera toxin (CT) and toxin coregulated pilus (TCP) in two different subsets of the epidemic strains of *Vibrio cholerae* (Zhu et al., 2002; Watnick et al., 2001; Lauriano et al., 2004).

On the basis of this we were eager to develop an understanding regarding the above mentioned signaling cascade that is functional in the present environmental isolates. In our study we observed that deletion in *hapR* and *flaA* as well as quorum sensing autoinducers deficiency did not cause any transition in colony morphology, EPS expression and biofilm formation in these environmental isolates. Hence our observations suggest that these non-O1/non-O139 *Vibrio cholerae* isolates do not follow the biofilm controlling HapR-mediated, flagella-mediated as well as quorum sensing autoinducer(s)-mediated signal transduction pathways those have been observed previously to take leading role in epidemic isolates.

Table 3 a : Serogrouping & antibiotic susceptibility of *Vibrio cholerae* environmental isolates.

Samples	O1 *ctxA & tcpA*	O139 *ctxA & tcpA*	Antibiogram
SB100	–	–	Amp^r,Chl^r, $Nalidixic^s$, $Strep^s$,$Norflox^r$, Polymixin B^S, Tetracycline ^{s}, Kan ^{s}
SB101	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, Tetracycline ^{s}, Kan ^{r}
SB102	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^S, Tetracycline ^{s}, Kan ^{s}
SB103	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, Tetracycline ^{s}, Kan ^{s}
SB104	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^S, $Tetracycline^s$, Kan ^{r}
SB105	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, $Tetracycline^s$, Kan ^{s}
SB106	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^S, Tetracycline ^{s}, Kan ^{s}
SB107	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, Tetracycline ^{s}, Kan ^{s}
SB108	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^S, Tetracycline ^{s}, Kan ^{r}
SB109	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, $Tetracycline^s$, Kan ^{r}
SB110	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, $Tetracycline^s$, Kan ^{s}
SB111	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, $Tetracycline^s$, Kan ^{r}
SB112	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, Tetracycline ^{s}, Kan ^{r}
SB113	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, $Tetracycline^s$, Kan ^{r}
SB114	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, Tetracycline ^{s}, Kan ^{r}
SB115	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, $Tetracycline^s$, Kan ^{r}
SB116	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, Tetracycline ^{s}, Kan ^{r}
SB117	–	–	Amp^r,Chl^r, $Nalidixic^s$,$Strep^s$,$Norflox^r$, Polymixin B^r, Tetracycline ^{s}, Kan ^{r}

Table 3 b : Pathogenicity of *Vibrio cholerae* environmental isolates.

Samples	Protease activity	Haemol ysin production
SB100	+	+
SB101	+	+
SB102	+	+
SB103	+	+
SB104	+	+
SB105	+	+
SB106	+	+
SB107	+	+
SB108	+	+
SB109	+	+
SB110	+	+
SB111	+	+
SB112	+	+
SB113	+	+

Table 3 c : Toxicity of extracellular products of *Vibrio cholerae* non-O1/ non-O139 environmental strains.

Samples	No. of dead adult mice after injection of intraperitoneal injection (n = 4)
SB100	3
SB101	4
SB102	3
SB103	3
SB104	3
SB105	2
SB106	4
SB107	3
SB108	4
SB109	3
SB110	2
SB111	3
SB112	3
SB113	4
SB114	3
SB115	2
SB116	3
SB117	2

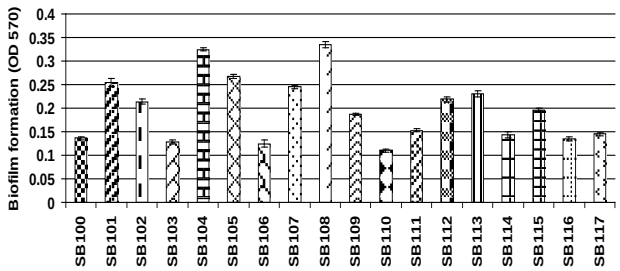

Fig.1. Biofilm formation by different *Vibrio cholerae* environmrental isolates at 30◦C for 24-hr Biofilm formation ability by the different *Vibrio cholerae* environmrental isolates were measured by biofilm formation assay. Biofilm formation by (A) SB100, SB101,SB102,SB103,SB104,SB105,SB106,SB107,SB108,SB109,SB110,SB111,SB112,SB113,SB114,SB115,SB116 and SB117 are presented. OD570 values indicate quantity of biofilm produced. Error bars are standard deviations.

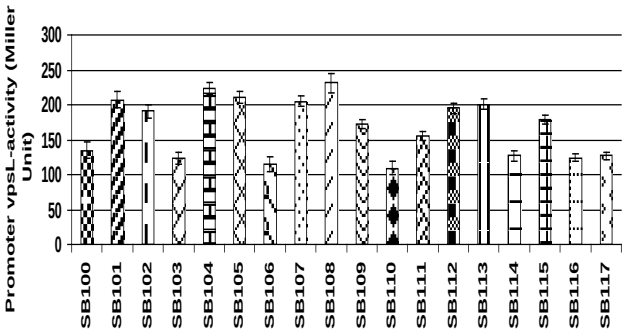

Fig.2 Comparison of EPS expression among different non-O1/non-O139 *Vibrio cholerae* environmental isolates. Transcription level of *vps*L was

examined as marker for *EPS* expression by β-galactosidase assay using *vpsL* promoter-lacZ fusion transcriptional reporter construct.

IV. Conclusion

The results of present investigation on isolated environmental *Vibrio cholerae* organisms those belonged to the non-O1/non-O139 serogruops and negative for both ctxA and tcpA have appeared to be significant as these organisms demonstrated certain new pattern in terms of antibiotic susceptibility. Moreover these organisms were found to be positive in protease activity, haemolysin production, potent in enteropathogenecity and biofilm forming ability. They also differed in cellular signaling mechanism controlling exopolysaccharide expression and biofilm structure as per prior description (Zhu et al., 2002; Watnick et al., 2001; Lauriano et al., 2004). We infer that the environmental clones of non-O1/non-O139 *Vibrio cholerae* those have demonstrated certain alternative behaviors than previous descriptions but have come out with potentially lethal virulence properties reflects further the significance of endemic reservoirs for future epidemics.

Acknowledgements

This work was supported by grant SR/SO/HS-43/2006 from Science and Engineering Research Council (SERC), Department of Science and Technology (DST), Government of India. We thank Dr. Sunando Bandyopadhyay for technical help.

References Références Referencias

1. Arita, M., Takeda, T., Honda, T., & Miwatani, T. (1986). Purification and characterization of *Vibrio cholerae* non-O1 heat-stable enterotoxin. *Infection & Immunity*, 52, 45-49.
2. Booth, B. A., Boesman-Finkelstein, M., & Finkelstein, R. A. (1984). *Vibrio cholerae* hemagglutinin/protease nicks cholera enterotoxin. *Infection & Immunity*, 45, 558-560.
3. Chakraborty, S., Mukhopadhyay, A. K., Bhadra, R. K., Ghosh, A. N., Mitra, R., Shimada, T., Yamasaki, S., Faruque, S. M., Takeda, Y., Colwell, R. R., & Nair, G. B. (2000). Virulence gene in environmental strains of *Vibrio cholerae. Applied & Environmental Microbiology*, 66, 4022-4028.
4. Chatterjee, S. N., & Chaudhuri, K. (2003). Lipopolysaccharides of *Vibrio cholerae*. I. Physical and chemical characterization. *Biochimica et Biophysica Acta,* 1639, 65-79.
5. Correa, N. E., Lauriano, C. M., McGee, R., & Klose, K. E. (2000). Phosphorylation of the flagellar regulatory protein FlrC is necessary for *Vibrio cholerae* motility and enhanced colonization. *Molecular Microbiology*, 35, 743-755.
6. Colwell R. R., & Nair, G. B. (2000). Virulence gene in environmental strains of *Vibrio cholerae. Applied and. Environmental Microbiology*, 66, 4022-4028.
7. Costerton, J. W., Lewandowski, Z., Caldwell, D. E., Korber, D. R., & Lappin- Scott, H. M. (1995). Microbial biofilms. *Annual Review of Microbiology,* 49, 711-745.
8. Crowther, R. S., Roomi, N. W., Fahim, R. E. F., & Forstner J. F. (1987). *Vibrio cholerae* metalloproteinase degrades intestinal mucin and facilitates enterotoxin-induced secretion from rat intestine. *Biochimica et Biophysica Acta*, 924, 393-402.
9. Datta-Roy, K., Bannerjee K., De, S. P., & Ghose, A. C. (1986). Comparative study of expression of hemagglutinins, hemolysins and enterotoxins by clinical and environmental isolates of non-O1 *Vibrio cholerae* in relation to their enteropathogenicity. *Applied & Environmental Microbiology*, 52, 875-879.
10. Donlan, R. M., & Costerton, J. W. (2002). Biofilms: survival mechanisms of clinically relevant microorganisms. *Clinical Microbiology Reviews*, 15, 167-193.
11. Dziejman, M., Serruto, D., Tam, V. C., Sturtevant, D., Diraphat, P., Faruque, S. M., Rahman, M. H., Heidelberg, J. F., Decker, J., Li, L., Montgomery, K. T., Grills, G., Kucherlapati, R., & Mekalanos, J. J. (2005). Genomic characterization of non-O1, non-O139 *Vibrio cholerae* reveals genes for a type III secretion system. *Proceedings of the National Academy of Science of United States of America,* 102, 3465-3470.
12. Finkelstein, R. A., Boesman-Finkelstein, M., & Holt, P. (1983). *Vibrio cholerae* hemagglutinin/lectin/ protease hydrolyzes fibronectin and ovomucin. Proceedings of the *National Academy of Science of United States of America*, 80, 1092-1095.
13. Ghosh, C., Nandy, R. K., Dashgupta, S. K., Nair, G. B., Hall, R. H., & Ghose, A. C. (1997). A search for cholera toxin (CT), toxin co regulated pilus (TCP) the regulatory element ToxR and other virulence factor in non-O1/non-O139 *Vibrio cholerae. Microbial Pathogenesis*, 22, 199-208.
14. Glass, R. I., Becker, S., Huq, M. I., Stoll, B. J., Khan, M. U., Merson, M. H., Lee, J. V., & Black, R. E. (1982). Endemic cholera in rural Bangladesh, 1966-1980. *American Journal of Epidemiology*, 116, 959-970.
15. Heidelberg, J. F., Eisen, J. A., Nelson, W. C., Clayton, R. A., Gwinn, M. L., Dodson, R. J., Haft, D. H., Hickey, E. K., Peterson, J. D., Umayam, L., Gill, S. R., Nelson, K. E., Read, T. D., Tettelin, H., Richardson, D., Ermolaeva, M. D., Vamathevan, J., Bass, S., Qin, H., Dragoi, I., Sellers, P., McDonald, L., Utterback, T., Fleishmann, R. D., Nierman, W. C., White, O., Salzberg, S. L., Smith, H. O., Colwell, R.

R., Mekalanos, J. J., Venter, J. C., & Fraser, C. M. (2000). DNA Sequence of both chromosomes of the cholera pathogen *Vibrio cholerae. Nature*, 406, 477-483.
16. Honda, T., Arita, M., Takeda, T., Yoh, M., & Miwatani, T. (1985) Non-O1 *Vibrio cholerae* produces two newly identified toxins related to *Vibrio parahaemolyticus* haemolysin and Escherichia coli heat-stable enterotoxin. *Lancet*, 2, 163-164.
17. Islam, S. S., & Shahid N. S. (1986). Morbidity and mortality in a diarrhoeal diseases hospital in Bangladesh. *Royal Society of Tropical Medicine and Hygiene*, 80, 748-752.
18. Joelsson, A., Liu, Z., & Zhu, J. (2006). Genetic and phenotypic diversity of quorum-sensing systems in clinical and environmental isolates of *Vibrio cholerae. Infection & Immunity*, 74, 1141-1147.
19. Janda, J. M., Powers, C., Bryant, R. G., & Abbott, S. L. (1988). Current perspectives on the epidemiology and pathogenesis of clinically significant *Vibrio* spp. *Clinical Microbiology Reviews*, 1, 245-267.
20. Kaper, J. B., Fasano, A., & Trucksis, M. (1994). Toxins of *Vibrio cholerae*. In I. K. Wachsmuth, P. A. Blake, & O. Olsvik (ed.), Vibrio *cholerae and cholera: molecular to global perspectives* (p.145-176). Washington, DC: ASM Press.
21. Kaper, J. B., Morris, J. G., & Levine, M. M. (1995). Cholera. *Clinical Microbiology Reviews*, 8, 48-86.
22. Karaolis, D. K., Johnson, J. A., Bailey, C. C., Boedeker, E. C., Kaper, J. B., & Reeves, P. R. (1998). A *Vibrio cholerae* pathogenicity island associated with epidemic and pandemic strains. *Proceedings of the National Academy of Science of United States of America* 95, 3134 3139.
23. Kiiyukia, C., Nakajima, A., Nakai, T., Muroga K., Kawakami, H., & Hashimoto, H. (1992). *Vibrio cholerae* non-O1 isolated from Ayu fish (plecoglossus altivelis) in Japan. *Applied & Environmental Microbiology,* 58, 3078-3082.
24. Kolter, R., & Losick, R. 1998. One for all and all for one. *Science*, 280, 226-227.
25. Kovacikova, G., & Skorupski, K. (2002). Regulation of virulence gene expression in *Vibrio cholerae* by quorum sensing: HapR functions at the *aphA* promoter. *Molecular Microbiology*, 46, 1135-1147.
26. Kumar, P., Jain, M., Goel, A. K., Bhadauria, S., Sharma, S. K., Kamboj, D. V., Singh, L., Ramamurthy, T., & Nair, G. B. (2009). A large cholera outbreak due to a new cholera toxin variant of the *Vibrio cholerae* O1 El Tor biotype in Orissa, Eastern India. *Journal of Medical Microbiology,* 58, 234-238
27. Kwok, S., Kellog, D. E., McKinney, N., Spasic, D., Goda, L., Levenson, C., & Sninsky, J. J. (1990). Effects of primer-template mismatches on the polymerase chain reaction: Human Immunodeficiency Virus 1 model studies. *Nucleic Acids Research*, 18, 999-1005.
28. Lauriano, C. M., Ghosh, C., Correa, N. E., & Klose, K. (2004). The sodium-driven flagellar motor controls exopolysaccharide expression in *Vibrio cholerae. Journal of Bacteriology*, 186, 4864-4874.
29. Matson, J. S., Yoo, H. J., Hakansson, K., & DiRita V. J. (2010). Polymyxin B resistance in El Tor *Vibrio cholerae* requires lipid acylation catalyzed by MsbB. *Journal of Bacteriology*, 192, 2044-2052.
30. McCarthy, S. A., & Khambaty, F. M. (1994). International dissemination of epidemic *Vibrio cholerae* by cargo ship ballast and other non potable waters. *Environmental Microbiology Review*, 51, 365-379.
31. McCormack, W. M., Mosley, W. H., Fahimuddin, M., & Benenson, A. S. (1969). Endemic cholera in rural East Pakistan. *American Journal of Epidemiology*, 89, 393-404.
32. Miller, J. H. (1992). *A short course in bacterial genetics* (2nd ed). New York, NY: Cold Spring Harbor Laboratory Press.
33. Miller, M. B., & Bassler, B. L. (2001). Quorum sensing in bacteria. *Annual Review of Microbiology*, 55, 165-199.
34. Nair, G. B., Oku, Y., Takeda, Y., Ghosh, A., Ghosh, R. K., Chattopadhyay, S., Paul, S. C., Kaper, J. B., & Takeda, T. (1988). Toxin profiles of *Vibrio cholerae* nonO1 from environmental source in Calcutta, India. *Applied and Environmental Microbiology*, 54, 3180-3182.
35. Norris, H. T. (1974). Cholera. In Barua D. and Burrows W. (ed.), *The pathology of cholera* (p. 160-188). Philadelphia, Pa: The W. B. Saunders Co.
36. Nakasone, N., Iwanaga, M., & Eeckels, R. (1987). Characterization of *Vibrio cholerae* O1 recently isolated in Bangladesh. *Transactions of the Royal Society of Tropical Medicine and Hygiene*, 81, 876–878.
37. Ogawa, A., Kato, J., Watanabe, H., Nair, G. B., & Takeda, T. (1990). Cloning and nucleotide sequence of a heat-stable enterotoxin gene from *Vibrio cholerae* non-O1 isolated from a patient with traveler's diarrhea. *Infection and Immunity*, 58, 3325-3329.
38. Okoh A. I., & Igbinosa E. O. (2010). Antibiotic susceptibility profiles of some *Vibrio* strains isolated from wastewater final effluents in a rural community of the Eastern Cape Province of South Africa. *Bio Medical Central Microbiology,* 10, 143.
39. Poyart, C., Pellegrini, E., Marceau, M., Baptista, M., Jaubert, F., Lamy, M. C., & Trieu-Cuot, P. (2003). Attenuated virulence of *Streptococcus agalactiae* deficient in D-alanyl-lipoteichoic acid is due to an

increased susceptibility to defensins and phagocytic cells. *Molecular Microbiology*, 49, 1615-1625

40. Rabbani, G. H., & Greenough, W. B. (1990). Cholera, In E. Lebenthal & M. Duffy (ed.), *Textbook of secretory diarrhe*a (p. 233-253). New York, NY: Raven Press.
41. Ramamurthy, T., Garg, S., Sharma, R., Bhattacharya., S. K., & Nair G. B. (1993) Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. *Lancet*, 341, 703-704.
42. Rashid, M. H., Rajanna, C., Ali, A., Karaolis, & D. K. R. (2003). Identification of genes involved in the switch between the smooth and rugose phenotypes of *Vibrio cholerae. FEMS Microbiology Letters*, 227, 113-119.
43. Rivera I. N. G., Chun, J., Huq A., Sackand, R. B., Colwell, R. R. (2001). Genotypes Associated with Virulence in Environmental Isolates of *Vibrio cholerae. Applied & Environmental Microbiology*, 67, 2421-2429.
44. Sambrook, J., & Russel, D. W. (2001). *Molecular cloning*: a laboratory manual (3rd ed). New York, NY: Cold Spring Harbor Laboratory Press.
45. Shinoda, S., Miyoshi, S., Tamanaka, H., & Miyoshi, N. N. (1985). Some properties of *Vibrio vulnificus* hemolysin. *Microbiology and Immunology*, 29, 583-590.
46. Siddique, A. K., Zaman, K., Baqui, A. H, Akram, K., Mutsuddy, P., Eusof, A., Haider, K., Islam, S., & Sack, R. B. (1992). Cholera epidemics in Bangladesh: 1985-1991. *Journal of Diarrheal Diseases Research*, 10, 79-86.
47. Siddique, A. K., Zaman K., & Y. M. (1989) Simultaneous outbreaks of contrasting drug resistant classic and El Tor *Vibrio cholerae* O1 in Bangladesh. *Lancet*, i, 396.
48. Somerville, J. E., Jr., Cassiano, L., & Darveau, R. P. (1999). *Escherichia coli msbB* gene as a virulence factor and a therapeutic target. *Infection and Immunity,* 67, 6583-6590.
49. Sharma, C., Thungapathra, M., & Ghosh, A. (1998). Molecular analysis of non-O1, non-O139 *Vibrio cholerae* associated with an unusual upsurge in the incidence of cholera-like disease in Calcutta, India. *Journal of Clinical Microbiology*, 36, 756-763.
50. Takao, T., Shimonishi, Y., Kobayashi, M., Nishimura, O., Arita, M., Takeda, T., Hondo, T., & Miwatani, T. (1985). Amino acid sequence of heat-stable enterotoxin produced by *Vibrio cholerae* non-O1. *FEBS Letters*, 193, 250-254.
51. Singh, D. V., Shukla, B. N., & Sanyal S. C. (1996). Haemolysin produced by *Vibrio cholerae* non-O1 is not enterotoxic. *Journal of Medical Microbiology*, 45, 35-39.
52. Simons, R. W., Houman, F., & Kleckner, N. (1987). Improved single and multicopy *lac*-based cloning vectors for protein and operon fusions. *Gene*, 53, 85-96.
53. Vaitkevicius, K., Lindmark, B., Ou, G., Song T., & Toma, C. (2006). A *Vibrio cholerae* protease needed for killing of Caenorhabditis elegans has a role in protection from natural predator grazing. *Proceedings of the National Academy of Science of United States of America,* 103, 9280-9285
54. Wai, S. N., Mizunoe, Y., Takade, A., Kawabata, S. I., & Yoshida, S. I. (1998). *Vibrio cholerae* O1strain TSI-4 produces the exopolysaccaharide materials that determine colony morpohology, stress resistance, and biofilm formation. *Applied and Environmental Microbiology,* 64, 3648-3655.
55. Waldor, M. K., & Mekalanos, J. J. (1996). Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science*, 272, 1910-1914.
56. Watnick P. I., & Kolter R. (1999). Steps in the development of a *Vibrio cholerae* El Tor biofilm. *Molecular Microbiology*, 34, 586-595.
57. Watnick, P. I., Lauriano, C. M., Klose, K. E., Croal, L., & Kolter, R. (2001). The absence of a flagellum leads to altered colony morphology, development and virulence in *Vibrio cholerae* O139. *Molecular Microbiology*, 39, 223-235.
58. WHO Scientific Working Group. (1980). Cholera and other Vibrio-associated diarrhoeas. Bulletin of the World Health Organization, 58, 353-374.
59. Yamamoto, K., Al-Omani, M., Honda, T., Takeda, Y., & Miwatani, T. (1984). Non-O1 *Vibrio cholerae* hemolysin: purification, partial characterization, and immunological relatedness to El Tor hemolysin. *Infection and Immunity* 45, 192-196.
60. Yamamoto, K., Wright A. C., Kaper, J. B., & Morris, J. G., Jr. (1990). The cytolysin gene of *Vibrio vulnificus*: Sequence and relationship to the *Vibrio cholerae* El Tor hemolysin gene. *Infection and Immunity*, 58, 2706-2709.
61. Yamanoi, H., Muroga, K., & Takahashi, S. (1980). Physiological characteristics and pathogenicity of NAG *Vibrio* isolated from diseased ayu. *Fish Pathology*, 15, 69-73.
62. Yildiz, F. H., & Schoolnik, G. K. (1999). *Vibrio cholerae* O1 El Tor: identification of a gene cluster required for the rugose colony type, exopolysaccharide production, chlorine resistance, and biofilm formation. *Proceedings of the National Academy of Science of United States of America*, 96, 4028-4033.
63. Yildiz, F. H. & Visick K. L. (2009). *Vibrio biofilms*: so much the same yet so different. *Trends in Microbiology,* 17, 109-118.
64. Yoshimura, S., Takao, T., Shimonishi, Y., Hara, S., Arita, M., Takeda, T., Imaishi, H., Honda, T., & Miwatani, T. (1986). A heat-stable enterotoxin of

Vibrio cholerae non-O1: chemical synthesis, and biological and physicochemical properties. *Biopolymers,* 25, S69-S83.

65. Zhu, J., Miller, M. B., Vance, R. E., Dziejman, M., Bassler, B. L., & Mekalanos, J. J. (2002). Quorum-sensing regulators control virulence gene expression in *Vibrio cholerae. Proceedings of the National Academy of Science of United States of America,* 99, 3129-3134.

This page is intentionally left blank