



UNIVERSITI PUTRA MALAYSIA

**MOLECULAR CHARACTERIZATION OF VIBRIO SPECIES
ISOLATED FROM SEAWATER**

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**MOLECULAR CHARACTERIZATION OF *VIBRIO* SPECIES ISOLATED
FROM SEAWATER**

By

YUHERMAN

**Thesis Submitted in Fulfilment of the Requirement for the degree of Doctor of
Philosophy in the Faculty of Food Science and Biotechnology
Universiti Putra Malaysia**

July 2001



DEDICATION

TO BOTH MY PARENTS

H. MOCHAMMAD DIN BIN BURHAN

AND

HJ. MARDIANA BINTI SALEH

TO MY SON

FACHRUL FARIZAN

TO MY UNCLE

Drs. ASNOL AMRI **AND** FAMILY

FOR THEIR MORAL SUPPORT AND ENCOURAGEMENT

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment
of the requirement for the degree of Doctor of Philosophy

**MOLECULAR CHARACTERIZATION OF *VIBRIO* SPECIES ISOLATED
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July 2001

Chairman: Associate Professor Dr. Son Radu

Faculty: Food Science and Biotechnology

A study was conducted to determine the prevalence of *Vibrio* species in seawater samples obtained from the coast of Malacca, Penang (Batu Feringgi, George Town), and Terengganu, respectively.

Four *Vibrio cholerae* O139, 10 *V. cholerae* non-O1, 7 *V. cholerae* O1, 160 *Vibrio parahaemolyticus*, and 57 *Vibrio vulnificus* strains were isolated from 240 seawater samples 4/32 (0.13%), 3/32 (0.09%), 2/32 (0.06%), and 24/32 (0.75%) seawater samples obtained from Malacca were positive for *V. cholerae* O139, *V. cholerae* non-O1, *V. cholerae* O1, and *V. parahaemolyticus* strains, respectively. 6/30 and 7/30 seawater samples obtained from Batu Feringgi and George Town beaches were positive for *V. parahaemolyticus* strains, respectively. All the *V. parahaemolyticus* strains were Kanagawa-negative. Fifty seven *V. vulnificus* strains of biotype 1 were isolated from 11 (18.33%) of 60 seawater samples



obtained around Marang beach (Terengganu). The antibiotic resistance patterns of all *Vibrio* species strains tested showed that all were resistant to one or more of the antibiotics tested. Of 15 antibiotics tested against all the *Vibrio* species strains, *V. cholerae* O139 serogroup, clinical *V. cholerae* and environmental *V. cholerae* have Antibiotic Resistance Index (ARI) values of 0.55, 0.56, and 0.57, respectively. 89, 18, 4, 8, and 4 strains of *V. parahaemolyticus*, *V. vulnificus*, *V. cholerae* O1, *V. cholerae* non-O1, and *V. cholerae* O139 serogroups harboured plasmid(s) with sizes ranging from 1.3 to 16 MDa, respectively. 85/120 and 29/40 isolates of *V. parahaemolyticus* isolated from Malacca, and Penang were positive for the presence of *toxR* gene, respectively, and none the strains were positive for the *tdh*, *trh*, and *ctx* genes. All clinical strains of *V. cholerae* O1 and non-O1 and environmental isolates of *V. cholerae* O139 were positive for the *ctx* gene. The Randomly Amplified Polymorphic DNA – Polymerase Chain Reaction assay of environmental and clinical *V. cholerae* O1 and non-O1 strains generated 15, 15, 7, and 9 RAPD-types with primers Gen 15003, Gen 15005, Gen 15007, and Gen 15008, respectively. All *V. cholerae* strains generated a total of 30 RAPD-types based on primers used, whereas 48 RAPD-types were observed among the 57 *V. vulnificus* strains. The RAPD-PCR of *V. parahaemolyticus* generated 133 and 137 RAPD-types obtained with primers Gen 15001 and Gen 15002, respectively. Combination of both these primers generated 154 types for 160 strains of *V. parahaemolyticus*. 28 distinct *V. parahaemolyticus* clusters and 20 single isolates were observed at similarity level of approximately 70% based on ERI consensus. 6 major clusters and 2 single isolates were observed at the same

similarity level among clinical and environmental isolates of *V. cholerae*. Multiple PFGE banding patterns were observed among 28/160 representative strains of *V. parahaemolyticus* with sizes ranging <48 to 340 kb. Seven different PFGE patterns were identified among the 14 strains of clinical *V. cholerae*. Twelve PFGE patterns were observed among 14 representative of 57 strains of *V. vulnificus*. ERIC-PCR and RAPD-PCR methods gave higher resolution among the three molecular methods that were used in characterization of the *Vibrio* species isolated from seawater. ERIC-PCR appeared to have the best discriminatory power, followed closely by RAPD-PCR and Pulsed-Field Gel Electrophoresis.



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**PENCIRIAN MOLIKULAR SPESIS *VIBRIO* YANG DIASINGKAN
DARIPADA AIR LAUT**

Oleh

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Suatu kajian telah dijalankan untuk mencirikan spesis *Vibrio* yang diasingkan daripada air laut dipantai Melaka, Penang (Batu Feringgi, George Town) dan Terengganu. Daripada 240 sampel air laut yang diuji, 4 *Vibrio cholerae* O139, 10 *V. cholerae* bukan-O1, 7 *V. cholerae* O1, 160 *Vibrio parahaemolyticus*, 57 *Vibrio vulnificus* diasingkan.

Kewujudan spesis *Vibrio* dalam sampel air laut daripada Melaka adalah sebanyak 4/32 (0.13%), 3/32 (0.09%), 2/32 (0.06%), 24/32 (0.75%) yang merupakan positif kepada kehadiran spesis *V. cholerae* O139, *V. cholerae* bukan-O1, *V. cholerae* O1 dan *V. parahaemolyticus*. Enam daripada 30 sampel air laut daripada pantai Batu Feringgi dan 7 daripada 30 sampel air laut daripada pantai George Town adalah positif dengan 16 dan 24 kultur *V. parahaemolyticus* dapat diasingkan. Kesemua

kultur *V. parahaemolyticus* adalah negatif-Kanagawa. Lima puluh tujuh kultur daripada biotip 1 untuk *V. vulnificus* diasingkan daripada 11 (18.33%) daripada 60 sampel air laut yang diperolehi daripada pantai Marang (Terengganu). Kesemua spesis *Vibrio* yang diuji untuk corak ketahanan antibiotik menunjukkan ketahanan terhadap satu atau lebih antibiotik. Daripada 15 antibiotik yang diuji terhadap spesis *Vibrio*, *V. cholerae* kumpulan sero O139, *V. cholerae* klinikal dan *V. cholerae* persekitaran menunjukkan nilai index ketahanan antibiotik sebanyak 0.55, 0.56 dan 0.57. Lapan puluh sembilan *V. parahaemolyticus*, 18 *V. vulnificus*, 4 *V. cholerae* O1, 8 *V. cholerae* bukan-O1 dan 4 *V. cholerae* kumpulan sero O139 mempunyai plasmid yang mempunyai julat saiz daripada 1.3 hingga 16 MegaDalton (MDa).

Penemuan gen virulen melalui pengeseian PCR untuk *V. parahaemolyticus* menunjukkan 75% (85/120) dan 73% (29/40) kultur *V. parahaemolyticus* daripada Melaka dan Penang memberikan bacaan positif kepada kehadiran gen *toxR*. Namun demikian, tiada bacaan positif diperolehi untuk 160 kultur *V. parahaemolyticus* untuk gen *tdh*, *trh* dan *ctx*. Kultur klinikal *V. cholerae* O1 dan bukan-O1 serta asingan daripada persekitaran untuk *V. cholerae* O139 adalah positif untuk gen *ctx*. Pengeseian RAPD-PCR untuk kultur klinikal dan persekitaran *V. cholerae* O1 dan bukan-O1 menghasilkan 15, 15, 7 dan 9 jenis RAPD dengan menggunakan primer Gen 15003, Gen 15005, Gen 15007 dan Gen 15008. Kesemua kultur *V. cholerae* menghasilkan 30 jenis RAPD untuk primer yang digunakan manakala 48 jenis RAPD diperhatikan dalam 57 kultur *V. vulnificus*. Keputusan RAPD-PCR untuk *V. parahaemolyticus* menghasilkan 133 dan 137 jenis RAPD dengan primer Gen

15001 dan Gen 15002. Gabungan kedua primer ini menghasilkan 154 jenis untuk 160 kultur *V. parahaemolyticus*.

Analisa corak ERIC-PCR dengan menggunakan dendrogram yang dihasilkan daripada perisian Gelcompar versi 4.1 menunjukkan 28 koloni *V. parahaemolyticus* yang spesifik dan 20 asingan diperhatikan pada peringkat yang sama sebanyak 70%. Dalam kultur *V. cholerae* yang diasingkan daripada sampel persekitaran dan klinikal, 6 koloni dan 2 asingan diperhatikan pada peringkat yang sama. Analisa corak ERIC-PCR dalam kultur *V. vulnificus* menghasilkan 12 kelompok yang utama dan 3 asingan pada peringkat yang sama.

Corak jalur PFGE diperhatikan dalam cap jari yang dihasilkan daripada 28 wakil daripada 160 kultur *V. parahaemolyticus* dengan julat saiz lebih daripada 48 hingga 340 kilobasa. Tujuh corak PFGE telah dikenalpasti daripada 14 kultur klinikal *V. cholerae*. Dua belas corak PFGE telah dipastikan daripada 14 wakil daripada 57 kultur *V. vulnificus*.

Kaedah ERIC-PCR dan RAPD-PCR menghasilkan resolusi yang lebih tinggi diantara tiga kaedah molikular yang digunakan untuk pencirian spesis *Vibrio* yang diasingkan dari air laut. ERIC-PCR lebih tinggi kuasa diskriminasi dan diikuti oleh RAPD-PCR dan PFGE.

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Materials and Methods	90
Sampling Methods	90
Isolation	90
Identification	91
Results	92
Discussion	93
IV ANTIBIOTIC SUSCEPTIBILITY TESTING AND PLASMID PROFILING	99
Introduction	99
Materials and Methods	101
Antibiotic Susceptibility Testing	101
Disc Diffusion Testing	102
Extraction of Plasmid DNA	103
Reagents	104
Results	105
Discussion	106
V DETECTION OF VIRULENT GENES AMONG <i>VIBRIO PARAHAEMOLYTICUS</i>, AND <i>VIBRIO CHOLERAE</i> O1, NON-O1, AND O139 STRAINS	121
Introduction	121
Materials and Methods	123
Bacterial Strains	123
Extraction of Genomic DNA and PCR Assay for <i>ctx</i> Gene in <i>Vibrio cholerae</i> O1, Non-O1, and O139 Strains	123
Extraction of Genomic DNA and PCR Assay for <i>toxR</i> , <i>tdh</i> , <i>trh</i> , and <i>ctx</i> Genes in <i>Vibrio parahaemolyticus</i> Strains	124
Results	125
Discussion	126
VI MOLECULAR CHARACTERIZATION OF THE <i>VIBRIO</i> SPECIES STRAINS USING RAPD-PCR, ERIC-PCR, AND PFGE	137
Introduction	137
Materials and Methods	142
Bacterial Strains	142
Genomic DNA Isolation	143
Randomly Amplified Polymorphic DNA – Polymerase Chain Reaction	143
Enterobacterial Repetitive Intergenic Consensus – Polymerase Chain Reaction	144
Genomic DNA Preparation for Pulsed-Field Gel Electrophoresis	146
<i>SpeI</i> Digestion	147
Analysis of PFGE Patterns	148
Results	148
Discussion	152
Randomly Amplified Polymorphic DNA – PCR	152

Enterobacterial Repetitive Intergenic Consensus – PCR ...	156
Pulsed-Field Gel Electrophoresis	160
GENERAL DISCUSSION	200
CONCLUSION	213
BIBLIOGRAPHY	218
CURRICULUM VITAE	250
PUBLICATIONS ARISING FROM WORK IN THIS THESIS...	251

LIST OF TABLES

Table		Page
1.	Differential characteristics of <i>Vibrio</i> species pathogenic for humans	77
2.	Reaction of <i>V. cholerae</i> in screening tests	78
3.	Comparison of epidemic- and non-epidemic- associated <i>Vibrio cholerae</i> strains	79
4.	Differentiation of clinical and El Tor biotypes of <i>Vibrio cholerae</i>	80
5.	Serotypes of <i>Vibrio cholerae</i> serogroup O1	81
6.	Appearance of <i>Vibrio</i> colonies on isolation agar medium after 24-h incubation	82
7.	<i>Vibrio</i> infection reported to CDC, by syndrome and complications, 1998	83
8.	Prevalence of <i>Vibrio</i> species isolated from seawater	97
9.	Percentage of antibiotic resistance index of <i>Vibrio</i> isolated from seawater and patients	112
10.	Antibiotic resistance patterns and plasmid profiling of <i>Vibrio parahaemolyticus</i> used in this study	113
11.	Antibiotic resistance patterns and plasmid profiling of <i>Vibrio cholerae</i> s used in this study	116
12.	Antibiotic resistance patterns and plasmid profiling of <i>Vibrio vulnificus</i> used in this study	117
13.	The results of the detection of virulent genes by using PCR on <i>Vibrio parahaemolyticus</i> strains	130
14.	The results of the detection of genes by using PCR on <i>Vibrio cholerae</i> strains	134
15.	The RAPD-PCR types of <i>Vibrio parahaemolyticus</i> strains	164
16.	The RAPD-PCR types of <i>Vibrio cholerae</i> strains	169
17.	The RAPD-PCR types of <i>Vibrio vulnificus</i> strains	170

LIST OF FIGURES

Table		Page
1.	A set of keys for biochemical identification of environmental <i>Vibrio</i> species based on Arginine(-) /Lysine (+) / Ornithine (+)	84
2.	Procedure for recovery of <i>Vibrio cholerae</i> strains	85
3.	Diagram of cholera toxin. The five subunits (B) of cholera toxin which mediate binding to the host cell receptor, ganglioside GM ₁ form a doughnut shape with the enzymacally active subunit (A) in The centre of the ring. The A subunit is composed of two parts, A1 and A2, which are bonded together by a disulfide bond. When the toxin interacts with the host cell the disulfide bond is reduced	86
4.	The basic PCR mechanism. (1a) A region of DNA is chosen for amplification. (1b) The strands are separated (denatured) by heating and then cooled in a million- to billion-fold excess of the primers. (1c) The temperature is raised to the optimum for a thermostable DNA polymerase and the primers are extended using the sequence of the template strands. (2a) Only one of the newly made strands is shown after a second round of heating. (2b) When cooled, primers bind to the newly made strand , which serves as a template. (2c) The newly made strand has two defined ends, each of which is determined, by the location of the primers	87
5.	Sampling sites: (A). Malacca beach (Malacca), (B). George Town beach (Penang), (C) Batu Feringgi (Penang), and (D) Marang beach (Terengganu)	98
6.	Plasmid extraction technique for Gram-negative bacteria	119
7.	Agarose (0.7%) gel electrophoresis of plasmid DNA from <i>V. parahaemolyticus</i> isolates no. 1 – 14	120
8.	Agarose (0.7%) gel electrophoresis of plasmid DNA from <i>V. parahaemolyticus</i> isolates no. 15 – 28	120
9.	Genomic DNA isolation for PCR assay	135
10.	Agarose (2%) gel electrophoresis of representative <i>toxR</i> positive <i>V. parahaemolyticus</i> strains	136

11. RAPD-PCR fingerprints of *V. cholerae* isolated from patients and outbreaks cases obtained with primers GEN 15003 (lanes :1[A], 2[B], 3[C], 4[D], 5[E], 6[E], 7[F], 8[G]); GEN 15005 (lanes: 9[A], 10[B], 11[C], 12[D], 13[E], 14[F]); GEN 15007 (lanes: 15[A], 16[B], 17[C]) and GEN 15008 (lanes: 18[A], 19[empty], 20[B], 21[C]). Lane S contains lambda ladder DNA molecular weight markers in basepairs (bp) 172
12. RAPD-PCR fingerprints of *V. cholerae* isolated from seawater obtained primers GEN 15003 (lanes: 1[H], 2[I], 3[J], 4[K], 5[L], 6[M], 7[N], 8[empty], 9[O]) and GEN 15005 (lanes: 10[G], 11[H], 12[I], 13[J], 14[K], 15[L], 16[empty], 17[M], 18[N], 19[O]). Lane S contains lambda ladder DNA molecular weight markers in basepairs (bp) 172
13. RAPD-PCR fingerprints of *V. cholerae* isolated from seawater obtained with primers GEN 15007 (lanes: 1 and 8 [D], 2 and 5 [E], 3[F], 4 and 7[G], 8[H], and GEN 15008 (lanes: 9 and 10 [D], 11[E], 12 and 13[F], 14[G], 15[H], 16 [I], 17, 18 and 19 [J]. Lane S contains lambda ladder DNA molecular weight markers in basepairs (bp) .. 173
14. RAPD-PCR analysis of *V. cholerae* O139 strains. Four kinds of arbitrary primers GEN 15003 (lanes: 2, 3, 4 and 5), GEN 15007 (lanes: 6, 7, 8, and 9), Gen 15008 (lanes: 10, 11, 12 and 13) and GEN 15009 (lanes: 14, 15, 16, and 17) were used for PCR. Lanes: 2, 6, 10 and 14 (VC2), 3, 7, 11 and 15 (VC15), 4, 8, 12 and 16 (VC27), 5, 9, 13, and 17 (VC38); 1 and 18, lambda DNA ladder size markers (bp) 173
15. RAPD-PCR fingerprints of the representative isolates *V. parahaemolyticus* no. 1 – 19 with primer GEN 15001. Lane S contains DNA molecular weight markers in kilobase pairs (kb) 174
16. RAPD-PCR fingerprints of the representative isolates *V. parahaemolyticus* no. 1 – 19 with primer GEN 15002. Lane S contains DNA molecular weight markers in kilobase pairs (kb) 174
17. RAPD-PCR fingerprints of the representative isolates of *V. vulnificus* no. 1 – 19 with primer GEN 15003. Lane S contains DNA molecular weight markers in kilobase pairs (kb) 175

18.	RAPD-PCR fingerprints of the representative isolates of <i>V. vulnificus</i> no. 1 – 19 with primer GEN 15009. Lane S contains DNA molecular weight markers in kilobase pairs (kb)	175
19.	Dendrogram illustrating the relationships among the 157 <i>Vibrio parahaemolyticus</i> strains analysed by PCR fingerprinting using primer GEN 15001. VPM: Malacca strains; VPP: Penang strains	176
20.	Dendrogram illustrating the relationships among the 150 <i>Vibrio parahaemolyticus</i> strains analysed by PCR fingerprinting using primer GEN 15002. VPM: Malacca strains; VPP: Penang strains	179
21.	Dendrogram illustrating the relationships among the 53 <i>Vibrio vulnificus</i> strains analysed by PCR fingerprinting using primer GEN 15003. Strains no. 23, 35, 46, and 54 are untypable	181
22.	Dendrogram illustrating the relationships among the 57 <i>Vibrio vulnificus</i> strains analysed by PCR fingerprinting using GEN 15009	182
23.	Dendrogram illustrating the relationships among the 19 representative strains of <i>V. cholerae</i> analysed by RAPD-PCR fingerprinting using primer GEN 15003	183
24.	Dendrogram illustrating the relationships among the 15 representative strains of <i>Vibrio cholerae</i> analysed by RAPD-PCR fingerprinting using primer GEN 15005 ...	184
25.	Dendrogram illustrating the relationships among the 15 representative strains of <i>Vibrio cholerae</i> analysed by RAPD-PCR fingerprinting using primer GEN 15007	185
26.	Dendrogram illustrating the relationships among the 18 representative of <i>Vibrio cholerae</i> strains analysed by RAPD-PCR fingerprinting using primer GEN 15008	186
27.	DNA fingerprinting of 14 strains of clinical <i>V. cholerae</i> O1 and non-O1 generated by ERIC-PCR amplification. The first lane is 1 kb ladder molecular size markers. Lanes 1 to 4 are clinical <i>V. cholerae</i> non-O1. Lanes 5 – 14 are clinical <i>V. cholerae</i> O1	187

28. DNA fingerprinting of 17 strains of environmental *V. cholerae* O1 and non-O1 generated by ERIC-PCR amplification. The first lane is 1 kb ladder molecular size markers. Lanes 1 to 8, 11, and 12 are environmental *V. cholerae* non-O1. Lanes 9, 10, and 13 to 17 are environmental *V. cholerae* O1 187
29. DNA fingerprinting of the representative strains of *V. parahaemolyticus* generated by ERIC-PCR amplification. The first lane is molecular size markers (1 kb ladder). Lanes 1 - 19 are *V. parahaemolyticus* strains from Malacca state (VPM 1 – VPM 19) 188
30. DNA fingerprinting of the representative strains of *V. vulnificus* generated by ERIC-PCR amplification. The first lane is molecular size markers (1 kb ladder). Lanes 1 – 19 are *V. vulnificus* strains from Terengganu state (VV1 – VV19) 188
31. Dendrogram of illustrating the relationships among the 148 *Vibrio parahaemolyticus* strains analysed by ERIC-PCR fingerprinting. Strains no. 27, 28, 29, 40, 44, 52, 53, 55, 56, 57, 142, and 157 are untypable 189
32. Genetic relationships among isolated *Vibrio cholerae* strains of different sources. EVC: Environmental *V. cholerae* strains; CVC: Clinical *V. cholerae* strains. The dendrogram was produced by clustering of the unweighted pair-group method with arithmetic means 191
33. Genetic relationships among isolated *Vibrio vulnificus* strains. Dendrogram was produced by clustering of the unweighted pair-group method with arithmetic means (UPGMA) 192
34. A flow-chart of the procedure of the simplified protocol for the preparation of DNA for PFGE analysis 193
35. PFGE patterns of *V. parahaemolyticus* strains after DNA digestion with *SpeI*. Representative samples isolated from Malacca. Lane M is molecular size markers (in kilobases); lanes 1 – 14 are examined *V. parahaemolyticus* (VPM1 – VPM14) 194
36. PFGE patterns of *V. parahaemolyticus* strains after DNA digestion with *SpeI*. Representative samples isolated from Penang. Lane M is molecular size markers (in kilobases); lanes 1 – 14 are representative *V. parahaemolyticus* (VPP121 – VPP125) 194

37.	PFGE patterns of overall clinical <i>V. cholerae</i> strains after DNA digestion with <i>SpeI</i> . Lane M is molecular size markers (in kilobases); lanes 1 – 14 are <i>V. cholerae</i> strains examined (CVC1 – CVC14)	195
38.	PFGE patterns of overall environmental <i>V. cholerae</i> strains after DNA digestion with <i>SpeI</i> . Lane M is molecular size markers (in kilobases); lanes 1 – 14 are environmental <i>V. cholerae</i> strains examined (EVC1 – EVC17). Isolates no. 1 to 8 have experienced the DNA degradation	195
39.	PFGE patterns of representative <i>V. vulnificus</i> strains after DNA digestion with <i>SpeI</i> . Lane M is molecular size markers (in kilobases); lanes 1 – 14 are representative <i>V. vulnificus</i> strains (VV1 – VV14)	196
40.	Genetic relationships among 25 representative strains of <i>V. parahaemolyticus</i> isolated from Malacca (VPM1 – VPM14) and Penang (VPP120 – VPP134). Similarity analysis was performed by using the Jeffrey coefficient and clustering was done by UPGMA. CVC: clinical <i>V. cholerae</i> strains; EVC: environmental <i>V. cholerae</i> strains	197
41.	Genetic relationships among 23 clinical and environmental <i>V. cholerae</i> strains isolated from Malacca. Similarity analysis was performed by using the Jeffrey coefficient and clustering was done by UPGMA. CVC: clinical <i>V. cholerae</i> strains; EVC: environmental <i>V. cholerae</i> strains	198
42.	PFGE patterns of the 14 representative of 57 <i>V. vulnificus</i> after DNA digestion with <i>SpeI</i> . Lane M is molecular size markers (in kilobases); lanes 1 – 14 are representative <i>V. vulnificus</i> strains (VV1 – VV14)	199

LIST OF ABBREVIATIONS

APW	Alkaline Peptone Water
ARI	Antibiotic Resistance Index
cAMP	Cyclic Adenosine MonoPhosphate
CDC	Center of Disease Control and Prevention
CFU	Colony-Forming Unit
CT	Cholera Toxin
CVC	Clinical <i>Vibrio cholerae</i>
DNA	Deoxyribo Nucleic Acid
EDTA	Ethylene Diamine Tetra Acetic
ERIC	Enterobacterial Repetitive Intergenic Consensus
EVC	Environmental <i>Vibrio cholerae</i>
GET	Glucose-EDTA-Tris base
GS	Gelatin Salt
IMR	Institute for Medical Research
KIA	Kligler Iron Agar
KP	Kanagawa Phenomenon
LIA	Lysine Iron Agar
MDa	Mega Dalton
min	minute(s)
mCPC	modified Cellobiose-Polymyxin B-colistin
NCCLS	National Committee for Clinical Laboratory Standard
O/F	Oxidase / Fermentation
ONPG	O-Nitrophenyl- β -Galactopyranoside
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis
RAPD	Randomly Amplified Polymorphic DNA
rpm	round per minute
SDS	Sodium Dodecyl Sulfate
sec	second(s)
SOD	Superoxidase Dismutase
<i>tdh</i>	Thermostable Direct Hemolysin
<i>trh</i>	Thermostable Direct Hemolysin-Related Hemolysin
TCBS	Thiosulfate Citrate Bile salts Sucrose
TCI	Thiosulfate Chloride Iodide
TSA	Trypticase Soy Agar
TSI	Triple Sugar Iron
TTGA	Taurocholate Tellurite Gerlatin Agar
VV	<i>Vibrio vulnificus</i>
VPP	<i>Vibrio parahaemolyticus</i> Penang
VPM	<i>Vibrio parahaemolyticus</i> Malacca
μ g	Micro-gram
μ l	Micro-liter

CHAPTER I

INTRODUCTION

Bacteria belonging to the genus *Vibrio* are widely distributed in the aquatic environment and are considered to be autochthonous bacteria in marine and estuarine waters (Nishibuchi and Kaper, 1985). There is considerable evidence that surface waters, especially estuary and lagoon systems with elevated salinity are natural habitats of *Vibrio* (Bockemuhl *et al.*, 1986). The *Vibrio* from seawater have attracted increasing attention since one of them is an important cause of food poisoning in Japan, where numerous outbreaks have followed consumption of raw fish dishes. In 1951, Japanese researchers discovered that *Vibrios* are a common cause of illness among people who eat fish caught in bacterially contaminated water. Since then, it has been found to cause about a quarter of all reported cases of diarrhea in Japan, where fish is often eaten raw. Outbreaks have also been reported around the world, including almost all coastal states of the United States, and epidemics have occurred in Latin America and Southeast Asia. In the nineteenth century, pandemic of Asiatic cholera occurred from the Far East to Africa, other parts of Asia, Europe, and North America. During the present century, the disease appears to have been more or less limited to India and surrounding areas although epidemics have occurred in other parts of the world, including Egypt, Indonesia, Korea and the Philippines (Wistreich and Lachtman, 1988). Today, cholera infection had been reported from India, Bangladesh, Nepal, Burma, Thailand, Malaysia, Saudia Arabia, China and Pakistan, Italia, Spain, Japan, Australia (Venkateswaran *et al.*, 1989, Amaro *et al.*, 1990, Albert, 1994,

Desmarchelier *et al.*, 1995, Barbieri *et al.*, 1999, Radu *et al.*, 1999). However, the geographic locations of cholera outbreaks have not changed dramatically for hundreds of years. In the USA, cholera was a severe epidemic disease, and the pattern was very similar from 1800 to 1900, until the introduction of safe drinking water. Where cholera has occurred, as in Bangladesh, the pattern even in 1998 has not changed much.

There have been seven pandemics of cholera in recorded history. Even though the etiological agents of the first four pandemics are not known since they occurred in the time before such agents could be recognized, the last three pandemics are known to be due to *Vibrio cholerae* serogroup O1. The current global epidemics of cholera are part of the tail of the seventh pandemic, which began in the 1960s (Colwell and Huq, 1999) Since 1883 when the causative agent, the Cholera Vibrio, was discovered by Robert Koch in epidemic in Egypt and India (Davis *et al.*, 1973), the disease continues to spread in other parts of the world. The latest case, large epidemic of cholera-like disease occurs in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. In this case, the causative agent or this organism was included into *Vibrio cholerae* non-O1. According to the Cholerae Working Group Report (1994), epidemics of cholerae caused by *Vibrio cholerae* O1 occur regularly in Bangladesh, however, until lately *Vibrio cholerae* non-O1 has been associated with sporadic cases of diarrhoeal disease in many parts of the world. This epidemic began in December 1992 in Southern Bangladesh and spread throughout the country. By the end of March 107,297 cases of diarrhea and 1473 death had been reported (Albert, 1994). This disease has also a close relationship with major public health problem confronting developing countries,