Stamford Journal of Pharmaceutical Sciences *S. J. Pharm. Sci.* 1(1&2): 69-75

Rapid Method for Species-Specific Identification and Determination of Toxigenicity of *Vibrio Cholerae* from Natural Aquatic Environment

Bidhan Chakraborty, Khadiza Zaman, .Md. Majibur Rahman
Department of Microbiology, Stamford University, Bangladesh
Stamford University Bangladesh

Corresponding Author
Prof.Dr.Md. Majibur Rahman
Department of Microbiology
Stamford University, Dhaka, Bangladesh.
Contact no# 8124566, 9116108. Ex-196.
E-mail: majibmdr@yahoo.com

Received- 22 November 2008 Accepted for Publication- 8 December 2008

ABSTRACT

Cholera caused by toxigenic *Vibrio cholerae* is a major public health problem confronting developing countries, where outbreaks occur in a regular seasonal pattern and are particularly associated with poverty and poor sanitation. It is generally accepted that seven distinct pandemics of cholera have occurred since the onset of the first pandemic in 1817. Again *Vibrio cholerae* is capable of surviving in aquatic environments for extended periods and is considered as autochthonous species in estuarine and brackish waters. Therefore, the present study was designed to isolate *V. cholerae* from natural environmental samples subsequently identified by conventional and molecular biological techniques. A total number of 10 isolates were included randomly in this study based on their initial identification. The serotypes of the isolates were determined by serological test (slide agglutination) and the number of serotypes O1, O139 and non-O1/O139 were 3, 2 and 5 respectively which were reconfirmed by PCR method. Finally, the toxigenicity of the isolates was analyzed by multiplex PCR method and five (5) isolates were found to contain the ctx gene, the major virulence factor of *V. cholerae*.

Key Words: *Vibrio cholerae*, Simplex PCR, Multiplex PCR, Serotypes, Toxigenicity.

INTRODUCTION

Cholera, an enteric diarrheal disease caused by the gram-negative bacterium *Vibrio cholerae*, continues to be a worldwide health concern. Two distinctive epidemiologic features of cholera are its tendency to appear in explosive outbreaks, often starting in several distinct foci simultaneously, and its propensity to cause true pandemics that progressively affect many countries in multiple continents over the course of many years. (Kaper et al., 1995). *V. cholerae*, the causative agent of cholera in humans, is classified into two serotypes: O1 and non O1 (Chatterjee et al., 1984). The O1 serogroup of *V. cholerae* is further classified into two biotypes, namely, the classical and El Tor biotypes. In 1993, *V. cholerae* serogroup O139 made an explosive appearance and caused a severe epidemic in the Indian continent (Ramamurthy et al., 1993). The major surface antigen employed in characterization of *V. cholerae* is the O antigen. According to the typing scheme of Sakazaki and Shimada, there are 139 different O groups. *V. cholerae* O1 and O139 are known to be the dominating and pathogenic strains. However, the pathogenecity of *Vibrio cholerae* is chiefly associated with the secretion of the *Cholera* toxin (CTX) which is a protein complex. CTX is responsible for the harmful effects of cholera infection. The structure of CTX is typical of the A-B subunit group of toxins in which each of the subunits has a specific function. The B subunit serves to bind the holotoxin to the eukaryotic cell receptor, and the A subunit possesses a specific enzymatic function that acts intracellular.

Recent studies of the aquatic environment have shown that *V. cholerae*, including strains of O1 and O139, are normal inhabitants of surface water, particularly brackish waters, and survive and multiply in association with zooplankton and phytoplankton quite independently of infected human beings (Huq et al., 1983). Identification of *V. cholerae* is usually achieved through a series of biochemical tests after their growth and isolation on a selective plating medium e.g., TCBS agar **(**Sakazaki, 1992). The process, however, is laborious and time-consuming and may be quite expensive for a laboratory. Further, close relatedness among *V. cholerae* and certain other members of the *Vibrio* spp. (e.g., *V. mimicus*) or *Aeromonas* spp. with respect to their biochemical properties has often made unambiguous identification of the organism quite difficult.

Hence, in this present study all the isolates primarily considered as *V. cholera* were characterized through molecular biological techniques based on PCR by amplifying the *ompW* gene, which encodes the exclusive and unique surface protein in *V. cholerae* which is often targeted for species-specific detection of *V. cholerae*. Finally, multiplex PCR was employed to reconfirm the serotypes by amplifying the *rfb* O1 and *rfb* O139 genes and to determine the toxigenicity of all the isolates by amplifying the *ctx* gene simultaneously.

METHODS AND MATERIALS

Sample collection and processing

All the samples were collected in the month of May 2008 from different locations of Mirpur. The samples were collected with aseptic technique using sterile glass conical flask. Following collection, the samples were enriched with Alkaline Peptone Water (APW) for about 6 hours at 37°C. The samples were then serially diluted (10-fold dilution) by using normal saline.

Isolation of V. cholerae

Following enrichment, 0.1µl of the samples were inoculated onto Thiosulphate Citrate Bile Salt (TCBS) and Taurocholate Tellurite Gelatin Agar (TTGA) media by a sterile glass rod through spread plate method. The culture plates were then incubated for 24-48 hours at 37°C. After incubation, characteristic colonies on TCBS and TTGA media were selected and then identified biochemically, serologically and by PCR method.

Biochemical Identification of V. cholerae

According to 'Microbiological Laboratory Manual' by Cappuccino G. and Sherman N., 1996 several biochemical tests were performed to identify the bacteria of interest.

Serological Identification of V. cholerae

In the study of *V. cholerae*, slide agglutination test was employed to differentiate the environmental isolates of *V. cholerae* as O1, O139 or non-O1/O139. For this purpose, 5µl of the antisera was taken on the slide and then the isolates were inoculated. Finally, agglutination was observed against light and the results were recorded.

Simplex PCR for rapid detection of V. cholerae

Simplex PCR assay was performed to detect the *ompW* gene for the confirmation of the *V. cholerae*. Primer sequence for *V. cholerae ompW* gene is listed in table-1. Samples (3 µl) were added to the PCR mixture to achieve a 25-µl final volume (3 µl of template DNA (lysate), 2.5 µl of each primer (10 pmol/µl), 2.5 µl of 2.5 mM deoxynucleoside triphosphates, 0.3 µl (5 U/µl) of *Taq* DNA polymerase (Takara Shuzo Co., Ltd.), 2.5 µl of 10X reaction buffer, 0.75 µl of 20 mM $MgCl_2$ (Extaq; Takara), and 10.95 µl of distilled water) and amplification conditions used were 5 min at 94°C for initial denaturation of DNA and 35 cycles, each consisting of denaturation at 94°C, annealing at 64°C, and extension at 72°C for 30 seconds each, with a final round of extension for 7 min at 72°C in a DNA RoboCycler gradient temperature cycler (Stratagene, La Jolla, Calif.).

Multiplex PCR for determination of serotypes and toxigenicity

Multiplex PCR assay was performed to detect the *rfb* O1*, rfb* O139 genes to confirm the serotypes of the isolates and also for *ctxA* gene to verify whether the isolates are toxigenic or not. Primer sequences for genes, e.g., *V. cholerae rfb* O1, *V. cholerae rfb* O139, and *ctxA*, are listed in table-1. Amplification with the three primer pairs (*rfb* O1, *rfb* O139, and *ctxA* [forward and reverse for each pair]) genes was performed simultaneously in 0.2-ml microcentrifuge tubes. Samples (2.5 µl) were added to the PCR mixture to achieve a 30-µl final volume (2.5 µl of template DNA (lysate), 0.5 µl of each primer for *ctxA* (10 pmol/µl),1.5 µl of each primer for *rfb*O1 and *rfb*O139 respectively, 2.5 µl of 2.5 mM deoxynucleoside triphosphates, 0.3 µl (5 U/µl) of *Taq* DNA polymerase (Takara Shuzo Co., Ltd.), 2.5 µl of 10X reaction buffer, 0.75 µl of 20 mM $MgCl_2$ (Extaq; Takara), and 14.45 µl of distilled water) and amplification conditions used were 5 min at 94°C for initial denaturation of DNA and 35 cycles, each consisting of denaturation at 94°C, annealing at 55°C, and extension at 72°C for 1 minute each, with a final round of extension for 7 min at 72°C in a DNA RoboCycler gradient temperature cycler (Stratagene, La Jolla, Calif).

Table1. Sequences of primers used in this study

Target gene	Sequence (5'-3')	Amplicon size (bp)	Reference
ctxA	*ctxA F*: CTC AGA CGG GAT TTG TTA GGC ACG *ctxA R*: TCT ATC TCT GTA GCC CCT ATT ACG	308	Fields et. al. (1992)
ompW	*terminal:* CAC CAA GAA GGT GAC TTT ATT GTG *internal:* GGT TTG TCG AAT TAG CTT CAC C	304	Singh et al. (2001)
rfb O1	O1*rfbF:* GTT TCA CTG AAC AGA TGG G O1*rfbR:* GGT CAT CTG TAA GTA CAA C	192	Singh et al. (2001)
rfb O139	O139*rfbF:* AGC CTC TTT ATT ACG GGT GG O139*rfbR:* GTC AAA CCC GAT CGT AAA GG	449	Singh et. al. (2001)

Agarose gel electrophoresis for detection of PCR products

After amplification, 6 µl of each reaction mixture was subjected to electrophoresis on a 1% agarose gel (11 by 14 cm) using a horizontal electrophoresis apparatus (Horizon 11.14; Life Technologies/Gibco-BRL). The gel containing the amplified DNA was stained with ethidium bromide and visualized with a UV transilluminator, and images of the transilluminator were digitized with a one-dimensional gel documentation system (Bio-Rad) and the DNA profile of the organism was determined.

RESULTS

Isolation of Vibrio cholerae

Following incubation period of 24-48 hours, typical colonies having the typical characteristics on TCBS and TTGA media were initially considered as *V. cholerae*.

Biochemical identification of Vibrio cholerae

Extensive biochemical tests were performed in order to measure the variability of biochemical behavior among the strains. Detailed biochemical study revealed that all the strains had the biochemical characteristics typical of *V. cholerae*.

Serology

All of the strains (n=10) with typical biochemical behaviors were subjected to serotyping for "O" antigen and "O139" antigen using O1 polyvalent antisera and O139 antisera (Polyvalent/Monovalent). The isolates were observed for visible clumping after the addition of antisera against light. Among the ten (10) isolates, three were found to be *V. cholerae* O1 (Env-06, Env-07, Env-10), two were found to be *V. cholerae* O139 and the rest of the isolates were considered to be *V. cholerae* non-O1/O139.

Table 2. Agglutination of the isolates against *V. cholerae* specific polyvalent antisera[☯].

Strain ID	Polyclonal antisera of	
	O1	O139
Env-01	-	-
Env-02	-	-
Env-03	-	+
Env-04	-	-
Env-05	-	+
Env-06	+	-
Env-07	+	-
Env-08	-	-
Env-09	-	-
Env-10	+	-

☯ Symbols: + : Positive, - : Negative

Confirmative identification of Vibrio cholerae by simplex PCR

Molecular method *ompW* PCR, was performed for the confirmation of the *V. cholerae*. All the strains tested were positive for the presence of *ompW* gene and hence confirmatively identified as *V. cholerae*.

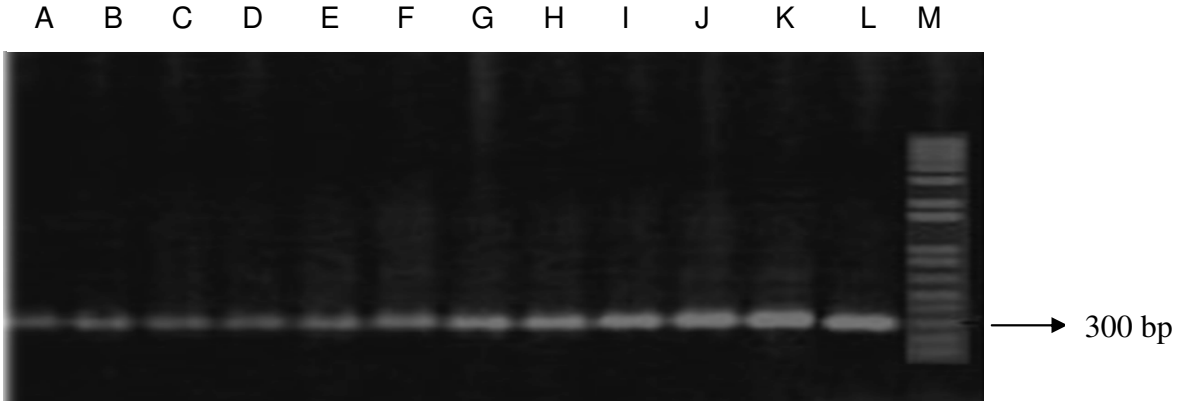

Figure-1. *Agarose gel electrophoresis of PCR amplicon of ompW.* Key lane: A= Env-01, B=Env-02, C=Env-03, D=Env-04, E=Env-05, F=Env-06, G=Env-07, H=Env-08, I=Env-09, J=Env-10, K= +ve control O1, L= +ve control O139, M= 1 Kb+, M= 1Kb+

Determination of serotypes and toxigenicity by multiplex PCR

Multiplex PCR assay was done for *in vitro* determination of the *rfb* O1*, rfb* O139 genes to confirm the serotypes and to determine the toxigenicity (*ctxA*) of the isolates.

DISCUSSION

V. cholerae is the bacteria that causes cholera, a potentially epidemic and life-threatening secretory diarrhea characterized by numerous, voluminous watery stools, often accompanied by vomiting, and resulting in hypovolemic shock and acidosis. Epidemics of cholera caused by toxigenic *V. cholerae* belonging to the O1 or O139 serogroups are a major public health problem in many developing countries of Asia, Africa, and Latin America (Faruque et al., 1998). Cholera has been categorized as one of the "emerging and reemerging infections" (Satcher, 1995) threatening many developing countries.

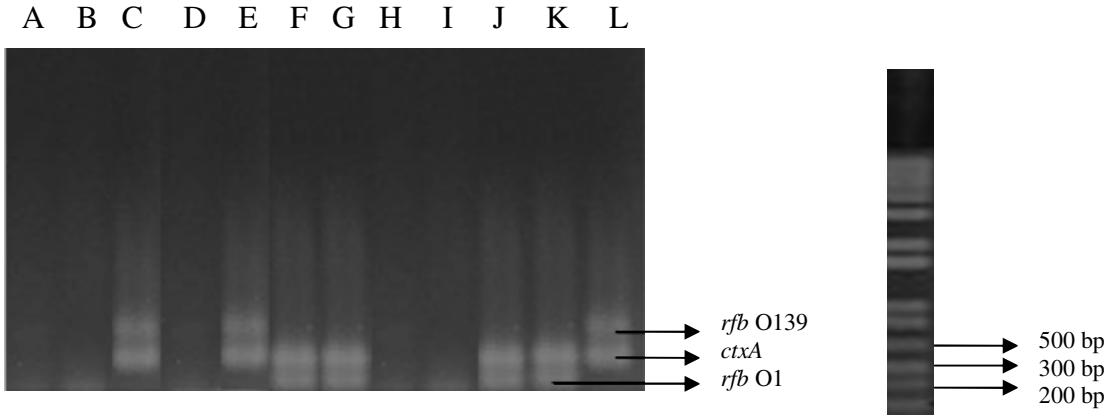

Figure-2. *Agarose gel electrophoresis of PCR amplicon of* O1 *rfb, ctxA and* O139 *rfb genes*. Key lane: A= Env-01, B=Env-02, C=Env-03, D=Env-04, E=Env-05, F=Env-06,G=Env-07, H=Env-08, I=Env-09, J=Env-10, K= +ve control O1, L= +ve control O139.

Contrary to the traditional belief that *V. cholerae* is a solely clinical bacterium that only survives in the aquatic environment for a short time, *V. cholerae* is now known to be indigenous to brackish waters (Colwell et al., 1992). Non-O1 strains are much more commonly isolated from the environment than are O1 strains, even in epidemic settings in which fecal contamination of the

environment might be expected. However, it is clear that CT-producing *V. cholerae* O1 can persist in the environment in the absence of known human disease (DePaola et al., 1992; Shandera et al., 1983). Periodic introduction of such environmental isolates into the human population through ingestion of uncooked or undercooked shellfish appears to be responsible for isolated foci of endemic disease along the U.S. Gulf Coast and in Australia (Blake et al., 1980; Lin et al., 1986; Lowry et al., 1989).

Table*3.* Gene of different isolates[®]

Strain ID	Lane no	*ctxA*
Env-01	A	-
Env-02	B	-
Env-03	C	+
Env-04	D	-
Env-05	E	+
Env-06	F	+
Env-07	G	+
Env-08	H	-
Env-09	I	-
Env-10	J	+

[®] Symbol: **+:** Positive, - **:** Negative

Environmental isolates may also have been responsible for the initial case clusters in the South American epidemic. Hence, isolates from environmental samples have the potential to spread endemics as well as epidemics and it plays a crucial role in the transmission of cholera. In assessing the public health significance, two critical properties of *V. cholerae* are taken into account. These include the production of CT, which is responsible for the severe diarrhea, and the possession of the O1 or O139 antigen, which acts as a marker of epidemic potential, since the actual determinant of such potential is not clearly known (Kaper et al., 1979). Hence the present study was planned to characterize *V. cholerae* by molecular techniques as well as to determine the presence of the genes described earlier. A total of ten (10) isolates were considered in this study randomly based on their primary identification by biochemical identification tests which were then further analyzed by molecular biological techniques for corresponding genes. Since the isolation and correct identification of *V. cholerae* is very crucial for the characterization purpose that was aimed at, the colonies having typical cultural characteristics were selected as presumptive *V. cholerae*, which were then subjected to biochemical tests for confirmation. Extensive biochemical tests were performed in order to measure the variability of biochemical behavior among the strains. However, all the strains showed the typical biochemical behavior characteristic of *V. cholerae* as compared to the control strains N16961. All the strains were positive for indole and gelatinase production and all isolates were positive for oxidase as well. All the strains could tolerate up to 6.5% of NaCl. Therefore all strains were further tested by PCR based molecular methods of species-specific PCR *ompW,* encoding outer membrane protein OmpW which is unique in *V. cholerae*, to confirmatively identify the strains as *V. cholerae* and all the isolates presented positive result for *ompW.*

Until 1992, *V. cholerae* belonging to serogroup O1 was considered to be the only causative agent of epidemic cholera (Colwell et al., 1992). *V. cholerae* as a species includes both pathogenic and nonpathogenic strains that vary in their virulence gene content. This bacterium contains a wide variety of strains and biotypes, receiving and transferring genes for toxins, colonization factors, antibiotic resistance, capsular polysaccharides that provide resistance to chlorine and new surface antigens, such as the O139 lipopolysaccharide and O antigen capsule. The *rfb* O1 and *rfb* O139 genes code for surface antigens O1 and O139 which indicate the potential of the isolates to introduce epidemic infections. Unlike *V. cholerae* O1, the non-O1 serogroups of *V. cholerae* do not have epidemic or pandemic potential (Blake et al., 1980). They have often been identified as the causative agents of sporadic cases (Hughes et al., 1978) and localized outbreaks (Dakin et al., 1974) of cholera-like diarrhea. The severity of *V. cholerae* non-O1-associated diarrhea is usually of a lesser magnitude than that of *V. cholerae* O1- associated diarrhea (Spira et al., 1979). Currently, the Indian subcontinent is experiencing an epidemic of clinical cholera caused by a strain of *V. cholerae* non-O1. This strain is serologically unrelated to the currently recognized O138 serogroup of *V. cholerae*. Therefore, it has been assigned to a new serogroup, i.e., O139, with the suggested name Bengal to refer to its first isolation from the coastal areas of the Bay of Bengal (Shimada et

al., 1993). The disease caused by *V. cholerae* O139 is as severe as the cholera caused by V. *cholerae* O1. From early January to the end of March 1993, about 107,297 people in Bangladesh have been affected by this strain, with an estimated 1,473 deaths (Albert et al., 1993). Even though there are striking similarities between *V. cholerae* O1 and O139, there are also difference, the most important of which is the possession of a polysaccharide capsule by *V. cholerae* O139 which is absent in *V. cholerae* O1 (Johnson et al., 1994). The major surface antigen employed in the characterization of *V. cholerae* is the O antigen. So, the strains that were primarily identified as *V. cholerae* were subjected to polyclonal O1 and O139 antisera, as there are no clear-cut phenotypic process to distinguish *V. cholerae* O1, O139 and non-O1/O139 except serology. Among the isolates, three (Env-06, Env-07 and Env-10) were identified as *V. cholerae* O1 and two (Env-03 and Env-05) were identified as *V. cholerae* O139. The rest of the isolates were considered as *V. cholerae* non-O1/O139. Traditional identification system is based on the cultural characteristics and the metabolic characteristics of an organism. Although it has a good potential for the characterization of microorganisms, but still it is not up to the mark. Rather, the recent developments in molecular biological techniques are found to be more effective in diagnostic microbiology as well as for research purposes. Extremely sensitive methods based on molecular biology principles are available for detecting pathogens and to demonstrate their toxigenicity.

Epidemic and pandemic strains of *V. cholerae* secrete cholera toxin (CT), the toxin responsible for the secretory diarrhea that is characteristic of the disease. CT is encoded by the *ctxAB* genes that are carried on a filamentous bacteriophage designated CTXΦ (Waldor et al., 1996). To determine whether the isolates were toxigenic or not, the isolates in this study were examined for the presence of *ctxA* by PCR based method. *ctxA* was amplified in five (5) isolates and the rest of the isolates were deprived from the gene. However, they are still in concern due to their potential to spread cholera.

CONCLUSION

Finally it can be concluded that the aim of the study was to isolate toxigenic *V. cholerae* from aquatic environment which was successfully accomplished. Among the ten isolates, five isolates were shown to contain the *ctx* gene, the chief virulence factor of *V. cholerae*. Hence, the sample site can be considered as contaminated and it is acting as a reservoir of pathogenic *V. cholerae* which can encourage endemic as well as epidemic infection in the area of interest.

ACKNOWLEDGEMENTS

The study was supported by grants from the department of Microbiology, Stamford University, Bangladesh. The helpful technical assistance of the microbiology laboratory is also acknowledged.

REFERENCES

Albert MJ, Siddique AK, Islam, MS, Faruque ASG, Ansaruzzaman M, Faruque S M, Sack R B. (1993). Large outbreak of clinical cholera due to *V. cholerae* non-O1 in Bangladesh. *Lancet*. 341: 704.

Barry AL, Thorsberry C. (1980) Susceptibility testing: diffusion disk procedure. In: Manual of Clinical Microbiology, 3rd Edition, Novick, R. (editor), pp. 436-474. VCH Publishing Co., New York.

Blake PA, Weaner RW. (1980) Diseases of humans (other than cholera) caused by vibrios. *Annu. Rev Microbiol*. 34: 341-367.

Blake PA, Allegra DT, Snyder JD, Barrett TJ, McFarland L, Caraway CT, Feeley JC, Craig JP, Lee JV, Puhr ND, Feldman, RA. (1980) Cholera—a possible endemic focus in the United States. *N Engl J Med.* 302: 305–309.

Chatterjee SN, Maiti M. (1984) Vibriophages and Vibriocins: physical, chemical and biological properties. *Adv Virus Res.* 29: 263-312.

Colwell RR, Spira WM. (1992) The ecology of *Vibrio cholerae*, p. 107-127. *In* D. Barua and W. B. Greenough (editor), Cholera. Plenum, New York, N.Y.

Cappuccino JG, Sherman N. (1996) *Microbiology, A Laboratory Manual*; 4th Ed. pp-186. The Benjamin-Cumming Publishing Company, Inc, Menlopark,California.

Dakin WPH, Howell DJ, Sutton RGA, O'Keefe MF, Thomas P. (1974) Gastroenteritis nonagglutinable (non-cholera) vibrios. *Med J Aust*. 2: 487-490.

DePaola A, Capers GM, Motes ML, Olsvik O, Fields PI, Wells J, Wachsmuth IK, Cebula TA, Koch WH, Khambaty F, Kothary MH, Payne WL, Wentz BA. (1992) Isolation of Latin American epidemic strain of *Vibrio cholerae* O1 from US Gulf Coast. *Lancet*. 339: 624.

Faruque SM, Albert MJ, Mekalanos JJ. (1998) Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. *Microbiol Mol Biol. Rev.* 62: 1301-1314.

Fields PI, PopovicT, Wachsmuth K, Olsvik Ø. (1992) Use of polymerase chain reaction for detection of toxigenic *Vibrio cholerae*Vibrio cholerae O1 strains from the Latin American cholera epidemic. *J Clin Microbiol*. 30:2118-2121.

Glass RI, Huq I, Alim ARMA, Yunus M. (1980) Emergence of multiple antibiotic- resistant *Vibrio cholerae* in Bangladesh. *J Infect Dis.* 142: 939–42.

Glass RI, Huq MI, Lee JV, Threlfall EJ, Khan MR, Alim AR, Rowe B, Gfross RJ. (1983) Plasmid-borne multiple drug resistance in *Vibrio cholerae* serogroup O1, biotype El Tor: evidence for a point-source outbreak in Bangladesh. *J Infect Dis.* 147: 204–209.

Hughes JM, Dannie MD, Hollis G, Eugene MS, Gangorosa MD, Weaner RE. (1978) Non-cholera vibrio infections in the United States. *Ann Intern Med.* 88: 602-606.

Huq A, Small EB, West PA, Huq MI, Rahman R, Colwell RR. (1983) Ecology of *V. cholerae* with special reference to planktonic crustacean copepods. *Appl Environ Microbiol*. 45: 275–283.

Johnson AJ, Salles CA, Panigrahi P, Albert MJ, Wright AC, Johnson RJ, Morris JG Jr. (1994) *Vibrio cholerae* O139 synonym Bengal is closely related to *Vibrio cholerae* O1 El Tor but has important differences. *Infect. Immun.* 62: 2108-2110.

Kaper J, Lockman H, Colwell RR, Joseph SW. (1979) Ecology, serology and enterotoxin production of *Vibrio cholerae* in Chesapeake Bay. *Appl. Environ. Microbiol*. 37: 91–103.

Kaper JB, Morris JG, Levine MM. (1995) Cholera. *Cli Microbiol Rev.* 8(1): 48-86.

Lin FY, Morris JG Jr., Kaper JB, Gross T, Michalski J, Morrison C, Libonati JP, Israel E. (1986) Persistence of cholera in the United States: isolation of *Vibrio cholerae* O1 from a patient with diarrhea in Maryland. *J Clin Microbiol.* 23: 624–626.

Lowry PW, Pavia AT, McFarland LM, Peltier BH, Barrett TJ, Bradford HB, Quan JM, Lynch J, Mathison JB, Gunn RA, Blake PA. (1989) Cholera in Louisiana: widening spectrum of seafood vehicles. *Arch Intern Med.* 149: 2079–2084.

Ramamurthy T, Garg R, Sharma R, Bhattacharya SK, Nair GB, Simada T, Takeda T, Karasawa T, Kurazano H, Pal A, Takeda Y. (1993) Emergence of novel strain of Vibrio cholerae with epidemic potential in southern and eastern India. *Lancet*. 314: 703-704.

Sakazaki R. (1992) Bacteriology of *Vibrio* and related organisms, p. 37-55. *In* D. Barua, and W. B. Greenough III (ed.), Cholera. Plenum Medical Book Company, New York, N.Y.

Satcher D. (1995) Emerging infections: getting ahead of the cure. *Emerg Infect Dis.* 1: 1-6.

Shandera WX, Hafkin B, Martin DL, Taylor JP, Maserang DL, Wells JG, Kelly M, Ghandi K, Kaper JB, Lee JV, Blake PA. (1983) Persistence of cholera in the United States. *Am J Trop. Med Hyg.* 32: 812–817.

Shimada T, Nair GB, Deb BC, Albert MJ, Sack RB. (1993) Outbreak of Vibrio cholerae non-O1 in India and Bangladesh. *Lancet.* 341: 1346-1347.

Singh DV, Matte MH, Matte GR, Jiang S, Sabeena F, Shukla BN, Sanyal SC, Huq A, Colwell RR. (2001) Molecular Analysis of Vibrio cholerae O1, O139, non-O1, and non-O139 Strains: Clonal Relationships between Clinical and Environmental Isolates. *Appl Environ Microbiol*. 67: 910-921.

Spira WM, Daniel RR. (1979) Biotype clusters found on the basis of virulence characteristics in non-O group I Vibrio cholerae, p. 440-457. In Proceedings of the 15th Joint Conference on Cholera, US-Japan Cooperative Medical Science Program, Bethesda, Md.

Waldor MK, Mekalanos JJ. (1996) Lysogenic conversion by a filamentous phage encoding cholera toxin. *Science*. 272: 1910–14.