CHAPTER · 2

REVIEW

OF

LITERATURE
2. REVIEW OF LITERATURE

2.1. Vibrios

Vibrio is the type genus of the family *Vibrionaceae* in the order X1 Vibrionales. The family *Vibrionaceae* includes opportunistic pathogens of humans and animals (Daniels et al., 2000; Thompson et al., 2004) as well as free living chemoheterotrophs and/or commensals of marine fauna (Guerinot and Patriquin, 1981; Grimes et al., 1985). The members of the family *Vibrionaceae* constitute a predominant heterotrophic bacterial group in aquatic environments (Simidu et al., 1977). The role of Vibrios in the marine environment has been shown to include biodegradation, nutrient regeneration and biogeochemical cycling (Colwell, 1994). It is now understood that vibrios play important roles in the health of humans and many different marine hosts, in addition to being an abundant and virtually ubiquitous component of the aquatic microbiota (Thompson et al., 2006).

2.2. Vibrios vis-à-vis Human disease

The genus *Vibrio* is a member of the family *Vibrionaceae* and consists of 44 recognized species (Bergeys manual of Systematic Bacteriology, 2005). There are 12 species that are routinely isolated from human clinical samples, and the diseases in which they are implicated include diarrhoeal disease, septicemia and wound infections (Janda et al., 1988; Holmberg, 1992; Farmer et al., 2003). As per Bergey's manual of Systematic Bacteriology (2005) twelve species of Vibrios occur in human clinical specimens (Table 2.1); 11 of these are apparently pathogenic to humans causing diarrhoea or extraintestinal infections. Of these 11, three species viz., *Vibrio cholerae*, *Vibrio parahaemolyticus*, and *Vibrio vulnificus*, account for the majority of *Vibrio* infections in humans. Pathogenic
vibrios cause 3 major syndromes of clinical illness: gastroenteritis, wound infections, and septicemia.

Table 2.1. Worldwide occurrence of Vibrio species in human clinical specimens*

<table>
<thead>
<tr>
<th>Species</th>
<th>Occurrence in human clinical specimen</th>
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<tbody>
<tr>
<td></td>
<td>Intestinal</td>
<td>Extraintestinal</td>
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<tr>
<td><em>V. cholerae</em></td>
<td>+++</td>
<td>+</td>
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<tr>
<td>Serogroups O1 and O139</td>
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<td>+</td>
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<tr>
<td>Serogroup non-O1</td>
<td>+</td>
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<tr>
<td><em>V. alginolyticus</em></td>
<td>-</td>
<td>+</td>
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<tr>
<td><em>V. cincinnatiensis</em></td>
<td>++</td>
<td>-</td>
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<tr>
<td><em>V. damsela</em></td>
<td>-</td>
<td>+</td>
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<tr>
<td><em>V. fluvialis</em></td>
<td>++</td>
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<tr>
<td><em>V. furnissii</em></td>
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<td><em>V. harveyi (V. carchariae)</em></td>
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<td><em>V. hollisae</em></td>
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<td><em>V. metschnikovii</em></td>
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<tr>
<td><em>V. mimicus</em></td>
<td>++</td>
<td>-</td>
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<td><em>V. parahaemolyticus</em></td>
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<td>+</td>
</tr>
<tr>
<td><em>V. vulnificus</em></td>
<td>+</td>
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* Source: Bergey’s manual of Systematic Bacteriology (2005)

The most frequently isolated enteric pathogens of this group are *V. cholerae*, the etiological agent of pandemic cholera and *V. parahaemolyticus*, a major cause of food borne disease, particularly in Japan and SE Asia (Joseph et al., 1982). Epidemiological data suggests that *V. mimicus*, *V. hollisae* and *V. fluvialis* can cause diarrhea or infection of gastrointestinal tract (Hlady and Klontz, 1996). Worldwide, large outbreaks are caused by toxigenic strains of serogroups O1 and O139 *V. cholerae* which produce the cholera toxin, but in the United States, non-toxigenic strains of *V. cholerae* predominate among the cases reported to the Centers for Disease Control and Prevention (CDC). *V. cholerae* accounted for 9.8% of all Vibrio isolates reported in the U.S to the CDC in 2004 (CDC, 2005).
2.2.1. *V. cholerae*: *V. cholerae* is the etiological agent of the dreaded waterborne disease, cholera. *V. cholerae* O1 is the primary causative agent (Morris and Black, 1985; Colwell, 1996; Mooi and Bik, 1997). Cholera epidemics have been reported in over 75 countries in South America, Africa and Asia during the past decade. In addition, each year sporadic cases are reported in other countries around the world. First pandemic of cholera was recorded in 1817. It is generally accepted that seven distinct pandemics of cholera have occurred since the onset of the first pandemic in 1817 (Pollitzer, 1959). The seventh pandemic began in Indonesia (Glass and Black, 1992), but cholera pandemics have usually begun in the Gangetic delta of the Indian subcontinent and then in other continents (Faruque et al., 1998; Siddique et al., 1992). In 1854, the Italian physician Filippo Pacini discovered the first Vibrio species i.e. *V. cholerae*. In the same period John snow studied the epidemiology of cholera and recommended the provision of pure tap water, free from contamination by sewers and house drains as an effective means of containing the dissemination of disease. During the 5th pandemic, Robert Koch isolated *V. cholerae* (Koch, 1884) from rice water stools of patients in Egypt in 1883 and in India in 1884. This cholerae strain is designated as the classical biotype of *V. cholerae*. The fifth pandemic (1881-1896) and 6th pandemic (1899-1923) were attributed to the classical biotype (Kaper et al., 1995). The cholera 7th pandemic started in 1961 was caused by another biotype of *V. cholerae*, the El Tor biotype. El Tor biotype was isolated from pilgrims in the El Tor quarantine camp. Except for the 7th pandemic which originated on the island of Sulawesi in Indonesia (Kamal, 1974), the other pandemics arose in the Indian subcontinent, usually the Ganges delta and spread to other continents and extending over many years (Chambers, 1938; Laval, 1989; Pollitzer, 1959).

2.2.1.1. Serotypes of *V. cholerae*: The antigenic structure of Vibrio species is defined on the major classes of bacterial antigens viz., somatic (O), capsular (K) and flagellar (F) which are associated with the bacterial lipopolysaccharide extracellular layer or capsule and flagellum, respectively. Strains of *V. cholerae* are divided into 2 serogroups designated as *V. cholerae* O1 and *V. cholerae* non O1. Non O1 *V. cholerae* which is morphologically and biochemically similar to *V. cholerae* O1, does not
agglutinate with group O1 specific antiserum (Colwell et al., 1984; Farmer et al., 1985). Within *V. cholerae* there are over 180 defined O antigens but serogroups O1 and O139 contain all strains that have caused all large epidemics and pandemics of cholera (Shimada et al., 1994; Yamai et al., 1997). Strains of *V. cholerae* O1 have been further differentiated into 3 serotypes designated Ogawa, Inaba and Hikojima, which have antigenic formulae of AB, AC and ABC types, respectively (Greenough, 1995). O antigen is the bacterium's major protective antigen and therefore changes in the O antigen of a preexisting epidemic strain may result in a new pathogen capable of causing diseases in populations immune to the original epidemic strain (Albert et al., 1993; Ramamurthy et al., 1993). For example *V. cholerae* O139 Bengal strain emerged from an O1 epidemic strain by genetic exchange of O-antigen biosynthesis regions (Berche et al., 1994; Mooi and Bik, 1997; Stroeher and Manning, 1997) and O139 strains cause disease in persons immune to O1 strains (Bhattacharya et al., 1993; Morris et al., 1995).

### 2.2.1.2. *V. cholerae* O139

*V. cholerae* O139 an emerging agent of epidemic cholera is another important cause of diarrhea (Morris et al., 1995). In late 1992 epidemics of severe acute watery diarrhea clinically resembling cholera and mainly affecting adults was reported in Madras, India and in Southern Bangladesh (Cholera Working Group, 1993; Ramamurthy et al., 1993). The epidemics later spread to other parts of both countries and to some of the neighbouring countries of the region (Chongsanguan et al., 1993; Nair et al., 1994; Faruque et al., 1997, Faruque et al., 1999). This bacterium did not belong to any of the 138 serogroups for *V. cholerae* described until then; the conclusion was that it belonged to a new group (Bhattacharya et al., 1993). This epidemic form of cholera like disease had been strongly associated with a strain of *V. cholerae* non O1 designated *V. cholerae* O139 Bengal (Albert et al., 1993). This clone shows striking similarity to *V. cholerae* O1 biotype El Tor but possesses a capsule like the non O1 Vibrios (Calia et al., 1994; Khan et al., 1995). It has been suggested that a second serogroup O139 has pandemic potential but O139 is currently restricted to epidemic disease in the Indian subcontinent and SE Asia. Genetic and phenotypic evidence strongly suggests that the O139 strain arose from a *V. cholerae* O1 strain probably El Tor biotype by horizontal gene transfer (Johnson et al., 1994; Waldor and...
The emergence of *V. cholerae* O139 Bengal in 1992, its initial rapid spread throughout Bangladesh and neighbouring countries and its propensity to replace the existing strains of *V. cholerae* O1 led experts to suspect *V. cholerae* O139 as the new pandemic strain of cholera (Albert et al., 1993; Iida et al., 1993; Ramamurthy et al., 1993; Rivas et al., 1993; Siddique et al., 1994). The epidemic causing strains of *V. cholerae* belong to the O1 or O139 serogroups and produce cholera toxin (CT) which is the major contributing factor for profuse diarrhoea (*Cholera gravis*) (Kaper et al., 1995).

2.2.1.3. Non O1 *V. cholerae*: Some strains of *V. cholerae* do not agglutinate with O1 antiserum but can still cause diarrheal illness. The most common presentation is the self-limited gastroenteritis, although certain strains, especially those that produce cholera toxin, can cause severe cholera-like disease (Datta-Roy et al., 1986). There were 130 cases of non-O1 *V. cholerae* infection (third most common Vibrio infection after *V. parahaemolyticus* and *V. vulnificus* infections) reported in Florida from 1981 to 1993 (Daniels and Shafaie, 2000). Non O1 *V. cholerae* was initially recognized as the causative agent of small outbreaks and sporadic cases of cholera like disease (Morris et al., 1981; Klontz, 1990; Morris et al., 1990; Bhattacharya et al., 1993). Isolates of *V. cholerae* belonging to serogroup other than O1 and O139 normally lack cholera toxin genes. *V. cholerae* non O1 serogroups are widely distributed in the aquatic environment and are free living in nature (Blake and Weaver, 1980; Wilson et al., 1981). Unlike *V. cholerae* O1, they do not usually have epidemic or pandemic potential (Blake and Weaver, 1980). However, they have often been identified as the causative agents of sporadic cases (McIntyre and Feeley, 1965; Hughes et al., 1978; Spira et al., 1978) and localized outbreaks (Aldova and Laznickova, 1968; Dakin et al., 1974) of cholera like diarrhoea which is sometimes accompanied by fever and blood and mucus in stools (Dakin et al., 1974; Hughes et al., 1978). However, unlike O1 strains, non-O1 strains of *V. cholerae* are commonly involved in invasive diseases such as septicaemia in immunocompromised hosts (Safrin et al., 1988; Lin et al., 1996). Non-toxigenic strains of *V. cholerae* O1 may be isolated from cases of diarrhoea and from the environment in areas where cholera is absent. These strains are usually haemolytic. Human infection attributed to non O1
*V. cholerae* is most often related to seafood ingestion, exposure to polluted fresh water, brackish water or sea water and foreign travel (DePaola, 1981; Morris *et al*., 1981; Bonner *et al*., 1983). In Italy a case of infection by *V. cholerae* O158 was reported (Filetici *et al*., 1997). In Italy, prevalent serovar was O40 (16%) followed by O6 (12%) and O2, O8, O41 and O64 and O107 (6% each) and all isolates were negative for CT gene (Filetici *et al*., 1997). Infection with non O1 *V. cholerae* due to exposure to contaminated water and ingestion of contaminated shellfish in various countries was reported (Gelbart, and Prabhudesai, 1986; Safrin *et al*., 1988; Dumler *et al*., 1989; Pitrak and Gindorf 1989). Strains of Non O1 *V. cholerae* greatly outnumber O1 strains in the environment and majority of these isolates lack the classical virulence factors such as cholera toxin and toxin co-regulated pilus (Baumann *et al*., 1984; Kaysner *et al*., 1987).

### 2.2.1.4. Sources of *V. cholerae*

*V. cholerae* is both a human pathogen and a natural inhabitant of aquatic environment (Colwell *et al*., 1977; Cottingham *et al*., 2003). Colwell *et al* (1981) hypothesized that *V. cholerae* is a member of the autochthonous i.e. naturally occurring microbial flora of brackish water and estuaries. Singleton *et al* (1982) stated that *V. cholerae* is an autochthonous member of the estuarine microbial community. The autochthonous existence of *V. cholerae* in aquatic environment of cholera endemic regions has been established in several studies (Huq *et al*., 1984; Colwell and Huq, 1994; Gil *et al*., 2004). 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 the brackish waters (Colwell and Spira, 1992). Lipp *et al* (2003) observed that it was highly unlikely that cholera in Peru arrived from other cholera affected regions of the world and thought that a more plausible explanation was that *V. cholerae* is autochthonous to Peruvian coastal, brackish and riverine waters and it is reasonable to examine those environmental conditions giving rise to both plankton blooms and increase in *V. cholerae* concentration. Colwell *et al* (1977) first hypothesized that coastal waters were an important reservoir of *V. cholerae*. The presence of *V. cholerae* O1 year round via its commensal association with plankton was established using direct detection methods (Huq *et al*., 1990). *V. cholerae* has been detected in seawater and other environment sources around the world, both in areas where cholera is endemic and in
cholera free area (Colwell et al., 1981; Kaysner et al., 1987; Jesudason et al., 2000; Huq et al., 2001; Jiang, 2001). *V. cholerae* has been isolated from continental water as well as from estuarine water and sea water (Colwell et al., 1977; Muller, 1977; Nacescu and Ciufecu, 1978; Bashford et al., 1979; Kaper et al., 1979; Colwell et al., 1981, Lee et al., 1982; West and Lee, 1982) in many geographical areas. Presently *V. cholerae* is considered a member of the autochthonous bacterial member of these habitats (Colwell et al., 1981; Kaneko and Colwell 1978; Kaper et al., 1979; Lee et al., 1982) and its presence is not correlated with commonly used coliforms as faecal indicators (Hood and Ness, 1982; Lee et al., 1982). It is now firmly established that *V. cholerae* is a natural inhabitant in diverse aquatic environments such as the ocean, estuaries, rivers and lakes (Garay et al., 1985; Myatt and Davis 1989; Venkateswaran et al., 1989a; Perez-Rosas and Hazen, 1989; Colwell and Spira, 1992). Water (and its associated microorganisms, animals and plants) is the natural habitat of the non-halophilic and marine Vibrios. Environmental factors regulating Vibrio concentrations and distribution in both fresh water and sea water ecosystems include the concentration of organic and inorganic chemicals, pH, temperature, salinity, oxygen tension and exposure to UV light (Chakraborty et al., 1997). The factors that govern the distribution of Vibrios include human, animal or plant hosts, inorganic nutrients and carbon sources availability, temperature, salinity, dissolved oxygen and depth below surface for the species that are found in ocean (Simidu and Tsukamoto, 1985). Independent of salinities Vibrio densities tend to increase in warmer waters when enough organic and inorganic nutrients are present for growth. *V. cholerae* positive sample (water) was positively correlated with air temperature (Lipp et al., 2003).

The microbial ecology of *V. cholerae* is best exemplified by the fact that *V. cholerae* can survive in 'free living state' in both fresh water and saline environments. It is widely distributed in habitats such as sewage, brackish water, estuaries, coastal inlets, polluted streams, rivers, ponds and lakes. *V. cholerae* can also persist in an epibiotic form associated with various microscopic life (Hood et al., 1984). Common microorganisms associated with *V. cholerae* include cyanobacteria, phytoplankton (diatoms, fresh water algae) and zooplankton (Huq et al., 1995; Chakraborty et al., 1997). *V. cholerae* may attach to the tissues of chitinous exoskeleton of crustaceans and production of enzyme chitinase might be important in this process. All these combined
niches provide a continuous source for the maintenance and dissemination of *V. cholerae* throughout the year. Dissolved organic matter during intense phytoplankton blooms has the potential to support explosive growth *V. cholerae* in seawater (Mourino-Perez *et al.*, 2003). Transmission of *V. cholerae* O1 and O139 to humans usually occurs through consumption of contaminated water and food (unwashed fruits, vegetables, raw seafood).

Water is recognized as the most important vehicle for cholera transmission. In addition, outbreaks of food borne cholera mainly associated with seafood including molluscan shellfish, crustaceans and fin fish are most often incriminated in food borne cholera (Albert *et al.*, 1997; Rabbani and Greenough, 1999). Seafood has been implicated in a number of cholera outbreaks. Toxigenic *V. cholerae* O1 was recovered from non potable (ballast, bilge and sewage) water from cargo ships (McCarthy and Khambaty, 1994). Aquatic birds may serve as vectors of *V. cholerae*; the organism was isolated from cloacal swabs and freshly voided droppings (Ogg *et al.*, 1989). *V. cholerae* biotype El Tor appears to concentrate on the surface of water hyacinth (*Eichhornia crassipes*) thereby enhancing its survival and its potential for transmission through waterways (Spira *et al.*, 1981). Similar association was observed between *V. cholerae* O139 and *Eichhornia crassipes* (Bhanumathi *et al.*, 2003). The specificity of attachment of *V. cholerae* to live copepods was confirmed by scanning electron microscopy which revealed that the oral region and egg sac were the most heavily colonized areas (Huq *et al.*, 1983). These microorganisms can survive in fresh water and sea water, either in a viable and culturable stage or by entering a viable but non culturable form (VBNC) (Grimes *et al.*, 1986). *V. cholerae* lives in association with crustacean zooplankton (Huq *et al.*, 1983; Heidelberg *et al.*, 2002) and has been detected in algae (Epstein, 1993; Islam *et al.*, 1999; Islam *et al.*, 2004). *V. cholerae* has been isolated from freshwater and marine macrophytes (Islam *et al.*, 1994) as well as from benthic animals like prawns, oysters (Tweedt *et al.*, 1981), crabs (Blake *et al.*, 1980) and chichironomid egg mass (Broza and Halpern, 2001) and has also been shown able to replicate intracellularly in free living amoebae (Abd *et al.*, 2005). *V. cholerae* can also grow in water as a free living organism in the planktonic phase (Venkateswaran *et al.*, 1989a; Mourino-Perez *et al.*, 2003; Worden *et al.*, 2006). Egg masses of the non biting midge (*Chironomus sp.*, diptera) harbour *V. cholerae* and acts as its sole carbon source thereby providing a possible natural reservoir for cholera.
bacterium (Wei and Hsu, 2001). Abd et al (2007) reported that *V.cholerae* O1 strains were facultative intracellular bacteria, able to survive and multiply symbiotically inside the aquatic free-living amoeba *Acanthamoeba castellanii*. The interaction showed a facultative intracellular behaviour of *V.cholerae* O1 classical and El Tor strains and a possible role of *A.castellanii* as an environmental host of *V.cholerae* species.

*V.cholerae* thus has both particulate associated existence and free living existence. *V.cholerae* has multiple lifestyles including a planktonic free swimming form, a sessile form attached to zooplankton and other aquatic flora and fauna (Colwell and Huq, 1994) and a pathogen of humans. It also enters VBNC (Roszak and Colwell 1987) under certain conditions. Crustacean zooplankton has frequently been reported to promote *V.cholerae* proliferation by serving as both a substratum and a substrate (Heidelberg et al., 2002; Huq et al., 1983). VBNC state is the state in which metabolically active cells cannot be cultured on microbiological media (Colwell and Huq, 1994a). VBNC cells of *V.cholerae* show a significant reduction in cell size and a morphological change from rods to cocci (Xu et al., 1982). Using fluorescent antibody techniques microbiologists have now identified a VBNC for *V.cholerae*. The wide spectrum of mucinases and chitinases secreted by *V. cholerae* may assist it in attaching and embedding in the walls of aquatic organisms (Epstein et al., 1993). Vibrio cells may enter VBNC state caused by nutrient starvation and physical stress. Microcosm studies showed that VBNC cells could remain viable in the environment for years and continue to be capable of causing disease (Colwell and Huq, 1994a). The ability of *V.cholerae* O1 to attach to and colonize exoskeleton (carapace) of shrimp and crabs (edible crustaceans) provides a potential means of survival in aquatic environments (Castro-Rosas and Escartin, 2002). Alam et al (2007) studied the Viable but nonculturable *V.cholerae* O1 in biofilms in the aquatic environment and their role in cholera transmission. In this study coccoid, nonculturable *V.cholerae* O1 in biofilms maintained for 495 days in Mathbaria (Bangladesh) in pond water became culturable upon animal passage and concluded that biofilms can act as a reservoir for *V.cholerae* O1 between epidemics because of its long-term viability in biofilms.

The most common sources of contamination include raw or undercooked shellfish, water, ice, rice, food and beverages from street vendors, and food left out at
room temperature for several hours (Blake et al., 1980; Pavia et al., 1987; Tauxe et al., 1988; St Louis et al., 1990). Foodborne transmission of cholera may be facilitated through the rapid growth of organisms in moist, alkaline foods held at ambient temperature.

The pandemic nature of *V. cholerae* indicates that this organism can adapt and survive in diverse environmental conditions (Kaper et al., 1995). Survival of *V. cholerae* O1 under low nutrient condition in aquatic environments has important implications in the epidemiology of cholera (Shiba et al., 1995). It has been shown that the bacterium can survive in a starvation medium for as long as 75 days without significant decrease in culturability (Baker et al., 1983). Natural occurrence of *V. cholerae* predominantly in the non-culturable state between epidemics and as aggregates of structured biofilms has been established (Alam et al., 2006). Adhesion to the surfaces both in the human intestine and in the aquatic environment plays an important role in *V. cholerae*’s success as a pathogen and an environmental organism (Kierek and Watnick, 2003). Cholera epidemics have been associated with heavy rainfall and increase in sea surface height (Lobitz et al., 2000; Lipp et al., 2002). Both of these conditions are predicted to alter the organic and inorganic compositions of an estuary which is the primary interface between humans and the marine environment (Reemtsma et al., 1993; Mahadevan and Subramanian, 1999; Padmavathi and Satyanarayana, 1999; Han and Webster, 2002). Analysis of environmental and clinical data have revealed significant correlations of water temperature, water depth, rainfall, conductivity and copepod counts with occurrence of cholera toxin producing bacteria (Huq et al., 2005). It has been established by remote sensing employing satellites, that sea surface temperature and sea surface height are correlated with cholera epidemic (Colwell, 1996; Lobitz et al., 2000). Ecological studies indicate that water temperature, salinity, concentration of organic substances and the potential tendency for association with sediments or chitinous surfaces of higher organisms significantly influence the occurrence and the number of these microorganisms in the environment (Venkateswaran et al., 1989). Temperature and quality of dissolved organic carbon had a significant influence on *V. cholerae* growth (Kirschner et al., 2008). Patel et al (2004) studied the survival of *V. cholerae* (non-O1, El Tor and classical strains) in industrially polluted water, with particular reference to iron concentrations and
suggested that chemical contamination of water may be one of the important factors instrumental in the subsequent ability of *V.cholerae* to persist, multiply and survive in the aquatic environment.

### 2.2.1.5. Cholera disease and predisposing factors

Infection with *V.cholerae* can cause profuse watery diarrhea, vomiting, and muscle cramps. Cholera is a dehydrating diarrhoeal illness that results in substantial loss of fluid and electrolytes and stool volumes may approach 1 L/h. (Hirschhorn et al., 1968; Pierce et al., 1969) Severe illness has been associated with high-dose exposure, low gastric acidity, and blood group O (Blake, 1993). Severe cholera may be characterized by "rice water" stools, loss of 10% or more of body weight, loss of normal skin turgor, dry mucous membranes, sunken eyes, lethargy, anuria, weak pulse, altered consciousness, and circulatory collapse. Severe infections may result in death if the dehydration is not treated aggressively with fluid and electrolyte replacement (Daniels and Shafaie, 2000)

Individuals with blood group O are at an increased risk of hospitalization due to classical and El Tor *V. cholerae* O1 as well as *V.cholerae* O139 (Barua and Pagueo, 1977; Chaudhuri and Das, 1978; Clemens et al., 1989; Faruque et al., 1994; Swerdlow et al., 1994). It has been hypothesized that *V. cholerae* infection is the evolutionary force behind the low prevalence of O blood group in Ganges river delta which is a historic and global epicenter of cholera (Glass et al., 1985; Kaper et al., 1995).

### 2.2.2. *V.parahaemolyticus*

*V.parahaemolyticus* was first identified as a cause of foodborne illness in 1950 (Fujino et al., 1953). *V.parahaemolyticus* is a sea food-borne halophilic pathogen that causes acute gastroenteritis in humans. *V.parahaemolyticus* has caused numerous cases of gastroenteritis, including many outbreaks. Cases are associated with the consumption of raw or undercooked shellfish such as oysters, shrimp, crabs and lobster. Early studies suggested an association between human strains causing diarrhoea and production of a positive hemolytic reaction on a special medium, the Wagatsuma Agar. This reaction, termed the Kanagawa phenomenon (KP), was positive for virtually all clinical isolates whereas most non-clinical strains were 'Kanagawa negative'.

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Subsequent studies demonstrated a direct correlation between ‘Kanagawa positive’ strains and the elaboration of a thermostable direct haemolysin (TDH). This haemolysin is enterotoxigenic in rabbit ileal loops and appears to alter ion flux in intestinal cells, thereby causing a secretory response and gastroenteritis (Nishibuchi and Kaper, 1995). Strains classified as ‘Kanagawa strong positive’ have two copies of the TDH gene (\(tdh1, tdh2\)) whereas strains classified as ‘Kanagawa weak’ have only a single copy. Kanagawa negative strains lack this gene (Nishibuchi and Kaper, 1995). In the mid 1980s however, Kanagawa negative strains associated with gastroenteritis began to appear from different geographic areas (Honda et al., 1987). It was later found that these Kanagawa negative strains produced a different haemolysin named ‘TDH related haemolysin’ (TRH). The gene for TRH (\(trh\)) has 70-80% homology to \(tdh\) and appears to be closely associated with urease expression (Nishibuchi and Kaper, 1995, Suthieukul et al., 1995). Thus kanagawa negative strains of \(V.parahaemolyticus\) can apparently also cause gastroenteritis if they have the \(trh\) gene. The \(tdh\) gene is also found in several unrelated Vibrios, including \(V.hollisae\), \(V.mimicus\) and \(V.cholerae\) non-O1 (Nishibuchi et al., 1996). This finding suggests that horizontal transfer of \(tdh\) genes to other species is a possible common enteropathogenic mechanism.

\(V.parahaemolyticus\) inhabits inshore coastal areas and estuaries and has only rarely been recovered from pelagic regions (Joseph et al., 1982). It has been isolated from various parts of the water column, sediment, zooplankton, shellfish and fish. Occasionally, \(V.parahaemolyticus\) has been recovered from fresh water in Indonesia and India (Chakraborty et al., 1997). It has also been recovered from inland bodies of water in the United States where the Na\(^+\) content is high. However salinity (specifically the concentration of Na\(^+\)) is the most important factor governing the environmental distribution of Vibrios. Although salinity is a critical parameter, it does not completely explain the environmental distribution of all Vibrios because halophilic species such as \(V.parahaemolyticus\) can survive in suboptimal Na\(^+\) concentrations (Bergey’s manual of Systematic Bacteriology, 2005). Like \(V.cholerae\), growth of \(V.parahaemolyticus\) in the marine environment is favoured by warmer temperature. \(V.parahaemolyticus\) has been isolated from a variety of marine animals including clam, oyster, lobster, scallop, sardine, squid, eel, crab and shrimp (Joseph et al., 1982). Most outbreaks of gastroenteritis caused
by *V. parahaemolyticus* have been linked to the consumption of crabs, shrimp, lobsters and oysters. In Japan, *V. parahaemolyticus* is a major cause of food poisoning and is associated with the ingestion of raw fish such as sashimi and sushi (Chakraborty et al., 1997).

The most common clinical manifestation of *V. parahaemolyticus* infection is gastroenteritis (Levine and Griffin, 1993). Acute watery diarrhea, abdominal cramps, and nausea usually characterize the illness. Between 1988 and 1997, a review of *V. parahaemolyticus* infections in the United States found that 59% of persons had gastroenteritis, 34% had wound infections, 5% had primary septicemia, and 2% had other sites of infections (Daniels et al., 2000). The infectious dose of *V. parahaemolyticus* is between $10^5$ and $10^7$ viable cells ingested (Sanyal and Sen, 1974). Sarakar et al (2003) observed that except for sharing the similar serovars, sewage and clinical strains of *V. parahaemolyticus* were genetically different. DePaola et al (2003) determined the potential virulence attributes, serotypes, and ribotypes for 178 pathogenic *V. parahaemolyticus* isolates from clinical, environmental, and food sources on the Pacific, Atlantic, and Gulf Coasts of the United States and from clinical sources in Asia and found that most of the environmental, food, and clinical isolates from the United States were positive for tdh, trh, and urease production. The fact that certain serotypes and ribotypes contained both clinical and environmental isolates while many others contained only environmental isolates implies that certain serotypes or ribotypes are more relevant for human disease. Phillips et al (2006) observed that total *V. parahaemolyticus* levels in oysters (*Crassostrea virginica*) increase as water temperatures increase and therefore, in the light of global warming, it has become increasingly important to efficiently monitor *V. parahaemolyticus* levels in market oysters. It was suggested that risk assessment may be accomplished by incorporating readily available remotely sensed (RS) satellite data on sea surface temperature (SST) into the current FDA risk assessment model. Pathogenic strains of *V. vulnificus* and *V. parahaemolyticus* which are natural inhabitants of estuarine environments world wide are often transmitted to humans through consumption of raw shellfish that flourish in the same estuaries (Andrews, 2004).
2.2.3. *V. vulnificus*: *V. vulnificus* was first identified and described by the Centers for Disease Control and Prevention (CDC) in 1976 (Hollis et al., 1976). *V. vulnificus* is an important pathogenic vibrio because of its invasiveness and the high fatality rates associated with infection. *V. vulnificus* causes wound infections and septicemia (Hollis et al., 1976; Klontz et al., 1988). Wound infections result from direct inoculation of the organism into traumatized cutaneous surfaces after contact with marine animals or the marine or estuary environment. Septicemia commonly develops in immunodeficient persons with hepatic disease who consume raw or improperly cooked oysters. On rare occasions, a classic secretory diarrhea typically observed with other Vibrio species, has been reported in patients with *V. vulnificus* in their faeces (Klontz et al., 1988), but the etiological role of *V. vulnificus* as a cause of diarrhoea has not proved. Other rare complications of *V. vulnificus* infection include meningitis, myositis, endometritis, peritonitis and ocular disease. Despite intense investigation, the pathogenic mechanisms of *V. vulnificus* are poorly defined. A unique, *V. vulnificus* cytotoxin-haemolysin apparently does not play a major role in microbial pathogenesis (Wright and Morris, 1991), although it is a highly sensitive and specific genetic marker for the species *V. vulnificus*. Other potential virulence factors include a capsule, iron-scavenging systems, and resistance to complement-mediated lysis.

*V. vulnificus* produces a number of enzymes (hyaluronidase, mucinase, DNAase, lipase, and protease) that may facilitate pathogenesis (Holmberg, 1992; Greenough, 1995). In addition, the presence of a capsule appears to be associated with invasive forms of *V. vulnificus*, since encapsulated forms are more commonly found among clinical isolates than among environmental isolates (Hayat et al., 1993). Persons with elevated transferrin-bound iron saturation (greater than 70%) or elevated ferritin levels, which include persons with hemochromatosis, thalassemia, or liver disease, are at increased risk for invasive infections. Primary septicemia refers to bloodstream infections that are acquired through ingestion of the organism through the GI tract. *V. vulnificus* primary septicemia infections are fatal in about 50% of the cases (Johnston et al., 1985; Klontz et al., 1988) Persons with known liver disease, particularly those patients with cirrhosis, are at high risk for *V. vulnificus* primary septicemia (Vollberg and Herrara, 1997). *V. vulnificus* can also cause an infection of the skin when open wounds are exposed to
warm seawater. These skin infections may lead to cellulitis, ulceration, necrotizing fasciitis, and sepsis. Because of the invasiveness of these wound infections, debridement of infected wounds is generally recommended to avoid limb amputation. *V. vulnificus* has been associated with other clinical syndromes, including pneumonia (Kelly and Avery, 1980), osteomyelitis (Vartian and Septimus, 1990), spontaneous bacterial peritonitis (Holcombe, 1991), eye infections (DiGaetano *et al*., 1989) and meningitis (Katz, 1988).

Three biogroups of *V. vulnificus* have also been described and all can infect humans (Bergeys manual of Systematic Bacteriology, 2005). Strains of *V. vulnificus* biogroup 2 have caused outbreaks in eels in Taiwan, Japan and Spain and apparently cause human wound infections (Tison *et al* 1982, Amaro and Biosca, 1996). *V. vulnificus* biogroup 3 were associated with human wound infections and exposure to cultured tilapia Bisharat *et al* (2005). The infection occurred when people purchased living, pond-raised, ‘Saint Peter’s fish’ (*Tilapia spp.*) from local vendors and then were inoculated with the organism through an existing skin break or trauma incurred while handling the fish. The halophilic strains of *V. vulnificus* biogroup 3 were able to grow in the fresh water inland ponds. These ponds had a high salt content because of the source of water and evaporation during the summer months. Lipp *et al* (2001) studied the occurrence and distribution of the human pathogen *V. vulnificus* in a subtropical Gulf of Mexico estuary and reported that although concentrations of *V. vulnificus* were positively correlated with temperature, salinity was a more important factor influencing variability of this organism. Levin (2005) stated that *V. vulnificus* is presently considered the most infectious and lethal of all human pathogenic vibrios. The organism requires at least 0.5% NaCl for growth, is naturally ubiquitous to marine coastal waters and shellfish, and is sensitive to refrigeration temperatures. Seminested reverse transcription-PCR (RT-PCR) has been used to detect viable but non-culturable (VBNC) cells of *V. vulnificus*.

*V. vulnificus* has been isolated from various locales around the world, including Europe and the Pacific and Atlantic, coasts of the United States (O’Neill *et al.*, 1990; Veenstra *et al.*, 1994; Chakraborty *et al.*, 1997). Thampuran and Surendran (1998) studied the incidence and distribution of *V. vulnificus* in marine and brackish-water fish and shellfish from coastal areas of Cochin on the west coast of India. *V. vulnificus* is typically found in waters having intermediate salinities (5-25ppt) and temperature up to
26°C (Motes et al., 1998); it does not grow at temperatures less than 10°C (Chakraborty et al., 1997). DePaola et al. (1994) isolated *V. vulnificus* from seawater, crustacean and estuarine fish from US waters in the Gulf of Mexico. The highest concentration of *V. vulnificus* in one study was found in the intestinal contents of bottom-feeding estuarine fish (sea catfish, sheepshead, Atlantic croaker) that consume mollusks and crustacean (DePaola et al., 1994); it is rarely recovered from offshore fish. The presence of *V. vulnificus* in shellfish may result from the constant filtering by these organisms of seawater containing Vibrios rather than the active multiplication of *V. vulnificus* in shellfish tissues (Kelly and Dinuzzo, 1985). Pathogenic strains of *V. vulnificus* and *V. parahaemolyticus* which are natural inhabitants of estuarine environments worldwide are often transmitted to humans through consumption of raw shellfish that flourish in the same estuaries (Andrews, 2004). *V. vulnificus* and *V. parahaemolyticus* are not transmitted person to person. Meilan et al. (2003) reported that *V. vulnificus* was found in 45% of water samples and 74% of oyster samples collected from Galveston Bay, USA. Densities of *V. vulnificus* in water and oysters were positively correlated with water temperature. No correlation was found between the intraspecific diversity among the isolated and sampling site or source of isolation. Linkous and Oliver (1999) described the nature of both the wound and primary septicemia infections and stated that this estuarine/marine bacterium occurs in high numbers in molluscan shellfish, primarily oysters, and its ingestion in raw oysters results in a ca. 60% mortality in those persons who are susceptible to this bacterium.

Jackson et al. (1997) showed that ca. 10³ *V. vulnificus* bacteria/gram of oyster and higher concentrations were associated with human infections. *V. vulnificus* poses a serious health threat to immunocompromised individuals and those with serum iron overload, with a fatality rate of approximately 50% (Hilton et al., 2006). Cirrhosis of the liver due to chronic alcoholism is considered a high risk factor for infection by this organism, presumably due to increased levels of serum iron released by damaged hepatocytes. An essential virulence factor is its capsular polysaccharide (CPS), which is responsible for a significant increase in virulence compared to nonencapsulated strains. However, this bacterium is known to vary the amount of CPS expressed on the cell surface, converting from an opaque colony phenotype to a translucent colony phenotype. Wong et al. (2005)
analysed *V. vulnificus* strains from environmental or clinical sources and observed that they were similar in virulence-associated phenotypes (protease activity, utilization of transferrin-bound iron, hemolysis, and inactivation in serum) and susceptibility to various stresses (4 and 52 °C, 0.1 and 10% NaCl, and pH 3.2). Virulence in mice was exhibited by 85% of the environmental strains and 95% of the clinical strains. *vvh* (*Vibrio vulnificus* hemolysin gene) was significantly more common in clinical strains than in environmental strains, and *vvh, flgF* (flagellar basal body rod protein gene), and *purH* (gene that encodes for a bifunctional polypeptide that controls the activities of 5-aminomidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase, AICARFT/IMPCHase) were more common in virulent strains than in nonviralent strains.

### 2.2.4. *V. alginolyticus*:

*V. alginolyticus* is a halophilic *Vibrio* first recognized as being pathogenic in humans in 1973 (Zen-Yoji *et al.*, 1973). *V. alginolyticus* is an important opportunistic bacterial pathogen for both human and aquatic animals (Reina and Heravs, 1993; Caruso *et al.*, 1998; Hervio-Heath *et al.*, 2003; George *et al.*, 2005; Xie *et al.*, 2005; Gonzalez-Escalona *et al.*, 2006). Wound infections account for 71% of *V. alginolyticus* infections (Hlady and Klontz, 1993). Ear infections are also seen with this organism. Gastroenteritis was thought to be a rare presentation of *V. alginolyticus* infection. *V. alginolyticus* was associated with chronic diarrhoea in a patient with AIDS (Caccemese and Rastegar, 1999), conjunctivitis (Lessner *et al.*, 1985) and post-traumatic intracranial infection (Opal and Saxon, 1986). *V. alginolyticus* inhabits coastal seawater environments and causes many clinical infections and tremendous damage in fish, shellfish and crustaceans culture. In South China, *V. alginolyticus* is found to be the dominant pathogen in seawater and have caused huge loss in marine aquaculture industry (Huang and He, 2002; Hu *et al.*, 2005; Xie *et al.*, 2005). Seawater is the normal habitat for *V. alginolyticus* and it has been isolated from seawater and seafood in many parts of the world (Kampelmacher *et al.*, 1972; Kristensen, 1974; Reali *et al.*, 1977; Vasconcelos *et al.*, 1975).
2.2.5. Other pathogenic Vibrio spp.:

*V. fluvialis* is a halophilic *Vibrio* first identified in 1975 in a patient with diarrhea in Bahrain (Furniss et al., 1977). *V. fluvialis* was identified in 40% of 177 samples collected from rivers, ponds, swamp, estuaries, sewage, sediment and crabs in Louisiana (Nishibuchi et al., 1983). *V. mimicus* is a non-halophilic *Vibrio* named according to its similarity to *V. cholerae*. *V. mimicus* can cause sporadic episodes of acute gastroenteritis and ear infections. *V. hollisae*, a halophilic *Vibrio* first described in 1982, most commonly causes gastroenteritis. *V. hollisae* is difficult to isolate, since it grows poorly on selective TCBS media and it needs to be isolated from colonies on a blood agar plate. *V. furnissii* was originally thought to be an aerogenic (able to produce gas from glucose) strain of *V. fluvialis*. In 1983, however, *V. furnissii* was shown to be a distinct species by genetic analysis (Brenner et al., 1983). Wong et al (1992) isolated *V. furnissii* from a relatively small percentage (7 to 12%) of oysters, clams, shrimps and crabs. *V. metschnikovii* was first described in 1888. It is often isolated from the environment but is rarely isolated from human specimens. *V. metschnikovii* is widely distributed in rivers, estuaries, sewage and has been isolated from the intestines of humans and other animals. *V. cincinnatiensis* is the most recently described pathogenic *Vibrio*. The only report of the pathogenic nature of this organism was by Bode et al (1986) who isolated *V. cincinnatiensis* from the cerebrospinal fluid and blood of a patient presenting with confusion to the University of Cincinnati in 1986.

2.3. Vibrios vis-à-vis Fish/Shrimp Disease

The general term Vibriosis is used to describe fulminant septicaemic infections produced by a number of *Vibrio* species in marine fish (Table 2.2). The etiological agent of the disease may be specific for a single type of aquatic life or may have a very broad host range (Hada et al., 1984; Ishimaru et al., 1995, 1996; Iwamoto et al., 1995; Borrego et al., 1996; Liu et al., 1996a; Yu et al., 1997). Vibrios are important bacterial pathogens for animals reared in aquaculture (Hjeltnes and Roberts 1993; Lightner and Redman, 1998; Austin and Austin, 1999; Bergh et al., 2001). *V. anguillarum*, *V. salmonicida*, and *V. vulnificus* are among the main bacterial pathogens of several fish species (Austin and
Austin, 1999). and *V. harveyi* is a major pathogen of shrimp, e.g., *Litopenaeus vannamei* and *Penaeus monodon* (Lavilla-Pitogo and de la Pena, 1998; Leano et al., 1998; Austin et al., 2003). Mortality caused by vibrios in reared fish and shellfish is very common during early larval stages and can occur suddenly, leading sometimes to death of the entire population (Ishimaru et al., 1995, 1996; Iwamoto et al., 1995; Borrego et al., 1996; Lee et al., 1996; Lambert et al., 1998; Novaoa et al., 1998; Hansen and Olafsen, 1999; Diggles et al., 2000; Olafsen, 2001). Lavilla-Pitogo and de la Pena (1998) have reported massive losses in shrimp cultures in Philippines due to a group of ‘luminous vibrios’ and observed that there was a decrease of nearly 60% in the survival of reared shrimp between 1992 and 1994, associated with the presence of luminous vibrios in rearing water. Lavilla-Pitogo and de la Pena (1998) recommended to farmers that shrimp rearing should not start unless luminous vibrios were absent. The rationale that all luminous vibrios are invariably associated with disease outbreaks in shrimp rearing contrasts with the results obtained by Fidopiastis et al. (1998, 2002), McFall-Ngai (2002), Oxley et al. (2002), and Ruby (1996) who have reported beneficial and/or harmless partnership between certain luminous vibrios e.g. *V. logei* and *V. fischeri* and host invertebrates.

**Table 2.2. Diseases in marine fish and invertebrates caused by or associated with Vibrio species*"**

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Disease (s)</th>
<th>Susceptible animals</th>
<th>Causative Vibrio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Whitespot disease</td>
<td>Kuruma prawns <em>(Penaeus japonicus)</em>&lt;br&gt;Tiger prawns <em>(Penaeus monodon)</em></td>
<td><em>V. alginolyticus</em></td>
</tr>
<tr>
<td>2</td>
<td>Terminal haemorrhagic septicaemia</td>
<td>Eels, ayu, rainbow trout, salmonids</td>
<td><em>V. anguillarum</em></td>
</tr>
<tr>
<td>3</td>
<td>Gastroenteritis</td>
<td>Groupers</td>
<td><em>V. harveyi</em></td>
</tr>
<tr>
<td>4</td>
<td>Vibriosis (larvae and juveniles)</td>
<td>Kuruma prawns</td>
<td><em>V. harveyi</em></td>
</tr>
<tr>
<td>5</td>
<td>Intestinal necrosis (larvae)</td>
<td>Flounders</td>
<td><em>V. ichthyoventeri</em></td>
</tr>
<tr>
<td>6</td>
<td>Brown spot disease</td>
<td>Kuruma prawns</td>
<td><em>V. penaeicida</em></td>
</tr>
<tr>
<td>7</td>
<td>Brown ring disease</td>
<td>Manila and fine clams</td>
<td><em>V. tapetis</em></td>
</tr>
<tr>
<td>8</td>
<td>Intestinal haemorrhage, skin discoloration</td>
<td>Japanese horse mackerel</td>
<td><em>V. trachuri</em></td>
</tr>
<tr>
<td>9</td>
<td>Disease larvae</td>
<td>Clams, oysters, scallops</td>
<td><em>V. pectenicida</em>, <em>V. tubiashii</em></td>
</tr>
</tbody>
</table>

*Source: Bergey’s manual of Systematic Bacteriology (2005)*
The rapid expansion of shrimp farming industry is plagued by diseases affecting shrimp survival and growth. Worldwide, penaeid shrimps are considered as a crustacean with high potential for intensive aquaculture. *P. monodon* (tiger shrimp) is the main shrimp product of Asia, with 50% of global shrimp production. Tiger shrimp is the largest shrimp with a fast growth rate in aquaculture conditions. They tolerate wide range of salinities but the hatchery survivals are low. With the recent progress in aquaculture, intensive systems used for marine organisms create an artificial environment that increases bacterial growth. Bacteria take advantage of ecological changes introduced in the aquaculture practice and may cause periodic disease. Most of the bacteria species are part of the autochthonous flora of marine organisms and their ecosystems and therefore a constant source of possible infection for crustaceans. Further different types of water treatment, the high shrimp densities, organic matter obtained (feeding, shrimp dead etc) perturb the bacterial community and stimulate the growth of opportunistic bacteria in shrimp tanks or ponds. In production of marine organisms the risk of a microbial infection is high, mainly at larval stages. Preventive measures recommended include adequate shrimp and water quality, routine shrimp evaluation, raking, tilling and removing pond bottom sediments and prolonged sun drying of ponds and disinfection with calcium hypochlorite. In shrimp, Vibriosis is the usual consequence of suboptimal environmental factors or poor management procedures in shrimp farming (Lightner, 1988; Brock, 1991).

Vibriosis has been studied for many years and has been reported to cause serious infections and low shrimp production (due to mortality, tissue lesion or necrosis, body malformation, low growth). The effect and severity are related to Vibrio species and doses; water, feed and shrimp quality and aquaculture management. Vibriosis is also known as penaeid bacterial septicaemia, penaeid vibriosis, luminescent vibriosis or red leg disease. Mass mortalities of *P. monodon* resulted from this disease. Hameed (1993) reported that an increase in vibrio population is one of the factors that reduce the survival rate of *P. indicus* larvae. Yasuda and Kitao (1980) observed low growth of shrimp larvae at protozoal stage when Vibrio sp were present in high level (10⁷ cfu/g) in water and shrimp gut. *V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. damselae, V. harveyi, V. penaeicida, V. anguillarum, V. nereis, V. tubiashi* and *V. flavialis* have been described as principal pathogenic vibrio species to penaeid shrimp. Gross observations, wet mounts,
histology and bacteria culture are the main diagnostic methods for vibriosis. In gross
observation, this infection is evidenced as black or brown cuticular lesions, necrosis,
opacity of musculature, black lymphoid organ and melanization of appendages. Large
number of bacteria in the haemolymph is observed in wet mounts. Necrosis and
inflammation of different organs (lymphoid organ, gills, heart and hepatopancreas) are
evaluated with histological methods.

Vibriosis is a major disease problem in shrimp aquaculture, causing high
mortality and severe economic loss in all producing countries (Brock and LeaMaster,
1992; Lightner, 1988; Mohney and Lightner, 1994). Shrimp aquaculture is an important
industry that experiences significant losses from Vibrio species, especially at the larval
and juvenile stages. Proteinaceous virulence factors, including alkaline proteases,
metalloproteases, cysteine proteases and alkaline serine proteases, have been identified as
important elements in Vibrio pathogenesis (Aguirre-Guzman et al., 2004). Vibrio spp are
most often considered opportunistic pathogens in shrimp, but primary disease caused by
highly virulent strains has also been reported (Takahashi et al., 1985; de la Pena et al.,
1992; Ishimaru et al., 1995). On the basis of phenotypic data, the major species causing
vibriosis in shrimp are V. alginolyticus, V. anguillarum, V. harveyi and V. parahaemolyticus
(Lightner, 1988; Jiravanichpaisal et al., 1994; Lightner, 1996). V. harveyi is a significant
pathogen of marine vertebrates and invertebrates. V. harveyi, which now includes
V. carchariae as a junior synonym, is a serious pathogen of marine fish and invertebrates,
particularly penaeid shrimp. In fish, the diseases include vasculitis, gastro-enteritis and
eye lesions. With shrimp, the pathogen is associated with luminous vibriosis. Yet, the
pathogenicity mechanisms are imprecisely understood, with likely mechanisms involving
the ability to attach and form biofilms, quorum sensing, various extracellular products
including proteases and haemolysins, lipopolysaccharide, and interaction with
bacteriophage and bacteriocin-like substances (Austin and Zhang, 2006). V. harveyi
infected black tiger prawn P. monodon showed bacterial invasions and multiplication in
the tubular lumens which was followed by necrosis of hepatopancreatic cells and the
thickened basal lamina, subsequent granulomatous encapsulation of the invaded tubules,
and production of granulation tissue around granulomatous lesions. Heavy bacterial
multiplication in the hepatopancreatic tubules caused systemic bacterial dissemination,
which resulted in marked necrosis in the heart and lymphoid organ (Jiravanichpaisal et al., 1994).

Jayasree et al (2006) surveyed diseases caused by Vibrio spp. in P. monodon culture ponds of coastal Andhra Pradesh and recorded the occurrence of five types of diseases: tail necrosis, shell disease, red disease, loose shell syndrome (LSS), and white gut disease (WGD). Among these, LSS, WGD, and red disease caused mass mortalities in shrimp culture ponds. Six species of Vibrio viz., V. harveyi, V. parahaemolyticus, V. alginolyticus, V. anguillarum, V. vulnificus, and V. splendidus were associated with the diseased shrimp. Babu et al (2001) reported alarming luminous disease in shrimp hatcheries in Andhra Pradesh and suggested that the most important strategy to protect shrimp larvae against luminescent vibriosis is by enhancing their natural defence system. Heavy mortalities in larvae of P. monodon in hatcheries of Kakinada and Visakhapatnam coasts due to Vibriosis was reported (Kumari and Babu, 2001). Jayasree et al (2001) carried out the identification and characterization of Vibrio spp. isolated from diseased shrimps from culture ponds of P. monodon in north coastal Andhra Pradesh. The loads in haemolymph varied from $0.7 \times 10^2$ to $5.8 \times 10^5$ cfu/ml and six species of Vibrio were identified from the diseased shrimps. V. harveyi was found to be the most predominant species occurring in all the diseased shrimps. V. alginolyticus and V. anguillarum were isolated from shrimps affected by loose shell, white gut and white spot syndromes, while V. parahaemolyticus was found in shrimps with white spot and loose shell. V. vulnificus and V. splendidus were of rare occurrence. Janakiram et al (2001) performed microbiological and histopathological study of the white gut disease of P. monodon from culture ponds of Visakhapatnam. The total Vibrio load in haemolymph of infected shrimps varied from $0.56 \times 10^3$ to $1.1 \times 10^4$ cfu/ml. Aravindan and Sheeja (2001) investigated the luminous bacterial disease in tiger shrimp post-larvae in a hatchery near Visakhapatnam and found that the gut of the host was found to support high density of V. harveyi than gills and exoskeleton. LC$_{50}$ experiments showed 50% mortality of P. monodon post-larvae with luminous bacterial concentration of $5.7 \times 10^6$ nos./ml at 43 hrs. V. parahaemolyticus, the causative agent of shell disease in the post-larval stages of Penaeus indicus inhabiting the backwater regions on the east coast of India were investigated (Aravindan et al., 2001)
Surendran et al (2000) studied the comparative microbial ecology of fresh water and brackish water prawn farms and observed that the total bacterial counts of the water, mud and the cultured prawn from fresh water farm registered lower values, compared with those from the brackish water farm. However indicator bacteria like total coliforms and faecal coliforms were high in number in the fresh water farm. Raghavan (2003) reported that no bacteria of significance to human health were found to be associated with any of the commercial feed samples analyzed, while farm-made feeds analyzed during the study showed a high incidence of various human pathogens such as *V. parahaemolyticus*, *V. cholerae*, *Escherichia coli* and *Staphylococcus aureus*. Felix (2000) studies the occurrence of pathogenic bacteria in shrimp farming systems of Tamil Nadu and observed that Vibrio spp. was the dominant flora and among the Vibrio isolates (n=278), *V. alginolyticus* was dominant comprising 28.8%, followed by *V. metschnikovii* and hypothesized that higher than usual nutrient content and salinity have resulted in 'blooms'of *Vibrio* spp. Abraham and Palaniappan (2004) studied the distribution of luminous bacteria in semi-intensive penaeid shrimp hatcheries of Tamil Nadu, India and found that *V. harveyi* was the dominant luminous species (94.05%). Luminous bacteria were found in the larval rearing tanks in considerable numbers ranging from log 0.70 to log 5.41/ml. The mean luminous bacterial counts showed an increase from eggs to mysis and decreased thereafter. The primary source of these bacteria in a shrimp hatchery was the faecal matter from brood stock, possibly at the time of spawning.

Otta et al (1999) studied the bacterial flora associated with shrimp culture ponds growing *P. monodon* in India. Total bacterial count ranged from $10^3$-$10^5$ cfu /ml and Vibrio count ranged from $10^1$-$10^4$ cfu/ml of pond water. *Vibrio* spp. was the largest group in all the ponds. *V. alginolyticus* accounted for 5.2 - 25% of the flora in various farms. *V. harveyi* constituted 5.2-36% of the flora. Representative strains of *V. alginolyticus* and *V. harveyi* showed low virulence to *P. monodon* larvae with LD$_{50}$ ranging from $10^6$ - $10^7$ cells. Kanagawa negative strains of *V. parahaemolyticus* were present in some of the farms. O1 serotype *V. cholerae* was absent and non O1 serotype *V. cholerae* and *V. vulnificus* were present in a few farms. Otta et al (2001) also investigated the bacterial flora of *P. monodon* shrimp hatcheries in India. The total plate counts of raw sea water on tryptic soya agar ranged from $10^2$ to $10^4$ /ml, whereas it ranged from $10^4$ to $10^6$ /ml in
larval tanks. In the larval tanks, the proportion of Vibrio species ranged from 50% to 73%, as compared to 31% in raw sea water. A mixed bacterial flora was observed in hatchery water but in the larval tanks, the flora in the larvae was predominantly made up of Vibrio species. Shome et al (1999) studied luminous V. harveyi isolated from P. monodon larvae reared in hatcheries in Andamans. The non-effectiveness of the antibiotics might be related to the tendency on V. harveyi forming biofilms in rearing tanks. Ponnuraj et al (1995) studied the mortality of shrimp (P. monodon) in culture ponds in Vedaranyam (Tamil Nadu) and the microbiological results indicated that the causative pathogen was V. parahaemolyticus. Alavandi et al (2006) carried out phenotypic and molecular typing of V. harveyi isolates and their pathogenicity to tiger shrimp larvae. Sucrose-fermenting biotypes of V. harveyi appeared to be associated with pathogenicity to larval shrimp. Higher temperature and salinity appeared to play a role on the onset of vibriosis and mortality in the challenged larval shrimp. Jayaprakash et al (2006a) studied Vibrios associated with Macrobrachium rosenbergii (De Man) larvae from three hatcheries on the Indian southwest coast and found that V. cholerae was the predominant species in the apparently healthy larval samples, whereas V. alginolyticus and V. vulnificus dominated during disease and morbidity. Vaseeharan and Ramasamy (2003) monitored the abundance of potentially pathogenic micro-organisms in P. monodon rearing hatcheries in India during 1996-1997 and noted that when the Vibrio-like-bacteria increases to 2 x 10^2 cfu, mortality of the post larvae occurs. Abundance of these microorganisms in hatchery samples indicated that they are opportunistic pathogens which can invade the shrimp tissue, subsequently cause disease when the post larvae were under stressful conditions. Sivasankar and Jayabalan (1994) studied the distribution of luminiscent bacterium V. harveyi in Netravathi estuary, Mangalore. Salinity had a greater influence on the distribution of V. harveyi than oxygen, temperature and pH.

Ni et al (1995) studied the Vibrio ecology of penaeids in ponds in China. Five species of Vibrio viz., V. alginolyticus, V. parahaemolyticus, V. vulnificus, V. fluvialis and V. mimicus were detected in the pond water and the prawn body with V. alginolyticus and V. parahaemolyticus as the dominant species for all ponds. Liu et al (1994) studied the flora variation of heterotrophic bacteria in giant tiger prawn (P. monodon) hatchery in Taiwan. At prior stages, the major bacterial flora were Gram positive strains, but after
Zoea III stage, the Gram negative bacteria become the main strains of which the *Vibrio* were dominant species. Wei and Hsu (2001) analysed water samples from *P. monodon* pond in Taiwan and found that the dominant species (47.5\%) belonged to the genus *Vibrio*. Li et al (2000) compared Vibrios isolated from shrimps in 5 different countries (China, Ecuador, Belgium, Mexico and Indonesia) and their results showed that the Vibrios in shrimps of different species from different countries are similar in distribution of the dominant species. *V. alginolyticus* and *V. harveyi* was detected in all the samples. *V. alginolyticus* was found in both healthy and diseased larvae. Hisbi et al (2000) noted that the dominant bacterial strains associated with shrimp *P. monodon* larvae in Indonesia were identified as *V. alginolyticus*, *V. damsela*, and *V. harveyi*. Vibrio species were found at different larval stages and in both diseased and healthy larvae. The study supported the idea that Vibrio species are part of the resident microflora in *P. monodon* larvae. Su et al (1994) noted that the quantity of *Vibrio* spp. and shrimp diseases did not seemingly relate to the changes of pH, DO, salinity, NH₄-N and H₂S, but connected directly with seasonal temperature.

Main pathogenic bacteria in shrimp larvae are mostly *V. harveyi* while in adults it is *V. parahaemolyticus* (Li et al., 2000). Sung et al (2001) studied the relationships between disease outbreak in cultured tiger shrimp (*P. monodon*) and the composition of Vibrio communities in pond water and shrimp hepatopancreas during cultivation. It was observed that for the initial 60 days after transfer, the composition of the Vibrio community in the pond water remained fairly diverse but subsequently decreases in species diversity were observed in ponds. Leano et al (1998) characterized the bacterial flora in the hepatopancreas of pond-reared *P. monodon* juveniles with luminous Vibriosis and showed that most (90.12\%) were Vibrio species, dominated by *V. harveyi* (27.91\%), *V. splendidus* II (13.37\%) and *V. parahaemolyticus* (10.46\%). Lavilla-Pitogo and de la Pena (1998) studied the mortalities of pond-cultured juvenile shrimp, *P. monodon*, associated with dominance of luminescent vibrios in the rearing environment. The histopathology of affected shrimps showed the hepatopancreas as the target organ of infection where severe inflammatory responses in the intertubular sinuses were seen. Esiobu and Yamazaki (2003) found that all healthy, live shrimp guts were heavily colonized by Vibrio species, especially by sub-species of *V. harveyi*. Abraham et al
(2003) studied the distribution and abundance of luminous bacteria with special reference to shrimp farming activities and concluded that *V. harveyi* was the dominant species, comprising greater than or equal to 82-97% of the total luminous population. *Vibrio* species comprise the most frequently encountered bacterial pathogens of cultivated shrimp, and *V. harveyi* is amongst the most virulent. However, not all isolates of *V. harveyi* are highly virulent. Some can be injected at high dose (10⁵-10⁷ cells per g shrimp body weight) without causing shrimp mortality, while other isolates are lethal at 10³ per g shrimp body weight or less. In addition, virulence is often lost upon continuous subculture. Indeed, recent work has suggested that 2 quite different bacteriophages, one from the family *Myoviridae* and the other from the family *Siphoviridae*, can change the phenotype of *V. harveyi* isolates from non-virulent to virulent (Flegel et al., 2005). Oakey and Owens (2000) reported a previously unreported bacteriophage extracted from a toxin-producing strain of *V. harveyi* isolated from moribund prawn larvae in tropical Australia and termed the bacteriophage VHML (*Vibrio Harveyi Myovirus Like*). Munro et al (2003) demonstrated that the presence of the bacteriophage VHML might confer virulence to *V. harveyi* strain. *V. parahaemolyticus* was found to be highly virulent to *P. monodon* with an LD₅₀ value of 1 x 10⁵ cfu/prawn and the extracellular proteases of the bacteria were also found to be toxic with an LD₅₀ value of 8 µg protein/prawn (Sudeesh and Xu, 2001). Nakayama et al (2006) observed that *V. harveyi* isolated from shrimp farm showed no luminescence but showed high pathogenicity based on toxicity test. Their study demonstrated that *V. harveyi* produces two kinds of toxins, haemolysin and protease toxin.

Kiyukia et al (1992) reported *V. cholerae* non O1 as a fish pathogen in Japan as it was isolated from diseased ayu fish (*Plecoglossus altivelis*). Causative agents for Vibriosis in fish included *V. anguillarum, V. cholerae* non O1, *V. damsela, V. vulnificus* and others (Austin and Austin, 1986). *V. cholerae* non O1 was reported as a pathogen of *P. chinensis* larvae (mysis stage) and the serotypes obtained were O5, 14, 26, 47 and 56 (Wang et al., 1997). Non O1 *V. cholerae* was isolated in aquarium water of fishes imported from extra EU countries (Barbieri et al., 1999). Association of *V. cholerae* with necrosis and ulceration of *M. rosenbergii* (De Man) broodstock was reported by John et al (1996). *V. cholerae* was the predominant species in the apparently healthy larval species
of *M. rosenbergii* (De Man) from the south west coast of India whereas *V. alginolyticus* and *V. vulnificus* dominated during disease and morbidity (Jayaprakash et al., 2006).

Gomez-Gil et al (1998) found a large population of vibrios, i.e., $10^5$ cfu/g and $10^4$ cfu/ml, respectively, in the hepatopancreas and hemolymph of healthy *L. vannamei*. Wang and Chen (2005) reported that in white shrimp *L. vannamei*, the mortality of *V. alginolyticus*-injected shrimp held in 5 ppt and 15 ppt salinity was significantly higher than that of shrimp held in 25 ppt and 35 ppt saline waters, and the mortality of *V. alginolyticus*-injected shrimp held in 5 ppt salinity was the highest. They concluded that the shrimp transferred from 25 ppt to low salinity levels (5 and 15 ppt) had reduced immune ability and decreased resistance against *V. alginolyticus* infection. Martin et al (2004) reported that *V. parahaemolyticus* and *V. harveyi* cause detachment of the epithelium from the midgut trunk of the penaeid shrimp *Sicyonia ingentis*. Saulnier et al (2004) reported that mortalities occurred in growout ponds of Penaeid shrimp in New Caledonia were due to the presence of *V. penaeicida* and *V. nigripulchritudo*. The *Vibrio* spp. isolated from the digestive tract of a population of healthy juvenile *L. vannamei* consisted of both sucrose and non-sucrose fermentors whereas the haemolymph contained only non-sucrose fermentors (Gomez-Gil et al., 1998).

Aguirre-Guzman et al (2001) studied the differences in the susceptibility of American white shrimp larval substages (*L. vannamei*) to four potentially pathogenic *Vibrio* species (*V. harveyi*, *V. parahaemolyticus*, *V. alginolyticus*, and *V. penaeicida*) and summarized that shrimp larvae demonstrated an age susceptibility that depends on the *Vibrio* species and dose level. Ding et al (2000) studied the interaction of virus and *V. alginolyticus* in the earlier stage of virus disease of *P. chinensis*. The results show that insidious infection of vibrio is advantageous to the infection of virus. The drop of prawns' immune potency is very important.

Extracellular products (ECPs) of the *V. alginolyticus* isolate were found toxic to silver sea bream (*Sparus sarba*) but also to cultured fibroblast cells derived from sea bream fin tissue (Li et al., 2003a). Zhang et al (2000) found that protease and phospholipase, including both acidic phospholipase and alkine phospholipase extracted from cultures of *V. parahaemolyticus* are more active than those of the others. Conforming to the result of activity of protease and phospholipase, lethal action of *V. parahaemolyticus* is
the strongest and that of *V. alginolyticus* is the weakest. *V. harveyi*, previously demonstrated to be virulent to *P. monodon* larvae were shown to produce proteinaceous exotoxins suggests that cysteine protease is the major toxin produced by the bacterium (Liu and Lee, 1999). Liu et al (1996) investigated the pathogenicity of different isolates of *V. harveyi* in tiger prawn, *P. monodon* and their results indicate that there are differences between penaeid and non-penaeid isolates of *V. harveyi* in pathogenicity and reveal that proteases, phospholipases, haemolysins or exotoxins might play leading roles in the pathogenicity of *V. harveyi* in the tiger prawn.

### 2.4. Vibrios vis-à-vis post-harvest quality

Food borne illness occurs when a person gets sick by eating food that has been contaminated with pathogenic microorganisms or their toxins. Of the 12 pathogenic Vibrio species, 8 species are known to be directly food associated.

Baffone et al (2000) examined fresh seafood products from Italy for the presence of Vibrio and recovered *V. alginolyticus* from 81.48% samples, *V. parahaemolyticus* from 4.8% samples and *V. cholerae* non O1 from 3.7% samples. Buck (1998) analysed seafood from retail outlets and natural habitats from Southern New England and Florida and observed that *V. alginolyticus* and *V. parahaemolyticus* were the most frequently isolated species although *V. fluvialis*, *V. damsela*, *V. cholerae* and *V. vulnificus* were also recovered. *V. cholerae* was isolated from various seafoods in Malaysia (Chen et al., 2004) and non O1/non O139 *V. cholerae* was isolated from Newport Bay, California (Jiang et al., 2003). Wong et al (1995) studied the occurrence of Vibrios in frozen seafood including peeled shrimp and fish and shrimp dumplings and found *V. alginolyticus* in 36% of the samples, *V. parahaemolyticus* in 15-18% of the samples, *V. cholerae* in 14.9% of the samples and *V. fluvialis* in 13.2% of the samples. Jaksic et al (2002) investigated the occurrence of Vibrio spp in sea fish, shrimps and bivalve mollusks harvested from the Adriatic Sea. *V. parahaemolyticus* was the predominant species detected (47.83%), followed by *V. vulnificus*.

Jeyasekaran and Ayyappan (2002) reported the presence of *V. cholerae* in farm reared tropical fresh water prawn (*M. rosenbergii*). Anand et al (2002) detected *V. cholerae* in shrimp samples landed in Tuticorin fishing harbour of Tamil Nadu, India.
Aravindan and Sheeja (2000) isolated *V. cholerae* in *P. monodon* during processing for export in Visakhapatnam region. Bhaskar et al (1998) reported that 8.3% of the cuttle fish (*Sepia sp*) studied contained *V. cholerae* Non O1 type. 10% of the squid (*Loligo sp*) examined contained *V. parahaemolyticus*. Dalsgaard et al (1996) reported the presence of Non O1 *V. cholerae* in cooked frozen shrimp products originating from farmed shrimp.

Prasad and Rao (1994) investigated the prevalence of pathogenic Vibrios in fresh, frozen and iced prawn and fish from Andhra Pradesh and found that 30% of *P. indicus* carried Vibrio species comprising of *V. parahaemolyticus*, *V. vulnificus*, *V. metschnikovii*, *V. cholerae* non O1 and *V. anguillarum*. Maximum number of isolates were found to be *V. cholerae* non O1 and the incidence of pathogenic Vibrios were lower in fish compared to prawn. Sudha et al (2002) reported that the order of dominance of Vibrio species in fish from pelagic and demersal habitats was *V. alginolyticus* > *V. orientalis* > *V. campbellii* > *V. mimicus* and their study revealed that Vibrios constitute a major portion of total bacterial flora in tropical fish and *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus* and *V. metschnikovii* were the pathogenic species isolated from fish. Thampuran et al (1996) studied the prevalence of pathogenic Vibrios in coastal water and fishes of Cochin and detected *V. alginolyticus*, *V. parahaemolyticus*, *V. vulnificus*, *V. mimicus*, *V. cincinnatiensis*, *V. damsela* and *V. metschnikovii*. Singh et al (1996) analysed 426 samples comprising 192 freshwater fish, 182 marine fish, 13 marine prawns, 13 freshwater prawns and 26 mollusks and isolated *V. parahaemolyticus*, *V. cholerae* non O1, *V. alginolyticus* from many samples. Sanjeev et al (2002) isolated *V. metschnikovii* in 16.67% of IQF squid, 8.82% of frozen prawns, 2.38% of frozen squid and 2% of IQF prawns. Sanjeev et al (2000) examined frozen fish products collected from 23 processing factories and found *V. cincinnatiensis* to be the dominant species (18.06%) followed by *V. alginolyticus* (15.18%), *V. parahaemolyticus* (9.42%), *V. vulnificus* (5.24%) etc. James and Iyer (1998) examined the quality of frozen squid and cuttlefish of the export trade and found that 8.3% of cuttlefish samples contained *V. cholerae* Non O1 and 10% squid samples had *V. parahaemolyticus*.

Nayyarahamed and Karunasagar (1994) studied the bacteria of public health significance in cultured shrimp (*P. monodon*) under different stages of farming operations and observed that Vibrios were present in all samples analysed. A poor correlation was
observed between the level of indicator organisms (coliforms) and the incidence of these pathogens indicating that these pathogens are a part of the natural microflora of the shrimp culture environment and suggested that potential pathogenic Vibrios could be normal inhabitants of the gut of cultured shrimp. Bhaskar and Setty (1994; 1998) studied the incidence of Vibrios of public health significance in the farming phase of tiger shrimp (*P. monodon*). *V. alginolyticus* was the most common Vibrio member (57% incidence). *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* were the other species encountered.

### 2.5. Identification of Vibrios

Thiosulfate-Citrate-Bile-Sucrose Agar (TCBS Agar) is used for isolating and cultivating enteropathogenic vibrios. TCBS Agar, prepared according to the formula of Kobayashi et al (1963), is a modification of the selective medium from Nakanishi (1963). All *Vibrio* spp. that are pathogenic to humans, except *V. hollisae*, will grow on TCBS Agar. TCBS agar is an ideal medium for the selective isolation and purification of vibrios (Thompson et al., 2004; Bergey’s manual of Systematic Bacteriology, 2005). This medium is recommended for isolating *Vibrio* spp. from stool specimens (McLaughlin, 1995) and is specified in Standard Methods for food testing (AOAC, 1995; Vanderzant and Splittstoesser, 1992; USFDA-BAM, 2001). Pfeffer and Oliver (2003) compared TCI agar (thiosulphate-chloride-iodide) with TCBS for the isolation of estuarine Vibrios and the results showed that a much larger number of colonies developed on TCBS agar than on TCI agar suggesting that TCBS agar is a superior medium when compared to TCI agar for the isolation of Vibrio species.

TCBS Agar is highly selective, meets the nutritional requirements of *Vibrio* spp., and allows vibrios to compete with intestinal flora. Yeast extract and peptone in the medium provide the nitrogen and vitamin requirements; sodium citrate, sodium thiosulfate and bile salts are selective agents which provide an alkaline pH to inhibit gram-positive organisms and suppress coliforms. Sucrose is the fermentable carbohydrate, and sodium chloride stimulates growth. Sodium thiosulfate is a sulfur source and acts with ferric citrate to indicate hydrogen sulfide production. Bromo Thymol Blue and Thymol Blue are pH indicators. Agar is a solidifying agent. Lotz et al (1983) examined thirty-one Vibrio species with 188 different strains of clinical, marine,
and stock origin on the TCBS media and advocated the widespread use of TCBS media to detect the vibrio infections associated with seafood ingestion or wounds exposed to seawater. The differentiating trait of sucrose helps in separating the pathogenic vibrios into two groups viz., generally sucrose positive (V.cholerae, V.metschnikovii, V.fluvialis, V.furnissi, V.alginolyticus and V.carlinae / V.harveyi) and generally sucrose negative (V.mimicus, V.hollisae, V.damselfa, V.parahaemolyticus and V.vulnificus). The salient differentiating features of the pathogenic Vibrios that enable their identification and confirmation is described in Bergey’s manual of Systematic Bacteriology (2005). Some isolates of Staphylococcus, Flavobacterium, Pseudoalteromonas, and Shewanella may show slight growth on TCBS (Thompson et al., 2004).

For the biochemical identification of vibrios, commercial kits reportedly gave poor or null comparative results for certain tests when compared with those obtained via classical Standard methods. Vandenberghe et al (2003) stated that Vibrio genus is a phenotypically diverse group which made their identification with the Biolog system difficult and unreliable. Dalsgaard et al (1996a) observed that API 20E assay (Bio Merieux, France) was not adequate for the identification of environmental isolates of V.vulnificus. O’Hara et al (2003) evaluated six commercial systems for the ability to identify the 12 species of Vibrio found in clinical samples and found that many isolates are not accurately identified by commercial methods, with the accuracy of systems ranging from 63.9% to 80.9%.

Several authors have reported simple schemes for the detection of all or any specific pathogenic vibrios. Choopun et al (2002) suggested that a combination of alkaline peptone enrichment followed by streaking on thiosulfate citrate bile salts sucrose agar and testing for arginine dihydrolase activity and esculin hydrolysis an effective rapid technique to screen for aquatic environmental V.cholerae. This technique provided 100% sensitivity and >70% specificity. Abraham et al (1999) developed a simple taxonomic key for identifying marine luminous bacteria based on 10 biochemical tests. Hoi et al (1998) reported that an improved selective medium, Cellobiose-Colistin (CC) agar, gave a significantly higher isolation rate of V.vulnificus from water and sediment samples than modified Cellobiose-PolymyxinB-colistin (mCPC) agar. Sanjeev et al (1998) concluded that for enumeration of V.parahaemolyticus, nutrient broth eosin-y medium gave
consistent results and hence was the best. Bhathena and Doctor (1995) suggested a two-tine serial enrichment technique, utilizing 8% salt-alkaline broth and salt-polymyxin broth (pH 8) for incubation periods of 8 h and 18 h, respectively, at 37°C resulted in the recovery of maximum numbers of V. parahaemolyticus.

The most widely used scheme for the identification of vibrios to the species level was proposed by Alsina and Blanch (1994, 1994a). They developed a practical set of keys for biochemical identification of environmental and clinical Vibrio species. They have been specially designed for environmental isolates, and can be used for strains that are Gram-negative, grow on TCBS medium and are facultative anaerobes. The keys are constituted by 28 tests and a maximum of 10 tests are needed for the most complicated identification. Alsina’s scheme were performed for species identification in numerous studies and proved useful (Martínez-Picado et al., 1996; Montes et al., 1999; Oxley et al., 2002; Ottaviani et al., 2003; Hjelm et al., 2004; Maugeri et al., 2004; Baffone et al., 2006). Croci et al (2007a) compared different biochemical and molecular methods for the identification of V. parahaemolyticus. Both in intra- and interlaboratory tests, the Alsina’s scheme showed the highest sensitivity. Among the biochemical identification methods tested, the Alsina’s scheme gave more reliable results.

A new scheme was proposed by Noguerola and Blanch (2008) which is not only an update of the key set used for the biochemical identification of Vibrio species (Alsina and Blanch 1994, 1994a.), but also an improvement in that it reflects the most recent systematic parameters (Thompson et al., 2004). Noguerola and Blanch’s scheme identifies Vibrio spp. with a set of dichotomous keys. A matrix of phenotypical results was developed based on the previous taxonomical studies and the first description manuscripts. A unification of results from various sources was also performed to integrate different taxonomical studies within the same data matrix. An initial identification key was defined using arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase tests, as well as defining eight different clusters. This key leads each cluster to a secondary key for species identification. Most of Vibrio spp. presented an identification threshold of 100%. No more than 14 tests are needed for even the most complicated identifications.
2.6. PCR (Standard, Multiplex and Real Time PCR) methods for the detection of Vibrios

Laboratory diagnosis of pathogenic vibrios has traditionally been based on phenotypic characteristics of these organisms, expressed as morphological, physiological, and biochemical properties, including antigenic composition. Phenotypic identification of Vibrio spp relies on time consuming techniques such as studies on the morphology and nutrition requirement that have limited discriminatory powers. Accurate phenotypic identification of Vibrio species is problematic, largely because of the great variability in biochemical characteristics (Thompson et al., 2004). The need for rapid, sensitive diagnostic methods led to the application of modern biotechnology, which yielded highly sensitive detection methods that employ DNA amplification methods based on the polymerase chain reaction (PCR). Saiki et al (1985) published the first experimental data on PCR, and ever since PCR technique (Mullis and Faloona, 1987) has tremendously influenced research in diverse areas of biological sciences leading to an unprecedented understanding of microorganisms. PCR, multiplex PCR, gene probes, and DNA fingerprinting techniques are now available to detect human pathogens in seafood and seawater. Several DNA molecular markers are now available for use in surveillance and investigation of food borne outbreaks that were previously difficult to detect. The techniques provide ways to screen for a broad range of agents in a single test. Its range of application is perceived to broaden in the near future for rapid differentiation of species. The organism of interest can be detected directly through PCR assays in a much shorter time than conventional culture methods. Molecular methods that utilize the polymerase chain reaction and nucleotide sequence determination overcome many of the limitations of phenotypic methods. Molecular techniques, particularly nucleotide sequence determination, provide data that are objectively scored to provide an unambiguous identification. Most importantly, methods that utilize the PCR can lead to identification of an isolate within hours and can be used on small quantities of cells, including those that are not viable or are otherwise unculturable.
PCR is an in vitro technique for the amplification of a specific region of genome. It is a rapid and simple means of producing relatively large number of copies of DNA molecules from minute quantities of source DNA. All living organisms viz., virus, bacteria, plants and animals have certain regions (sequences) in their genome that are specific to that particular organism. These specific regions are short stretches of DNA, which may be a gene or simply a stretch of nucleotide bases. This stretch of DNA is known as target sequence. PCR is a cyclic process in which the target sequence is enzymatically amplified and nearly a million new DNA strands containing the target sequence are synthesized by the end of the cyclic process. One of the important components for the success of the PCR are the ‘primers’. They are typically short, single stranded oligonucleotides which are complementary to the section of DNA which flank either side of the target DNA or gene. The specificity of the primers determines the success or failure of the PCR. There are three major steps in a PCR cycle, which are repeated for 30 or 40 cycles. First step is template denaturation by heating at 94°C in which the double stranded DNA uncoils and forms two single strands of DNA. Second step is primer annealing (at 54°C), in which the primers attach to the flanking DNA section of the target sequence on the template and forms a stable bond. In this step a little piece of double stranded DNA is formed. Third step is primer extension (at 72°C). This temperature is ideal for the working of *taq* polymerase. The bases complementary to the target sequence are coupled to the primer on the 3’ end. The three steps of template denaturation, primer annealing and primer extension comprise a single cycle of PCR. After 30 cycles one can expect a billion copies of the target gene. PCR, multiplex PCR and Real time PCR methods are now available to detect pathogenic vibrios.

### 2.6.1. PCR methods for pathogenic vibrio species

A few of the PCR methods developed or used for the detection of pathogenic Vibrios viz., *V.cholerae*, *V.parahaemolyticus*, *V.vulnificus*, *V.alginolyticus* and *V.harveyi* are listed below (Table 2.3). These PCR methods target specific regions which may be a gene or simply a stretch of nucleotide bases specific to that particular pathogenic Vibrio species. In some cases, multiplex PCR methods that can detect two or more genes of the same organism in a single PCR assay have been used.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Type of PCR</th>
<th>Target gene / sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td>PCR</td>
<td>ctx</td>
<td>Karunasagar et al (1995)</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td>PCR</td>
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<td>Multiplex PCR</td>
<td>tdh, trh</td>
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<td>pR72H sequence</td>
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<td>gyrB</td>
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<td>PCR</td>
<td>toxR</td>
<td>Vongxay et al (2006)</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td>PCR</td>
<td>pR72H</td>
<td>Croci et al (2007)</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td>PCR</td>
<td>tll gene</td>
<td>Rekha et al (2008)</td>
</tr>
<tr>
<td><em>V. parahaemolyticus</em></td>
<td>PCR</td>
<td>tdh</td>
<td>Karunasagar et al (1996)</td>
</tr>
<tr>
<td><em>V. vulnificus</em></td>
<td>PCR</td>
<td>gyrB</td>
<td>Kumar et al (2006)</td>
</tr>
<tr>
<td><em>V. vulnificus</em></td>
<td>PCR</td>
<td>vvhA</td>
<td>Parvathi et al (2005)</td>
</tr>
<tr>
<td><em>V. vulnificus</em></td>
<td>PCR</td>
<td>hemolysin /cytolysin gene</td>
<td>Coleman et al (1996)</td>
</tr>
<tr>
<td><em>V. vulnificus</em></td>
<td>Multiplex PCR</td>
<td>viuB, vvh</td>
<td>Panicker et al (2004b)</td>
</tr>
</tbody>
</table>
2.6.2. Multiplex methods targeting more than one organism

Multiplex PCR methods that can detect two or more pathogens in a single PCR assay have been used by several researchers. The target sequences for amplification were usually species-specific regions of the pathogens or unique genes of each pathogen. A few multiplex PCR methods involving pathogenic vibrios are tabulated below (Table 2.4).

Table 2.4. Multiplex-PCR methods targeting *V.cholerae*, *V.parahaemolyticus*, *V.vulnificus*

<table>
<thead>
<tr>
<th>Target Organisms</th>
<th>Target gene / sequence</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V.cholerae</em>, <em>V.parahaemolyticus</em>, <em>V.vulnificus</em>, <em>V.mimicus</em></td>
<td><em>hsp60</em> for <em>V. vulnificus</em>, <em>sodB</em> for <em>V. cholerae</em> and <em>V. mimicus</em>; <em>flaE</em> sequence of <em>V. parahaemolyticus</em></td>
<td>Tarr et al (2007)</td>
</tr>
<tr>
<td><em>V.vulnificus</em>, <em>V.parahaemolyticus</em>, <em>V.cholerae</em></td>
<td><em>vvhA</em> gene; <em>pR72H</em> DNA; <em>16S</em>-23S rRNA intergenic spacer region (ISR)</td>
<td>Hervio-Heath et al (2003)</td>
</tr>
<tr>
<td><em>V.vulnificus</em>, <em>V.cholerae</em>, <em>V.parahaemolyticus</em></td>
<td><em>vvh</em>, <em>viuB</em> for <em>V. vulnificus</em>, <em>ompU</em>, <em>toxR</em>, <em>tcpI</em>, <em>hlyA</em> for <em>V. cholerae</em>, <em>tll</em>, <em>tdh</em>, <em>trh</em>, open reading frame 8 for <em>V. parahaemolyticus</em></td>
<td>Panicker et al (2004)</td>
</tr>
<tr>
<td><em>Salmonella enterica</em> serotype Typhimurium, <em>V.vulnificus</em>, <em>V.cholerae</em></td>
<td><em>hns</em>, <em>spvB</em>, <em>vvh</em>, <em>ctx</em>, <em>tll</em></td>
<td>Lee et al (2003)</td>
</tr>
</tbody>
</table>
## 2.6.3. Real-Time PCR

Real-Time PCR is a quantitative PCR that has completely revolutionized the detection of RNA and DNA. Traditional PCR has advanced from detection at the endpoint of the reaction to detection while the reaction is occurring. Real-Time PCR tools allow for the detection of PCR amplification during the early phases of the reaction. Measuring the kinetics of the reaction in the early phases of PCR provides a distinct advantage over traditional PCR detection. Traditional PCR methods use agarose gels for detection of PCR amplification at end of the reaction, while Real-Time PCR collects data in the exponential phase. An increase in the reporter fluorescent signal in Real-Time PCR is directly proportional to the number of amplicons generated. No post-PCR steps like running of gel is required. Non-specific amplification can be detected by melt curve analysis of PCR products. The starting copy number of the target is determined by monitoring when PCR product was first detected; the higher the starting copy number of the target the sooner a significant increase in the fluorescence is detected. The fluorescence data is continuously analysed by the machine which displays the results eliminating the need for post-PCR processing.

<table>
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<tbody>
<tr>
<td>---------------------</td>
<td>---------------------------------------------------------------</td>
<td>-------------------------------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>V. cholerae and V.parahaemolyticus</td>
<td>collagenase gene</td>
<td>DiPinto et al (2005)</td>
<td></td>
</tr>
</tbody>
</table>
Four basic tools are used in Real-Time PCR viz., DNA-binding dyes, Molecular beacons, hybridization probes and hydrolysis probes. The simplest method uses fluorescent dyes that bind specifically to double stranded DNA. DNA-binding dye based Real-Time PCR method involves detection of the binding of a fluorescent dye (SYBR Green) to DNA. SYBR Green dye is non-sequence specific fluorescent intercalating agent. It does not bind to single stranded DNA (ssDNA). SYBR green is a fluorogenic minor groove binding dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA (dsDNA). During elongation, increasing amounts of the dye bind to the nascent double-stranded DNA. When monitored in real-time, this results in an increase in the fluorescence signal that can be observed during the polymerization step and that falls of when the DNA is denatured. Consequently fluorescent measurements at the end of the elongation step of every PCR cycle are performed to monitor the increasing amount of amplified DNA. Fluorescence values are recorded during every cycle and represent the amount of product amplified to that point in the amplification reaction. The more the template present at the beginning of the amplification reaction, the fewer number of cycles it takes to reach a point in which the fluorescence signal is first recorded as statistically significant above background. This point is defined as Ct (cycle threshold) and will always occur during the exponential phase of amplification. This method obviates the need for target specific fluorescent probes but its specificity is determined entirely by its primers. Non-specific amplifications require follow-up assays (melting point curve or dissociation analysis) for amplicon identification. Normally SYBR green is used in singleplex reactions, however when coupled with melting point analysis, it can be used for multiplex reactions. Melt curve analysis ensures specificity of the amplified PCR products. Melt curve analysis of each sample is performed by plotting the first negative derivative of the fluorescence (F) with respect to temperature (T) against temperature [(-dF/dT) vs T] and should show a single melting maximum for each sample indicating specific amplification without primer-dimer.

Real-Time PCR methods were developed and used for the detection of pathogenic vibrios. A few of the methods used for \textit{V. cholerae} and other pathogenic vibrios are listed below (Table 2.5).
### Table 2.5. Real-Time PCR methods for *V. cholerae, V. parahaemolyticus, V. vulnificus and V. alginolyticus*

<table>
<thead>
<tr>
<th>Organism</th>
<th>Real Time PCR method</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>V. cholerae</em></td>
<td>SYBR Green</td>
<td>Gubala (2006)</td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td>TaqMan PCR</td>
<td>Lyon (2001)</td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td>Molecular beacon</td>
<td>Fykse et al (2007)</td>
</tr>
<tr>
<td><em>V. cholerae</em></td>
<td></td>
<td>Fedio et al (2007)</td>
</tr>
<tr>
<td><em>V. vulnificus</em></td>
<td>SYBR Green</td>
<td>Panicker et al (2004a)</td>
</tr>
</tbody>
</table>

#### 2.7. Growth Kinetics and Enzymatic activities of Vibrios

Opportunistic pathogens must adapt to potentially stressful environmental changes while living freely in water, upon colonization of the gut of the shrimp and upon infection of such diverse hosts as shrimps, fish and humans. The expression and activity of various enzymes play a crucial role in determining the ability of pathogenic Vibrios to survive and cause infection in susceptible hosts. Beleneva and Maslennikova (2005) reported that strains of *Vibrio* spp. produced DNAses, RNAses, alkaline phosphatases, chitinases, proteinases, amylases and lipases. Dhevendaran and Georgekutty (1998) reported that majority of *Vibrio* spp produced lipase, protease and arylsulfatase. Baffone et al (2001) studied the enzymatic activities (urease, lipase, gelatinase, and haemolysin) of *V. alginolyticus, V. parahaemolyticus, V. cholerae non-01, V. vulnificus, V. fluvialis, V. furnissii and V. metschnikovii*. Lipase and gelatinase activities were observed in 100% of the strains. Henderson and Millar (1998) noticed that Vibrio species produce a phospholipase B capable of hydrolyzing both intact phospholipids and lysophospholipids. Saramma et al (1994) studied the amylase production by Vibrio species and recorded that the percentage of amylolytic population was maximum in the genus Vibrio (82.12%).
Sodium chloride could be replaced by potassium chloride or magnesium chloride without affecting growth and enzyme production.

Sudha et al (1998) studied the effect of temperature on growth and biochemical properties of selected species of pathogenic vibrios viz., *V. parahaemolyticus, V. vulnificus, V. alginolyticus, V. mimicus* and *V. harveyi* at 4, 15, 28 ± 2, 37 and 42°C in Trypticase Soy Broth with 3% NaCl. *V. parahaemolyticus* and *V. alginolyticus* exhibited growth at 42°C. All the species studied grew slowly at 15°C, but failed to grow at 4°C. Observations of the biochemical activity were in accordance with the growth except at 15°C where, although there was growth, most of the biochemical reactions gave negative results. Altermark et al (2007) noted that the optimal conditions for enzymatic activity coincide well with the corresponding optimal requirements for growth of the organisms.

Chitin is one of the most abundant polymers in nature. Chitin, a highly insoluble polymer of N acetyl glucosamine, is produced in massive quantities in the marine environment (eg. exoskeleton of crustaceans). Marine bacteria rapidly catabolize chitin for survival in aquatic ecosystems. Osawa and Koga (1995) showed that *V. parahaemolyticus, V. alginolyticus, V. mimicus* were positive for chitinase and chitobiase activities, and capable of utilizing N acetyl glucosamine as a sole source of carbon and nitrogen. Meibom et al (2004) described the chitin utilization pathway for *V. cholerae* and noticed that environmental and clinical *V. cholerae* strains become naturally competent after growth on chitin, suggesting that growth within the marine environment on its natural hosts may stimulate horizontal gene transfer. Castro-Rosas and Escartin (2002) observed that both attachment and colonization on the shrimp exoskeleton were optimal at a salinity of 1.0 to 1.5%, a pH of 6.0 to 7.0, and a temperature of 37°C. The ability of *V. cholerae* O1 to attach to and colonize exoskeletons of edible crustaceans provides a potential means of survival in aquatic environments. Munro and Colwell (1996) showed that *V. cholerae* ATCC 14035 can remain in the culturable state in seawater for a relatively long time, i.e. sufficiently long to be carried by ocean currents to widely distant geographical locations. Wong et al (1995) examined the survival of *V. alginolyticus, V. parahaemolyticus, V. cholerae* and *V. fluvialis* in tryptic soy broth (TSB) supplemented with 1% sodium chloride (NaCl). Survival of *V. cholerae* at low temperatures was increased by the addition of 0.5% of heated pyrophosphate and
metaphosphate, probably by decreasing the lethality of the cold injury to the cells. Measures should be taken to minimize the risk from pathogenic vibrios in frozen seafoods, especially if phosphates are used and psychrotrophic strains are present. Jahid et al (2006) concluded that polyphosphate protects *V. cholerae* from environmental stresses under phosphate limitation conditions. It has been proposed that toxigenic *V. cholerae* can survive in estuaries and brackish waters in which phosphorus and/or nitrogen can be a limiting nutrient. Chang et al (1995) investigated the survival of *V. cholerae* non-O1 and *V. mimicus* and found out that the survival time was the shortest in fresh water and longest in seawater at 4°C. Catalase (*katG*) was expressed by *V. vulnificus* only in warm estuarine waters (20°C). Since catalase plays a key role in the culturability of *V. vulnificus* in complex (H2O2-rich) media, the loss of catalase activity may be considered a cold shock response that contributes to the viable but nonculturable state (Thompson et al., 2006). Huelsmann et al (2003) detected albuminase, caseinase, elastase, collagenase and gelatinase in wild strains of *V. vulnificus*. Marco-Noales et al (1999) noted that under optimal conditions of salinity and temperature, *V. vulnificus* was able to survive in the free-living form for at least 3 years. Bryan et al (1999) reported that cold-adaptive "protective" proteins may enhance survival and tolerance at cold temperatures. Paludan-Mueller et al (1996) suggested that starvation for carbon or phosphorus induces maintenance of culturability of *V. vulnificus* incubated at low temperature via the synthesis of distinct sets of starvation-specific proteins. Tanaka et al (2008) observed the accumulation of cadaverine following acid adaptation of *V. parahaemolyticus* and suggested that lysine decarboxylase plays a role in the adaptive acid tolerance response. Magalhaes et al (2000) monitored the survival of *V. parahaemolyticus* in lobster homogenates inoculated with the bacteria, and incubated at -25°C, 6°C and 28°C. The greatest survival of the bacterium was at ambient (28°C) temperature and the cultures kept at 6°C were viable until the end of the incubation period. Yao et al (2000) concluded that *V. parahaemolyticus* can enter the viable but nonculturable state under certain conditions. *V. parahaemolyticus* could reach the nonculturable stage in 50 to 80 days during starvation at 3.5°C. Kanagawa-negative strain lost culturability more slowly than Kanagawa-positive strain at low temperature. These surviving cells were capable of growth and multiplication with limited nutrients at an
extraordinary rate when the temperature was upshifted (Jiang and Chai, 1996). Manefield et al (2000) stated that the expression of luminescence in the V. harveyi pathogenic to P. monodon is regulated by an intercellular quorum sensing mechanism involving the synthesis and detection of two signaling molecules, one of which is N-hydroxy butanoyl-L-homoserine lactone and the other is uncharacterized. These results suggest that intercellular signaling antagonists have potential utility in the control of V. harveyi prawn infections. Cai and Cheng (2006) studied the extra-cellular enzyme-producing abilities of Vibrios isolated from abalone (Haliotis diversicolor) intestines and from water and the results showed that 78.6%, 21.4%, 64.3%, 64.3%, and 92.9% of intestinal strains were producing protease, lipase, phospholipase, amylase and gelatinase, respectively, while in farming water there were 63.6%, 54.5%, 72.7%, 100%, and 54.5% of strains producing protease, lipase, phospholipase, amylase and gelatinase, respectively. Overall, abilities of producing extra-cellular enzymes were greater among isolates from the farming water than those from abalone intestines, which indicated that supplementation of probiotics is necessary for abalone to improve the bacterial communities in abalone intestine.

2.8. Control of Vibrios

The growth of pathogenic vibrios can be inhibited or destroyed by physical agents, namely low and high temperature, irradiation, electric current, microwaves, high pressure, pH etc., and chemical agents like chlorine, \( \text{H}_2\text{O}_2 \), sorbate etc..

**Cold:** Several psychrotrophic strains of V. mimicus, V. fluvialis and V. parahaemolyticus have been isolated from seafoods and they survive well at 4, 10 and 30°C (Wong et al., 1994). Growth of V. parahaemolyticus was observed when held for even short periods of time under improper refrigeration (Oliver and Kaper, 2001)

**Heat:** All Vibrio spp are sensitive to heat, although a wide range of thermal inactivation rates have been reported. Thorough heating of shellfish to an internal temperature of at least 60°C for several minutes should be sufficient to eliminate pathogenic vibrios (West, 1989)

**Irradiation:** Doses of 3 kGy of gamma irradiation eliminated vibrios from frozen shrimp (Rashid et al., 1992). Dixon and Rodrick (1992) detected V. vulnificus in the non-irradiated control shell-stock oysters, however, it was not detected in any of the shell
stock after 1.0, 2.0 and 5.0 kiloGrays of exposure. Cultures of virulent and avirulent
*V. vulnificus* in phosphate buffered saline were quite radiosensitive as no colony forming
units could be detected after 0.5 kilogram exposure.

**UV radiation:** Wang et al (2004) found that *V. vulnificus* was the most sensitive to UV
and Fe\(^{3+}\) treatments. Abraham and Palaniappan (2000) noted that U-V damage of
luminoous vibrio was repaired on subsequent exposure (24 h) of irradiated bacteria to
visible light.

**Electric Current:** Park et al (2003) inactivated bacteria in seawater by low-amperage
electric current and found that *V. parahaemolyticus* was completely eliminated in 100 ms
by a 0.5-A, 12-V direct current.

**Microwave:** The vegetative cells of *S. typhi* and *V. cholerae* were totally destroyed from
an initial count of 10\(^7\) cfu/g to a nondetectable level after microwave treatment
(Muzaddadi and Nayak, 2000).

**High Pressure:** High hydrostatic pressure (30,000 to 50,000 lb/in\(^2\)) applied to oysters
reduced *V. vulnificus* populations by 6 log cfu/g after 10 min and in the case of
*V. parahaemolyticus* up to 9 log cfu/g in 30 seconds (Calik et al., 2001; Kilgen, 2000).
Cook (2003) studied the sensitivity of Vibrio species to High-Pressure Processing and
observed that *V. vulnificus* was the species that was most sensitive to treatment at 200
MPa (MPa = Mega pascal; 1 MPa = 145.0377 psi) with a decimal reduction time [D] of
26 s. *V. cholerae* was the species that was most resistant to treatment at 200 MPa (D =
149 s). The O3:K6 serotype of *V. parahaemolyticus* was more resistant to pressure than
other serotypes of *V. parahaemolyticus*. Calik et al (2001) reported that the optimum
conditions for reducing *V. parahaemolyticus* counts from 10\(^9\)cfu/ml to 10\(^1\) cfu/ml was
50000 psi for 30 seconds.

**Chlorine:** Sousa et al (2001) studied the effects of chlorine on cells of *V. cholerae* and
their results strongly indicate that 8 ppm chlorine was effective in killing viable cells
from pure cultures. Venugopal et al (2000) studied the survival of *V. parahaemolyticus* in
presence of Chlorine and observed that a minimum level of 0.5 ppm of available chlorine
was able to reduce the count of both Kanagawa positive (K+) and Kanagawa negative (K-)
*V. parahaemolyticus* in PBS by 90% within 5 min and complete killing of both was
achieved in 20 and 30 min, respectively. Thampuran et al (2006) noticed that in shrimp
meat contaminated with varying levels of \textit{V.cholerae} exposed to chlorine, a chlorine level of 4ppm could effect complete destruction of a \textit{V.cholerae} population of $10^3$ cfu/ml in 10 min. On headless shrimp with shell-on, 7ppm was required to destroy $10^3$ cells/g of \textit{V.cholerae} within 10 min.

**Hydrogen peroxide:** Srisapoom et al (1999) tested the effectiveness of hydrogen peroxide in controlling \textit{V.alginolyticus}, \textit{V.harveyi}, \textit{V.parahaemolyticus} and \textit{V.vulnificus} in Mueller Hinton Broth with 1.5% NaCl and found that \textit{V.harveyi} could be eliminated at 1.2 ppm after 6 hrs of exposure while it took 3 hrs at 7.48 ppm for \textit{V.parahaemolyticus}.

**Sorbates:** Many organic acids are used as food preservatives. The most active are acetic, lactic, propionic, sorbic and benzoic acids. Citric, caprylic, malic, fumaric and other organic acids have limited activity but are used primarily for flavourings. The antimicrobial activity of organic acids is related to pH, and the undissociated form of the acid is primarily responsible for antimicrobial activity. Sorbic acid is a trans-trans, unsaturated monocarboxylic fatty acid which is slightly soluble in water (0.16g/100ml) at 20°C. The potassium salt of sorbic acid is readily soluble in water (58.2g/100ml at 20°C). With a pKa of 4.75, activity is greatest at pH less than 6.0 to 6.5. The undissociated form is 10 to 600 times more effective than the dissociated form (Eklund, 1983). Bacteria inhibited by sorbates include \textit{Acinetobacter}, \textit{Bacillus}, \textit{Campylobacter}, \textit{Clostridium}, \textit{E.coli} O157:H7, \textit{Listeria monocytogenes}, \textit{Pseudomonas}, \textit{Salmonella}, \textit{Staphylococcus}, \textit{Vibrio spp} and \textit{Yersinia enterocolitica}. Sorbic acid inhibits primarily catalase-producing bacteria (York and Vaughn, 1955). Sorbate is applied to foods by direct application, dipping, spraying, dusting or incorporation into packaging. One of the primary targets of sorbic acid in vegetative cells appears to be the cytoplasmic membrane. Sorbic acid inhibits amino acid uptake. It reduces the cytoplasmic membrane electrochemical gradient and also inhibits dehydrogenases involved in fatty acid oxidation. Sorbate reacts with the thiol group of cysteine and by this mechanism inactivates sulfhydryl enzymes. \textit{V.parahaemolyticus} is inhibited by 0.1% sorbic acid (Oliver and Kaper, 2001).

**Citrate:** Inhibition by citrate may be due to chelation. Buchanan and Golden (1994) found that while undissociated citric acid is inhibitory against \textit{Listeria monocytogenes}, the dissociated molecules protectes the organism. Mata et al (1994) tested the killing effect of lime juice (ceviche) on \textit{V.cholerae} O1 El Tor and the effect was evident within 5
of exposure of vibrios to lime juice, with reductions of more than 99.9 % of the initial bacterial mass. After 2 h of marination of fish with lime juice (the minimum recommended), no vibrios were detected in the lowest working dilutions (1:10, 1:100). Ma et al (2005) reported that citric acid inhibits *V.parahaemolyticus* and the MIC was 0.0008 g/mL.

**Phosphates:** Gram positive bacteria are generally more susceptible to phosphates than are Gram negative bacteria. 0.5% STPP was found inhibitory to *S.aureus* (Lee et al., 1994). The ability of polyphosphates to chelate metal ions appears to play an important role in their antimicrobial activity. Presence of magnesium reverses inhibition of Gram-positive bacteria by polyphosphates. Polyphosphates inhibit cell division by blocking cell septation. Although polyphosphates are highly inhibitory to a variety of food borne pathogens, Oliver and Kaper (2001) observed that 1% tripolyphosphate has no lethal effect on *V.vulnificus*.

### 2.9. DNA Fingerprinting of pathogenic Vibrios with special reference to *Vibrio cholerae*

The whole-genome sequences of three vibrios, i.e., *V.cholerae* (Heidelberg et al., 2000), *V.parahaemolyticus* (Makino et al., 2003), and *V.vulnificus* (Chen et al., 2003) are completed. The origin of the two chromosomes in vibrios was addressed by Heidelberg et al (2000) wherein they argued that an ancestral protovibrio with a single (large) chromosome captured a mega-plasmid, which, in turn, evolved into the small chromosome. Comparative genomic analysis of the complete genome sequences for several *V. cholerae* strains, both clinical and environmental isolates, is under way to define what constitutes a pathogenic strain. A high functional diversity (i.e., plasmid addiction, phage relatedness, metabolism, and information processing) was found among cassettes recovered from vibrios isolated from aquaculture facilities and surface seawater despite the fact that 70% of these elements could not be assigned a function (Thompson et al., 2006).

The paradigm of cholera epidemics held until recently was that the disease originated in a particular region of the globe and then spread to other places via human
contact and/or contaminated material (Mintz et al., 1994; Wachsmuth et al., 1994). It has now been pointed out that the genetic backgrounds of environmental and clinical *V. cholerae* strains are quite similar and that pathogenic strains may arise from nontoxigenic strains within the aquatic environment (Mintz et al., 1994; Faruque et al., 1998a; Chakraborty et al., 2000; Sechi et al., 2000; Singh et al., 2001; Brazil et al., 2002; Li et al., 2002). *V.cholerae*, once a harmless environmental organism, has become pathogenic via multiple horizontal gene transfers (Heidelberg et al., 2000). Nevertheless, it has been demonstrated by studies in the last few years that horizontal gene transfer has contributed to several important characteristics of vibrios, such as pathogenicity and ecological niches (Boyd et al., 2000, Karaolis et al., 1994, 1995, 1998; Rowe-Magnus et al., 2001, 2002, 2002a, 2002b; Waldor and Mekalanos, 1996). The genes for cholera toxin (CT), the most important virulence factor of *V.cholerae*, have long been thought to be encoded in the chromosome of the bacterium. Waldor and Mekalanos (1996) reported that these genes are actually encoded in the genome of a newly identified bacteriophage. Comparative genome analysis has revealed a variety of genomic events, including mutations, chromosomal rearrangements, loss of genes by decay or deletion, and gene acquisitions through duplication or horizontal transfer (Kaper and Hacker, 1999; Makino et al., 2003). All of these events may be driving forces in evolution and speciation of vibrios (Hacker and Kaper, 2000; Ochman et al., 2000; Hacker and Carmiel, 2001; Ochman and Moran, 2001; Hacker et al., 2003).

Molecular typing methods were employed to study the genetic heterogeneity of pathogenic Vibrios. George et al (2005) investigated the genetic heterogeneity among *V.alginolyticus* isolated from shrimp farms by PCR fingerprinting. DNA polymorphism of *V.alginolyticus* isolated from culture environment, diseased fish and penaeid shrimp was studied by Chen et al (2002). Sudheesh et al (2002) employed Random Amplified Polymorphic DNA-PCR (RAPD-PCR) for typing of *V.parahaemolyticus* and *V.alginolyticus* isolated from cultured shrimps. Maluping et al (2005) employed three PCR-based techniques viz., RAPD-PCR, enterobacterial repetitive intergenic consensus sequence PCR (ERIC-PCR) and repetitive extragenic palindromic PCR (REP-PCR) for the analysis of genetic variability among *V.parahaemolyticus* strains isolated from shrimps (*P.monodon*) and from the environments where these shrimps are being
cultivated. They demonstrated genetic variability within the *V. parahaemolyticus* strains. In addition, RAPD, ERIC and REP-PCR are suitable rapid typing methods for *V. parahaemolyticus*. All three methods have good discriminative ability and can be used as a rapid means of comparing *V. parahaemolyticus* strains for epidemiological investigation. Based on the results of this study, it was concluded that REP-PCR is inferior to RAPD and ERIC-PCR, owing to the fact that it is less reproducible. Moreover, the REP-PCR analysis yielded a relatively small number of products. This may suggests that the REP sequences may not be widely distributed in the *V. parahaemolyticus* genome.

Intraspecific diversity of *V. vulnificus* strains was analysed using Pulsed-Field Gel Electrophoresis Analysis (Wong *et al.*, 2004), RAPD-PCR (Lin *et al.*, 2003; Chatzidaki-Livanis *et al.*, 2006) and AP-PCR (Vickery *et al.*, 2000). Hernandez and Olmos (2004) employed RAPD to type *V. harveyi*. Dalsgaard *et al.* (1995) applied ribotyping for differentiating *V. cholerae* non-O1 isolated from shrimp farms in Thailand and observed that there was no correlation between specific ribotype distributions and the locations of the shrimp farms. Ribotyping appears to be a suitable method for differentiating environmental *V. cholerae* non-O1 strains, and comparison of ribotype patterns showed a high degree of genetic divergence within *V. cholerae* non-O1.

*V. cholerae* O1 or O139 isolates from cholera patients form tight clusters within the species (Beltran *et al.*, 1999; Farfán *et al.*, 2000; Stine *et al.*, 2000). Keymer *et al.* (2007) studied the genomic and phenotypic diversity of coastal *V. cholerae* strains and observed that autochthonous environmental isolates of this species routinely display more extensive genetic diversity than the primarily clonal pathogenic strains. Jiang *et al.* (2000) determined genetic diversity of *V. cholerae* by amplified fragment length polymorphism (AFLP) fingerprinting and suggested that the population structure of *V. cholerae* undergoes a shift in genotype that is linked to changes in environmental conditions. Chokesajjawatee *et al.* (2008) determined clonality and relatedness of *V. cholerae* isolates by genomic fingerprinting, using long-range Repetitive Element Sequence-Based PCR. Kumar *et al.* (2007) performed genetic characterization of *V. cholerae* strains by Inter Simple Sequence Repeat-PCR. Zo *et al.* (2002) analysed the diversity, relatedness, and ecological interactions of toxigenic *V. cholerae* O1 populations in two distinctive habitats, the human intestine and the aquatic environment using ERIC-PCR and concluded that the
resulting population structure supports the hypothesis that spatial and temporal fluctuations in the composition of toxigenic _V.cholerae_ populations in the aquatic environment can cause shifts in the dynamics of the disease.

The most discriminative typing schemes may be those that analyze several loci evenly scattered in the chromosome, because they are most likely to reflect overall genomic DNA polymorphism. Methods employed to monitor the presence of toxigenic _V.cholerae_ strains in environmental and clinical samples include multilocus enzyme electrophoresis (MEE) (Desmarchelier _et al._, 1988; Chen _et al._, 1991; Salles and Momen 1991; Wachsmuth _et al._, 1993), ribotyping (Koblavi _et al._, 1990; Popovic _et al._, 1993; Karaolis _et al._, 1994) and pulsed-field gel electrophoresis (PFGE) (Cameron _et al._, 1994). MEE by indexing allelic variation in sets of randomly selected structural genes of the chromosomal genome provides a basis for estimating overall levels of single-locus and multilocus genotypic variation in populations and species (Selander _et al._, 1986). MEE was used to examine the relationships between _V.cholerae_ strains; they were grouped into 73 zymovars (strain or group of strains with the same alleles) by using 13 structural loci (Salles and Momen, 1991) and into 10 electrophoretic types by using 16 enzyme loci (Chen _et al._, 1991). MEE requires microbial cultivation and specialized reagents and it has been limited to research use. In addition, toxigenic _V.cholerae_ _O_1 El Tor isolates can only be differentiated into four clonal groups on the basis of MEE (Wachsmuth _et al._, 1993).

Genotyping of _V.cholerae_ _O_1, i.e., construction of molecular genetic maps by analyzing the segregation of restriction fragment length polymorphisms (RFLPs) among the progeny of strains with different restriction enzymes, can be highly informative (Kaper _et al._, 1981; Yam _et al._, 1989; 1991; Wachsmuth _et al._, 1993). Koblavi _et al._ (1990) developed an rRNA RFLP, or ribotyping, assay based on _BglII_ cleavage of whole-cell DNA to study a collection of 89 _V.cholerae_ _O_1 isolates. Ribotyping involves restriction endonuclease digestion, Southern blotting, an autoradiography of hybridized ribosomal DNA and requires several days. A total of 17 rRNA gene restriction patterns were observed. However, no correlation between serotype and rRNA gene restriction pattern was obtained. The molecular epidemiology of _V.cholerae_ isolates in Latin America has been studied by using RFLP of rRNA, _ctx_ genes, MEE, etc. It was
established that there are at least four distinct toxigenic El Tor *V. cholerae* O1 clones (Wachsmuth et al., 1993).

PFGE was found to be more discriminating than the MEE or ribotyping schemes described previously (Cameron et al., 1994). PFGE requires tedious cell preparation in agarose-embedded plugs and lengthy electrophoretic separations. Furthermore, the PFGE patterns of *V. cholerae* O1 may be too numerous and analysis of these patterns may be too complex to be used in a general typing scheme (Cameron et al., 1994).

Despite the extensive applicability of these techniques, their use has been limited since they are time consuming and labour intensive. However, PCR can be performed with only one instrument for rapid detection of specific sequences of nucleic acid (Saiki et al., 1988; Koch et al., 1993).

Randomly amplified polymorphic DNA (RAPD) technique (Welsh and McClelland, 1990; Williams et al., 1990) employs single short primers (decamers) with arbitrary nucleotide sequences in a polymerase chain reaction (PCR) to randomly amplify genome DNA, which subsequently generates strain specific arrays of amplified DNA fragments. This DNA polymorphism assay is based on amplification of random DNA segments with single primers of arbitrary nucleotide sequence, i.e., random amplified polymorphic DNA-PCR, arbitrary primer PCR. Arbitrary primer PCR and priming efficiency in various PCR experiments carried out by other investigators were also found to be low in sensitivity and did not allow distinction of strains within a species (Calia et al., 1994; Salles et al., 1994). RAPD with short primers may be particularly vulnerable to artefactual variations due to slight differences in PCR conditions (Ellsworth et al., 1993, Okuda et al., 1997; Wong et al., 1999) and is complicated by variations in band intensity and the lack of reproducibility of certain minor bands (Samore et al., 1996).

PCR typing methods using specific primers designed on the basis of the repeated and conserved sequences in bacteria and more stringent annealing conditions display more promising fingerprints than RAPD analysis (Liu et al., 1995). PCR-mediated strain fingerprinting is based on the targeting of repeated DNA sequences with outwardly directed oligonucleotide primers. The general methodology is referred to as repetitive element sequence-based PCR or rep-PCR. Fingerprinting by rep-PCR is a useful tool for DNA-based epidemiological assessment of clonal realationship among bacterial isolates.
Primers aimed at prokaryotic repetitive extragenic palindromes (REP) or enterobacterial repetitive intergenic consensus (ERIC) have proven to be valuable for discriminating isolates of a variety of eubacterial species (Versalovic et al., 1991). Repetitive DNA sequences offer a rational basis for the synthesis of oligonucleotide probes and primers useful for typing bacterial pathogens (Versalovic et al., 1991). The approaches applied by Versalovic et al. (1991) are a simple and useful alternative to the other methods used to date, because ERIC primers are highly specific and the procedure rapidly distinguishes toxigenic strains from nontoxigenic strains of *V. cholerae*. Multiple colonies isolated from the same culture, as well as repeated isolation of the same strain over time, revealed a consistent pattern, demonstrating that the fingerprint is stable and specific to a given bacterial strain (Lupski and Weinstock, 1992). Besides ERIC-PCR, PCR methods based on the highly conserved ribosomal gene spacer sequence (RS) and the 38-bp repetitive extragenic palindromic sequence (REP) in *Enterobacteriaceae* and other bacteria have been used for the typing of pathogenic bacteria (Stern et al., 1984; Stubbs et al., 1999).

### 2.9.1. ERIC-PCR: Enterobacterial repetitive intergenic consensus (ERIC)

ERIC sequences are 126 bp long and appear to be restricted to transcribed regions of the genome, either in intergenic regions of polycistronic operons or in untranslated regions upstream or downstream of open reading frames. ERIC sequences are novel and highly conserved at the nucleotide sequence level, but their chromosomal locations differ between species (Hulton et al., 1991). The ERIC sequence in *V. cholerae* has been identified and is located near the hemolysin gene, apparently "hitchhiking" with the hemolysin gene (Hulton et al., 1991). The hemolytic property of biotype El Tor has been shown to be less strong in *V. cholerae* strains isolated during the course of an epidemic (Barret and Blake, 1981), i.e., as the epidemic progresses, and is interconvertible within hemolytic and nonhemolytic variants (Goldberg and Murphy, 1984). It is possible, by ERIC-PCR, to generate a characteristic genomic fingerprint for given bacterial species, including *V. mimicus*, *V. vulnificus*, *V. parahaemolyticus*, *V. campbelli*, *V. mediterranei*. 

(Woods et al., 1993).
Valgino1yticus, Escherichia coli, Shigella sonnei, Shigella dysenteriae, Shigella boydii, Shigella flexneri, and Bacillus subtilis, which can be used to distinguish patterns of particular strains (Rivera et al., 1995a, Versalovic et al., 1991). ERIC-PCR also allows clear distinction among bacterial species and strains containing these repetitive elements (Versalovic et al., 1991). Dispersed repetitive DNA sequences have been described for eubacteria. Oligonucleotides matching enterobacterial repetitive intergenic consensus (ERIC) sequences (Hulton et al., 1991) were synthesized, tested, and compared with ERIC-PCR primers for the amplification of eubacterial genomic DNA (Versalovic et al., 1991). The distribution of dispersed repetitive DNA sequences in the genomes of a number of gram-negative soil bacteria using conserved primers corresponding to ERIC sequences by PCR was examined by de Bruijn (1992).

Rivera et al (1995) studied ERIC sequence polymorphism in V.cholerae strains isolated before and after the cholera epidemic in Brazil, along with epidemic strains from Peru, Mexico, and India, by PCR. A total of 17 fingerprint patterns (FPs) were detected in the V. cholerae strains examined; 96.7% of the toxigenic V.cholerae O1 strains and 100% of the O139 serogroup strains were found to belong to the same FP group comprising four fragments (FP1). The nontoxigenic V.cholerae O1 also yielded four fragments but constituted a different FP group (FP2). A total of 15 different patterns were observed among the V.cholerae non-O1 strains. Two patterns were observed most frequently for V.cholerae non-O1 strains, 25% of which have FP3, with five fragments, and 16.7% of which have FP4, with two fragments. Three fragments, 1.75, 0.79, and 0.5 kb, were found to be common to both toxigenic and nontoxigenic V.cholerae O1 strains as well as to group FP3, containing V.cholerae non-O1 strains. Two fragments of group FP3, 1.3 and 1.0 kb, were present in FP1 and FP2, respectively. The 0.5-kb fragment was common to all strains and serogroups of V.cholerae analyzed. It is concluded from the results of this study, based on DNA FPs of environmental isolates, that it is possible to detect an emerging virulent strain in a cholera-endemic region. ERIC-PCR constitutes a powerful tool for determination of the virulence potential of V. cholerae O1 strains isolated in surveillance programs and for molecular epidemiological investigations. By using ERIC-PCR, as described in this study, it is possible to differentiate toxigenic (FP1) from nontoxigenic (FP2) strains of V. cholerae O1. It was speculated that the application
2.9.3. RS-PCR: Ribosomal Gene Spacer Sequence (RS)

Spacer regions within the 16S and 23S genes in prokaryotic rRNA genetic loci exhibit significant length and sequence polymorphisms in different species and are flanked by highly conserved sequences (Jensen et al., 1993). Multiple copies of these loci occur in bacteria (Srivastava et al., 1990). Therefore, amplification using primers designed on the basis of these flanking sequences will generate polymorphic fingerprints which can be used to distinguish bacterial strains at the species and subspecies levels (Jensen et al., 1993; Al-Saif et al., 1998; Bidet et al., 2000). RS-PCR has been applied to typing of species from many genera, including Listeria, Staphylococcus, and Salmonella (Jensen et al., 1993, Lagatolla et al., 1996). The 16S-23S rRNA intergenic spacer regions of V. parahaemolyticus contain different tRNA compositions, and similarities in the nucleotide sequences of the noncoding regions flanked by the tRNA genes have been noted (Maeda et al., 2000).

Wong and Lin (2001) designed and evaluated three rapid PCR typing methods for V. parahaemolyticus using primers designed on the basis of the following specific sequences: RS, REP and ERIC sequences. Typing patterns and clustering analysis indicated that these methods apparently differentiated V. parahaemolyticus strains from reference strains of interspecific E.coli, V.cholerae, and V.vulnificus and were also valuable in subspecies typing of this pathogen. Forty domestic strains of V. parahaemolyticus, representing a wide range of PFGE patterns, were grouped into 15, 27, and 27 patterns, with discrimination indexes of 0.91, 0.97, and 0.98, by RS-PCR, REP-PCR and ERIC-PCR, respectively. The discriminative abilities of these PCR methods closely approached or even exceeded those of PFGE and ribotyping. REP-PCR is preferable to ERIC-PCR because of the greater reproducibility of its fingerprints, while RS-PCR may be a practical method because it generates fewer amplification bands and patterns than the alternatives.
2.10. Cholera toxin and ctx genes

Cholera enterotoxin (CT) is a major virulence determinant of *V. cholerae* O1 (Finkelstein, 1988). *V. cholerae* strains that carry the *ctx* genes in the CTX genetic element can produce CT and these strains are termed as toxigenic strains. Toxigenic strains are responsible for cholera epidemics. The pathogenesis of cholera is a complex process and the major virulence factors of *V. cholerae* are the CT encoded by the *ctxAB* genes and toxin co-regulated pilus (TCP), encoded by the *tcpA* gene (Kaper et al., 1995). The *toxR* gene encodes transcription factor that directly regulates the expression of CT (Miller et al., 1987). CT is the primary virulence factor produced by *V. cholerae*. CT is a potent A-B type exotoxin encoded by the *ctxAB* operon. CT encoded by *ctxAB* is responsible for the severe diarrheal symptoms elicited by the bacterium (Kaper et al., 1995) and TCP encoded by *tcpA* is responsible for the efficient colonization of the human intestine tract by the bacteria (Taylor et al., 1987; Tacket et al., 1998). The O139 strains of *V. cholerae* produced CT (Nair and Takeda, 1993). However, the expression of CT is rare in other serogroups of *V. cholerae* non-O1 (Said et al., 1994). *V. cholerae* O1 strains (particularly those with the potential to produce CT) in the environment could become a major public health concern.

Two critical regions of DNA on the chromosomes of both *V. cholerae* O1 and O139 are responsible for the pandemic potential of these strains to cause cholera in susceptible populations. Both regions are located on pathogenicity islands (Groisman and Ochman, 1996). The first island designated ctx? is a 7000 – 9700bp region encoding at least six genes (Waldor and Mekalanos 1996). The most important of these gene products is cholera toxin; an oligomeric protein (mol wt. 84,000 daltons) composed of 5 B subunits (ctxB) and one A subunit (ctxA). The B subunit binds holotoxin to the cell receptor whereas the A subunit provides toxigenic activity intracellularly after proteolytic cleavage into 2 peptides A1 and A2. Activation of A peptide results in ADP-ribosyltransferase activity leading to altered ion transport and hypersecretion of water and Cl into the lumen of the intestine. The structural genes for the *ctx* element resides on a filamentous phage ctx? (Waldor and Mekalanos, 1996). This indicates that toxigenic *V. cholerae* can arise de novo by horizontal gene transfer, which presumably occurs in the
gastrointestinal tract. Other genes located in this region include an accessory cholera toxin (ace), a zonula occludens toxin (zot), core encoded pilin (cep) and an open reading frame of unknown function (Janda, 1998). An RTX toxin gene cluster (haemolysins/leukotoxin) in V.cholera El Tor is tightly linked to the cholera toxin prophage but is enzymatically independent of the ctx element (Lin et al., 1999). The toxin RTXA exhibits cytotoxic activity against HEp-2 cells. The second pathogenicity island is designated VPI and is associated with epidemic and pandemic strain of V.cholerae. VPI is 39.5kb in size and contains two TOX R regulated genes; a regulator of virulence genes (Tox T) and a gene cluster containing essential colonization factors including the TCP (Karaolis et al., 1998). There is genetic evidence that this island can be transferred from V.cholerae O1 to non O1 strains. The tcp gene encodes for a 20.5Kda protein that forms bundles of long filamentous pili 6-7nm in dia (Kaper et al., 1995; Janda, 1998). Both in vitro and in vivo experimentation indicates that TCP is essential for colonization and therefore infectivity. In classical biotype strains of V.cholerae O1 there is a CTX prophage on each of the two V.cholerae chromosomes (Mekalanos, 1983; Trucksis et al., 1998) whereas in the El Tor biotype strains of V.cholerae O1 the CTX prophages are tandemly arranged on the larger of the 2 chromosomes (Mekalanos et al., 1983; Pearson et al., 1993).

The genes for the virulence determinants (CT, TCP) belong to a network of genes (the Tox R regulon) whose expression is modulated by transcriptional regulators encoded by the toxRS, tcpPH and tox T genes. Biochemical and genetic analysis coupled with studies in experimental animals and human volunteers revealed many details of cholera pathogenesis. Orally ingested bacteria survive passage through the stomach and then use motility and chemotaxis function to adhere to and penetrate the mucous coat of the upper intestinal epithelium. Vibrios must then coordinately express two sets of genes encoded by the tcp and ctx operons. Expression of both the tcp and ctx operons is regulated by two membrane proteins Tox R and Tox S (DiRita, 1992). Tox T is the most downstream regulator of the Tox R regulon in that it can activate ctx and tcp promoters independently once Tox T expression has occurred (DiRita et al., 1991, 1996). However two other membrane proteins TCP and Tcp H are also required to work synergistically with Tox R and Tox S to activate Tox T transcription (Ha"se and Mekalanos, 1998). The regulation is
in response to various environmental signals. These signals include physical parameters such as temperature and pH as well as physiological signals such as cell density, growth phase and motility (Bina et al., 2003).

ctx strains are rarely detected in V.cholerae isolates from environmental samples. However, there are atypical environmental strains that possess the ctx gene. A DNA probe study showed that a small percentage of environmental strains of V.cholerae Non O1 have the ctx gene (Nair et al., 1988).

V.cholerae O139, an emerging agent of epidemic cholera is another important cause of diarrhoea. Like serogroup O1 strain, O139 strain carry genes encoded by the ctx operon and TCP (Hall et al., 1994, Janda, 1998). However, these two groups differ in several ways. The number of copies of ctxA genes and their arrangement in O139 strain differ from that of serogroup O1. O139 are encapsulated and lack specific genes (rfb R and rfb S) involved in O1 antigen synthesis (Johnson et al., 1994). Serogroup O139 appears to have resulted from a number of genetic rearrangements in an O1 strain including deletion of the O1 rfb region and acquisition by horizontal transfer from a non O1 strain of a 35kb DNA region that encodes the surface polysaccharide (Bik et al., 1995; Comstock et al., 1996). Genetic and phenotypic evidence strongly suggests that the O139 strain arose from a V.cholerae O1 strain probably El Tor biotype by horizontal gene transfer (Johnson et al., 1994; Waldor and Mekalanos, 1994; Bik et al., 1995, 1996; Comstock et al., 1996). V. cholerae Non O1 and Non O139 strains can acquire genes for toxin production by transduction and therefore have been hypothesized to be the source of new epidemic and pandemic clones, the toxigenic O 139 serogroups having arisen from recombination with toxigenic O1 strains (Faruque et al., 2000a).

CTX2 is found in all epidemic V.cholerae isolates but is rarely recovered from the non O1 / non O139 V. cholerae environmental isolates (Albert, 1996). It is a common belief that environmental strains do not produce CT and therefore are of negligible importance in epidemic potential (Twedt et al., 1981; DePaola et al., 1983; Kaysner et al., 1987; Faruque et al., 1998). Virulence genes including ctxAB were found among environmental strains from Calcutta, India (Chakraborty et al., 2000). Occurrence of ctxA was found among 10% on non O1 / non O139 environmental isolates from Brazil (Rivera et al., 2001). Clinical toxigenic V.cholerae isolates are closely related to non-toxigenic
Environmental strains (Jiang et al., 2000a) and CT genes are highly mobile among environmental isolates. The spread of CT genes in the environment can be facilitated by the exposure of CTXε-positive strains to sunlight (Faruque et al., 2000). It is presently unclear whether the CT genes among these environmental strains are expressed or what their biological and ecological function is in the aquatic environment.

In order to determine the prevalence of horizontal transfer of VPI? and CTX? among non epidemic (Non O1 and Non O139 serogroups), 300 strains of V.cholerae of both clinical and environmental origin were screened for tcpA and ctxAB and suggested that potentially pathogenic non epidemic strains most likely evolved by sequential horizontal acquisition of the VPI? and CTX? independently (Li et al., 2003). Horizontal gene transfer plays an important role in the evolution of pathogenic bacteria. All three of the known V. cholerae virulence markers (CTX?, VPI and O antigen biosynthesis regions) are believed to have been acquired by horizontal gene transfer. More than 95% of the strains belonging to serogroup O1 and O139 produce CT whereas more than 95% of strains belonging to non O1 non O139 serogroups do not produce CT (Kaper et al., 1995). Reports on the incidence of CT among environmental isolates of V.cholerae are rare (Nair et al., 1988). Covacci et al (1997) hypothesized that some pathogenic bacteria have evolved from non-pathogenic strains of the same species via horizontal transfer of virulence genes.