BIOCHEMICAL AND MOLECULAR CHARACTERIZATION OF PATHOGENIC VIBRIOS FROM HATCHERIES AND AQUACULTURE FARMS

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This is to certify that this thesis entitled 'Biochemical and molecular characterization of pathogenic vibrios from hatcheries and aquaculture farms' embodies the result of original work conducted by Badireddy Madhusudana Rao under my supervision and guidance from November 2004 to December 2008. I further certify that no part of this thesis has previously formed the basis for the award to the candidate, of any degree, diploma, associateship, fellowship or other similar title of this or any other University or society. He has passed the Ph.D qualifying examination of the Cochin University of Science and Technology, held in April 2006.

Place : Cochin

Date: 28-01-2010

(**Dr P. K. SURENDRAN**)

DECLARATION

I hereby declare that thesis is a record of bonafide research carried out by me under the supervision and guidance of Dr P.K. Surendran, my supervising guide, and it has not previously formed the basis of award of any degree, diploma, associateship, fellowship or other similar title or recognition to me, from this or any other University or society.

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Date: 28/01/2010

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B. Madhusudana Rao

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CHAPTER · 1

INTRODUCTION

1. Introduction

Vibrio are important during hatchery rearing, aquaculture phase and post-harvest quality of shrimps. *Vibrio spp* are of concern to shrimp farmers and hatchery operators because certain species can cause Vibriosis. Vibrio species are of concern to humans because certain species cause serious diseases. Bergey's manual of Systematic Bacteriology (2005) lists 44 species under the genus Vibrio, of which 12 are pathogenic to humans viz., *V.cholerae, V.vulnificus*, *V.parahaemolyticus*, *V.furnissi*, *V.metschnikovii, V.cincinnatiensis*, *V.alginolyticus*, *V.mimicus*, *V.fluvialis*, *V.hollisae*, *V.damsela* and *V.harveyi*. Vibrios considered pathogenic to shrimps include *V.harveyi, V.alginolyticus, V.parahaemolyticus, V.vulnificus, V.proteolyticus, V.fischeri, V.anguillarum* and *V.splendidus*. Vibrios related to post harvest shrimp qualities are mainly *V.cholerae*, *V.parahaemolyticus* and *V.vulnificus*.

Indian marine exports witnessed impressive growth from 37,175 tons in 1970 to 5,41,701 tons in 2007-08 (Fig. 1.1). In terms of value, the increase was from Rs. 35.54 crores in 1970 to Rs. 7620.92 crores in 2007-08. These exports have generated valuable foreign exchange which increased from US $ 47.38 millions (1970) to US $ 1899.09 millions (2007-08) (MPEDA, 2005; MPEDA 2008).

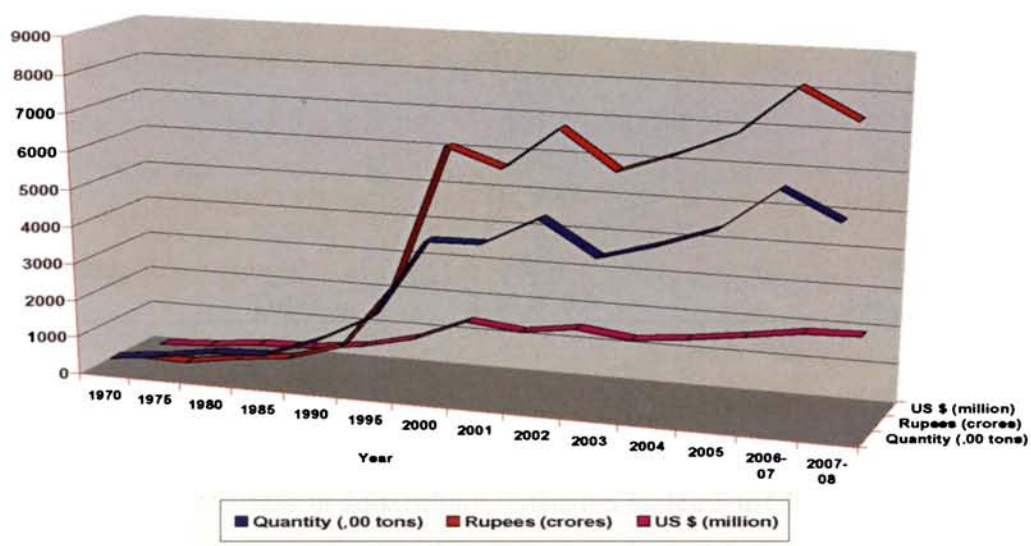


Fig 1.1. Growth of Indian Marine Exports

Frozen shrimp constituted a significant part of the marine exports. The quantity of frozen shrimp exported from India in 2007-08 was 1,36,223 tons which had realized US $ 980.62 million in foreign exchange earning (Rs. 3941.62crores).

World wide, penaeid shrimps are considered a crustacean with high potential for intensive aquaculture. *Penaeus 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. During the year 2007-08, a total of 1,06,165 MT of shrimp was produced from a culture area of 1,22,078.80 ha. Andhra Pradesh was the leading state (Table 1.1), both in terms of area under culture (50,396 ha) and shrimp production (56,557 MT).

Table 1.1. State wise details of shrimp production (2007-08)*

State	Area under culture (ha)	Production (MT)
Andhra Pradesh	50,396	56,557
West Bengal	49,236	28,000
Kerala	7597.86	5902.57
Orissa	6286	5410.4
Karnataka	3577	2119
Tamil Nadu	2729.7	3437.74
Gujarat	1659.84	3148.9
Goa	840	643
Maharastra	756.4	946.37
Total	**1,22,078.8**	**1,06,164.98**

* Source : MPEDA Annual Report 2007-08

With the progress in aquaculture, intensive systems used for shrimp aquaculture create an artificial environment that increases bacterial growth. To maintain the productivity of such an intensive aquaculture, high inputs of fish protein have to be employed for feeding, together with high levels of water exchange and the massive use of antibiotics/ probiotics / chemicals. It seems that the combination of these conditions favours the proliferation of vibrios and enhances their virulence and disease prevalence.

Bacteria take advantage of ecological changes introduced in the aquaculture practice and may cause periodic disease. Most of the bacterial species are part of the autochthonous flora of ecosystems and therefore a constant source of possible infection for crustaceans. The risk of a microbial infection is high, mainly at larval stages. The effect and severity are related to Vibrio species and dose /water, feed, shrimp quality and aquaculture management.

Liu et al (1994) observed that in giant tiger prawn (*P.monodon*) hatchery., at prior stages, the major bacterial flora were Gram positive strains, but after Zoea III stage, the Gram negative bacteria become the main bacterial flora of which the Vibrio were the dominant species. 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). Yasuda and Kitao (1980) observed low growth of shrimp larvae at protozoal stage when *Vibrio species* were present in high level (10^7 cfu/g) in water and shrimp gut. Nayyarahamed and Karunasagar (1994) studied the microbiology of cultured shrimps in India by analyzing the microbial load of water, sediment and cultured shrimp (*P. monodon*) and their results suggested that potential pathogens like *V.cholerae, V.parahaemolyticus* and *V.vulnificus* could be normal inhabitants of the gut of cultured shrimp. Selvin and Lipton (2003) reported that *V.alginolyticus* was associated with white spot disease of *P.monodon*. 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*. Jayaprakash et al (2006a) studied Vibrios associated with *Macrobrachium rosenbergii* (De Man) larvae from three hatcheries on the southwest coast of India 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. Ni et al (1995) detected five species of Vibrio viz., *V.alginolyticus, V.parahaaemolyticus, V.vulnificus, V.fluvialis* and *V.mimicus* in pond water and the prawn body with *V.alginolyticus* and *V.parahaemolyticus* as the dominant species for all ponds. 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, 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 species. *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* and 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. 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.

V. cholerae was the predominant species in the apparently healthy larval species of *M. rosenbergii* (De Man) whereas *V. alginolyticus* and *V. vulnificus* dominated during disease and morbidity (Jayaprakash *et al.*, 2006a). Gomez-Gil et al.(1998) found a wealth of vibrios, i.e., 10^5 cfu/g and 10^4 cfu/ml, respectively, in the hepatopancreas and hemolymph of healthy *Litopenaeus vannamei*. Wang and Chen (2005) concluded that the shrimp transferred from 25 ppt salinity water to low salinity levels (5 and 15 ppt) had reduced immune ability and decreased resistance against *V. alginolyticus* infection. The Vibrio spp. isolated from the digestive tract of a population of healthy juvenile *L. vannamei* consisted of both sucrose and non-sucrose fermenters whereas the haemolymph contained only non-sucrose fermenters (Gomez-Gil *et al.*, 1998).

Consumption of seafood can occasionally result in food-borne illnesses due to the proliferation of indigenous pathogens like Vibrio (Chen, 1995). Of the 12 pathogenic *Vibrio species*, 8 species are known to be directly food associated (Oliver and Kaper, 2001). Dalsgraad et al (1995) isolated 143 *V.cholerae* non O1 strains from shrimp farms in Thailand. *V.cholerae* non O1 strains are far more frequently isolated from the environmental sources than O1 strains and appear to constitute part of the microflora of prawns (Nair *et al.*, 1991). Jeyasekaran and Ayyappan (2002) reported the presence of

V.cholerae in farm reared tropical fresh water prawn (*M. rosenbergii*). Aravindan and Sheeja (2000) isolated *V.cholerae* in *P.monodon* during processing for export in Visakhapatnam region. Dalsgaard *et al.*, 1996) reported the presence of Non O1 *V.cholerae* in cooked frozen shrimp products originating from shrimp, produced by aquaculture. 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). *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. *V. parahaemolyticus* has been isolated from various parts of the water column, sediment, zooplankton, shellfish and fish. *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). 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).

European Union (EU) was the largest market for Indian marine exports during the year 2007-08 (Fig. 1.2a) with a percentage share of 35% in US $ realization followed by Japan (16.1%), USA (13.3%), China (13.3%), South East Asia (7.5%), Middle East (5%) and other countries (10%). Quantity wise, EU was the main destination for Indian marine exports (27%) in 2007-08 (Fig. 1.2b) followed by China (26%), Japan (12%), South East Asia (12%), USA (7%), Middle East (5%) and other countries (11%) (MPEDA, 2008).

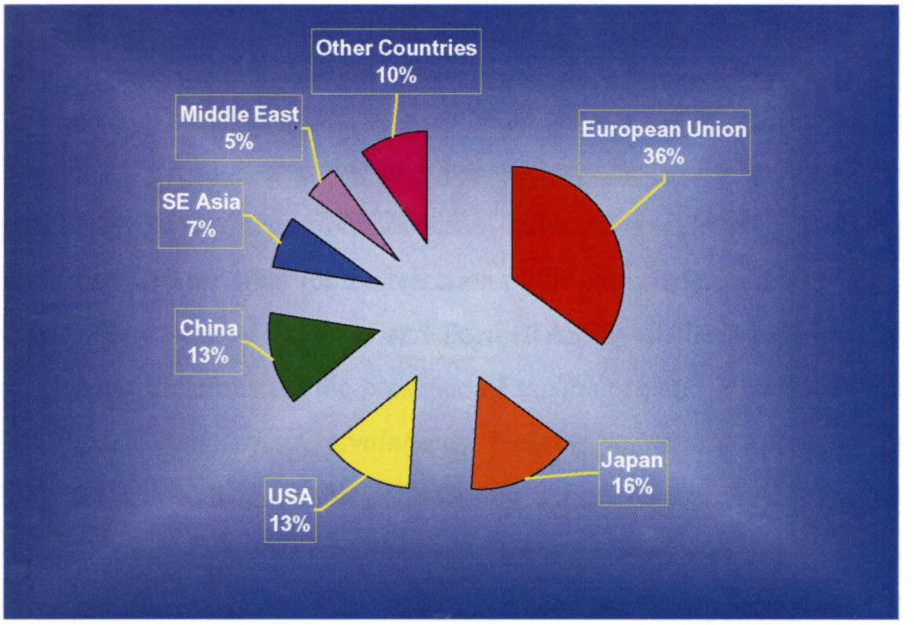


Fig. 1.2a. Indian Marine Exports -2007-08 (US $ realization)

Source : MPEDA, 2008

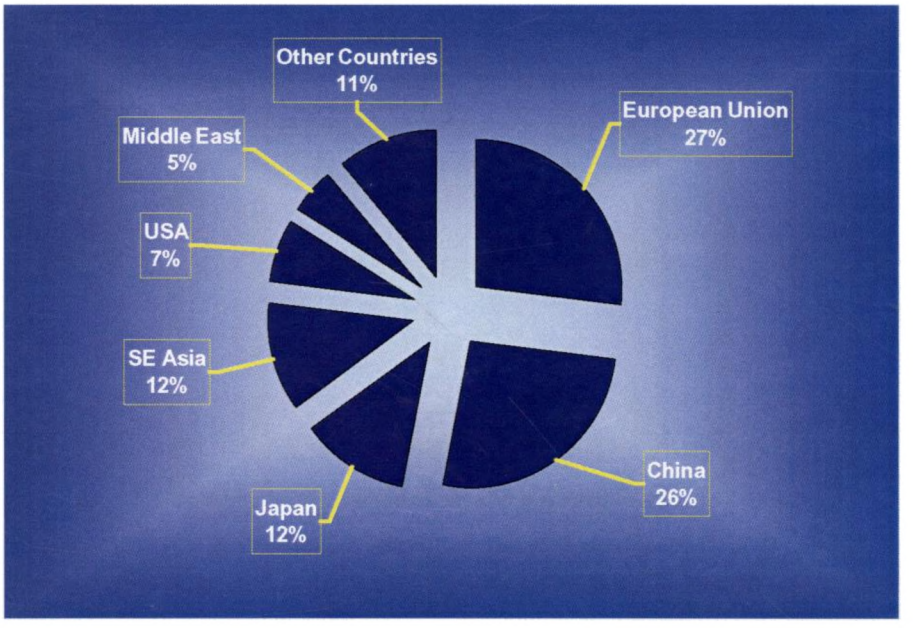


Fig. 1.2b. Indian Marine Exports – 2007-08 (Quantity wise)

Source : MPEDA, 2008

Consumers' greatest concern is the quality of food they eat. Strict quality guidelines have been laid by the importing nations, for the food products that enter their

markets. The microbiological quality requirement for export of frozen shrimp products is that *V.cholerae*, *V.parahaemolyticus* and *V.vulnificus* should be absent in 25g of the processed shrimp (Export Inspection Council of India, 1995). The mere presence of these pathogenic Vibrios is sufficient for the rejection of the exported product.

Rapid Alert System for Food and Feed (RASFF) of the European Commission has issued alert notifications for the presence of *V.cholerae* and *V.cholerae* Non O1 and Non O139, *V.parahaemolyticus*, *V.vulnificus*, *V.alginolyticus* and *V.fluvialis* in shrimps imported by the EU countries (Table 1.2). During the period 1999 to 2008, a total of 210 alert notifications were issued vis-à-vis shrimp and fish. The presence of *V.parahaemolyticus*, *V.cholerae*, *V.vulnificus* was the sole reason for rejection in 113, 55 and 3 instances, respectively. However, in many cases, the alert notifications were issued due to the presence two or more *Vibrio species* in the imported product.

Table 1.2. RASFF notifications regarding the detection of Vibrio in processed fish and shrimp products imported into EU countries*

Year	Total Vibrio notifications	VC	VP	VC + VP	VC+VP+VV	VC+VP+VA	VV	VP+VA	VC+VV	Other Vibrios
1999	08	2	1	2	0	0	0	3	0	0
2000	30	14	8	5	1	1	0	0	0	1
2001	36	12	16	5	0	0	1	0	1	1
2002	35	5	24	2	1	0	0	0	1	2
2003	32	4	27	0	0	0	1	0	0	0
2004	36	5	27	4	0	0	0	0	0	0
2005	19	8	7	4	0	0	0	0	0	0
2006	01	1	0	0	0	0	0	0	0	0
2007	04	0	3	0	0	0	0	1	0	0
2008	09	4		2	1	0	1	0	0	0
					1 (+VF)					
Total	210	55	113	24	4	1	3	4	2	4

VC : *V.cholerae*; VP : *V.parahaemolyticus*; VV : *V.vulnificus*; VA : *V.alginolyticus*; VF : *V.fluvialis*

*Source: (http://ec.europa.eu/food/food/rapidalert/rasff_portal_database_en.htm).

RASSF notifications were issued with respect to *P.monodon* and other shrimp exported to EU from India. Recent rejections vis-à-vis Vibrios in black tiger shrimps were mainly due to the presence of *V.cholerae* and *V. parahaemolyticus* (Table 1.3). The export rejections cause serious economic loss to the shrimp industry and might harm the brand image of the shrimp products from the country.

(http://ec.europa.eu/food/food/rapidalert/rasff_portal_database_en.htm).

Table 1.3. RASFF notifications vis-à-vis the detection of Vibrios from processed seafood from India*

Year	Notification Date	Notification Number	Notifying Country	Cause of rejection
2005	03/11/2005	2005.778	Norway	*Vibrio cholerae* NON O:1/NON O:139 (presence) in black tiger shrimps (*Penaeus monodon*)
	03/11/2005	2005.772	Norway	*Vibrio cholerae* NON O:1/NON O:139 (presence) in headless shell on black tiger shrimps (*Penaeus monodon*)
2007	29/01/2007	2007.AFZ	Denmark	*Vibrio parahaemolyticus* (presence /25g) in head on black tiger shrimps
	13/12/2007 30/03/2005	2005.ATL	France	*Vibrio parahaemolyticus* (presence of pathogenic strain) in frozen black tiger shrimps (*Penaeus monodon*)
2008	29/05/2008 20/05/2008	2008.0583	Denmark	*Vibrio vulnificus* and high number of aerobic plate counts (Pseudomonas dominated) in chilled shrimps (*Metapenaeus spp*)
	12/06/2008 03/03/2008	2008.AKB	Norway	*Vibrio cholerae*, *Vibrio cholerae* NON O:1/NON O:139, *Vibrio fluvialis*, *Vibrio parahaemolyticus* and *Vibrio vulnificus* in frozen raw black tiger shrimps (*Penaeus monodon*)
	27/06/2008 12/06/2008	2008.AXE	Norway	*Vibrio cholerae* NON O:1/NON O:139 (presence in 1/10 samples) in frozen black tiger shrimps
	30/07/2008 22/07/2008	2008.BDH	Norway	*Vibrio cholerae* NON O:1/NON O:139 and *Vibrio parahaemolyticus* in frozen black tiger shrimps from India

30/07/2008 24/07/2008	2008.BDQ	Norway	*Vibrio cholerae* NON O:1/NON O:139 and *Vibrio parahaemolyticus* in frozen black tiger shrimps
20/08/2008 06/08/2008	2008.BFP	Norway	*Vibrio cholerae* NON O:1/NON O:139 and prohibited substances nitrofuran (metabolite) furazolidone (AOZ) (7.5 µg/kg - ppb) and nitrofuran (metabolite) nitrofurazone (SEM) (0.65 µg/kg - ppb) in frozen black tiger shrimps

*Source: (http://ec.europa.eu/food/food/rapidalert/rasff_portal_database_en.htm).

There is a need for an independent study on the incidence of different pathogenic vibrios in shrimp aquaculture and investigate their biochemical characteristics to have a better understanding about the growth and survival of these organisms in the shrimp aquaculture niche. PCR based methods (conventional PCR, duplex PCR, multiplex-PCR and Real Time PCR) for the detection of the pathogenic Vibrios is important for rapid post-harvest quality assessment. Studies on the genetic heterogeneity among the specific pathogenic vibrio species isolated from shrimp aquaculture system provides valuable information on the extent of genetic diversity of the pathogenic vibrios, the shrimp aquaculture system.

The present study was undertaken with this goal. The following aspects were investigated in detail.

- ✓ Study the incidence of pathogenic *Vibrios spp.* in *Penaeus monodon* shrimp hatcheries and aquaculture farms.
- ✓ Biochemical investigations of the pathogenic *Vibrio spp* isolated from *P.monodon* hatchery and aquaculture environments.
- ✓ Assess the effect of salt (NaCl) on the growth and enzymatic activities of pathogenic *Vibrio spp.*
- ✓ Study the effect of preservatives/ chemicals on the growth of pathogenic *Vibrio spp.*
- ✓ Employ polymerase chain reaction (PCR) methods for the detection of pathogenic *Vibrio spp.*

- ✓ Develop a duplex-PCR for the simultaneous detection of *V. cholerae* and differentiation of cholera toxin producing *V. cholerae* isolates.
- ✓ Develop a pathogenic Vibrio-Multiplex PCR for the detection of pathogenic Vibrios viz., *V.cholerae*, *V. cholerae* (*ctx*), *V. alginolyticus*, *V. vulnificus* and *V.parahaemolyticus*.
- ✓ Study the genetic diversity of *V. cholerae* using three PCR typing methods based on enterobacterial repetitive intergenic consensus (ERIC) sequences, ribosomal gene spacer (RS) sequence and repetitive extragenic palindromic (REP) sequences.

About this thesis:

In this thesis, the investigation has been dealt in the following manner.

A detailed study was made on the total bacterial counts, *E.coli* and total vibrio loads in water and post-larvae samples from *P.monodon* shrimp hatcheries and pond water, pond sediment and shrimp samples from aquaculture farms. Qualitative analysis of the Vibrios was performed to determine the incidence of pathogenic *Vibrio spp* in the aquaculture system. Biochemical properties of the pathogenic *Vibrio spp.* were investigated in detail and special stress was given to assess the influence of salt on the growth and enzymatic activities of the pathogenic *Vibrio spp.*, as salt plays an important role in the distribution of Vibrios in the environment. The effect of certain chemicals on the growth of pathogenic Vibrio spp. was studied so as to devise strategies for their control.

In the next part, PCR methods were employed for the rapid detection of pathogenic *Vibrio spp.* A duplex-PCR was developed for the simultaneous detection of *V. cholerae* and differentiation of cholera toxin producing *V. cholerae* isolates which can help in monitoring the incidence of choleratoxigenic strains of V.cholerae in food and environmental samples. A pathogenic Vibrio-Multiplex PCR was developed for the detection of pathogenic Vibrios viz., *V.cholerae*, *V. cholerae* (*ctx*), *V. alginolyticus*, *V. vulnificus* and *V.parahaemolyticus* which can help in identifying these human pathogenic Vibrios in a single PCR reaction tube. The genetic diversity of *V. cholerae* was studied using three PCR typing methods based ERIC-PCR, RS-PCR and REP-PCR as this

information provides an insight into the extent of genetic heterogeneity in *V.cholerae* in the black tiger shrimp aquaculture system .

The thesis is presented in 4 chapters. In chapter-1, introduction is given. In chapter-2, a review of literature is presented. A detailed review on the role of vibrios in human disease, shrimp disease and post-harvest quality is given initially followed by a review on the identification of vibrios with special emphasis on PCR, multiplex and Real Time PCR methods. DNA fingerprinting of pathogenic vibrios with special reference to *V. cholerae* is also reviewed.

Chapter-3 is the Material and Methods section. Details pertaining to the samples analyzed, bacteriological media, type cultures, PCR components, primers, equipment used and all the methods employed are presented.

In chapter-4, results and discussion are presented. Results are presented in tables, by graphical representation of data and by use of relevant photographs. The results obtained in this study are discussed with those of previous relevant studies.

A summary of the work presented in the thesis is given in chapter 5. A detailed bibliography of all the citations made in the thesis is given at the end. An annexure giving the composition of bacteriological media and test reagents is given. A list of publications by the author is also appended.

CHAPTER · 2

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Vibrios

Vibrio is the type genus of the family *Vibrionaceae* in the order XI Vibrionales. The family *Vibrionaceae* includes opportunistic pathogens of humans and animals (Daniels *et al.*, 2000; Thompson *et al.*, 2004) as well as free living chemohetrotrophs 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 *Vibrionaeceae* 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*

Species	Occurrence in human clinical specimen	
	Intestinal	**Extraintestinal**
V.cholerae		
Serogroups O1 and O139	++++	+
Serogroup non-O1	++	++
V.alginolyticus	+	++
V.cincinnatiensis	-	+
V.damsela	-	++
V.fluvialis	++	-
V.furnissii	++	-
V.harveyi (V.carchariae)	-	+
V.hollisae	++	-
V.metschnikovii	-	+
V.mimicus	++	+
V. parahaemolyticus	++++	+
V.vulnificus	+	+++

* 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 diarrohea 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 Fillippo 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 form 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 O1strains (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 diarrohea (Morris *et al.*, 1995). In late 1992 epidemics of severe acute watery diarrohea 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 (Chongsa-nguan *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

Mekalanos, 1994; Bik *et al.*, 1995, Bik *et al.*, 1996; Comstock *et al.*, 1996). 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 produce cholera toxin (CT) which is the major contributing factors for profuse diarrohea (*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. vulinificus* 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 a 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 (Twedt *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 morpholocial 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 Paguio, 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.parahaemolyitcus* is a sea food-borne halophilic pathogen that causes acute gastroenteritis in humans. *V.parahaemolyitcus* 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'.

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 illeal 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, tdh2x*) 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. Ocassionally, *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 diarrohea 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 in 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 world wide 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. 10^3 *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-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase, AICARFT/IMPCHase) were more common in virulent strains than in nonvirulent 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; Yii *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; Novoa *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*

S.No.	Disease (s)	Susceptible animals	Causative Vibrio
1	Whitespot disease	Kuruma prawns (*Penaeus japonicus)* Tiger prawns (*Penaeus monodon*)	*V.alginolyticus*
2	Terminal haemorhhagic septicaemia	Eels, ayu, rainbow trout, salmonids	*V.anguillarum*
3	Gastroenteritis	Groupers	*V.harveyi*
4	Vibriosis (larvae and juveniles)	Kuruma prawns	*V.harveyi*
5	Intestinal necrosis (larvae)	Flounders	*V.icthyoenteri*
6	Brown spot disease	Kuruma prawns	*V.penaeicida*
7	Brown ring disease	Manila and fine clams	*V.tapetis*
8	Intestinal haemorhhage, skin discoloration	Japanese horse mackerel	*V.trachuri*
9	Disease larvae	Clams, oysters, scallops	*V.pectenicida, V.tubiashii*

*Source: Bergey's manual of Systematic Bacteriology (2005)

The rapid expansion of shrimp farming industry is plagued by diseases affecting shrimp survival and growth. World wide, 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 ecosytems 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, luminecent 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 (10^7 cfu/g) in water and shrimp gut. *V.parahaemolyticus, V.vulnificus, V.alginolyticus, V.damsela, V.harveyi, V.penaecida, V.anguillarum, V.neresis, V.tubiashi and V.fluvialis* have been desribed 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 (lymhoid organ, gills, heart and hepatopancreas) are evaluated with histological methods.

Vibriosis is a major disease problem in shrimp aqauculture, 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 Visakhapatanam 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 x 10^2 to 5.8 x 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 x10^3 1.1 x 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 x 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 micro-organisms 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 lumniscent 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.parahaaemolyticus. 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, NH_4-N and H_2S, 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 (10^5-10^7 cells per g shrimp body weight) without causing shrimp mortality, while other isolates are lethal at 10^3 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 LD_{50}value of 1 x 10^5 cfu/prawn and the extracellular proteases of the bacteria were also found to be toxic with an LD_{50} 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.

Kiiyukia 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 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 phospholiase, including both acidic phospholiase and alkine phospholiase extracted from cultures of *V. parahaemolyticus* are more active than those of the others. Conforming to the result of activity of protease and phospholiase, 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 dominace 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.cincinnetiensis, 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 01 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 sea water. 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.carchariae / V.harveyi*) and generally sucrose negative (*V.mimicus, V.hollisae, V.damsela, 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-time 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 spcies 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 perfomed for species identification in numerous studies and proved useful (Marti´nez-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 2.3. PCR methods for *V.cholerae*, *V.parahaemolyticus*, *V.vulnificus*, *V.alginolyticus* and *V.harveyi*

Organism	Type of PCR	Target gene / sequence	Reference
V.cholerae	PCR	*ctxA, tcpA, hlyA, rtxA, toxR, stn-sto*	Fraga et al (2007)
V.cholerae	PCR	*ompU,toxR, ctx, zot, tcp, hlyA*	Karunasagar et al (2003)
V.cholerae	Multiplex PCR	*ctx A, ace, zot, tcpA and toxR.*	Jing et al (2003)
V.cholerae	Quadraplex PCR	*wbe* and/or *wbf, ctxA, tcpA, toxR*	Khuntia et al (2008)
V.cholerae	PCR	*ctx*	Karunasagar et al (1995)
V.cholerae	PCR	*ctxAB*	USFDA-BAM (2001)
V. parahaemolyticus	Muliplex PCR	*tdh, toxRS*	Okura et al (2003)
V.parahaemolyticus	Multiplex PCR	*tdh, trh.*	DePaola et al (2003a)
V.parahaemolyticus	PCR	tdh	Hara-Kudo et al (2003)
V.parahaemolyticus	PCR	*pR72H* sequence	Robert-Pillot et al (2002)
V.parahaemolyticus	PCR	*gyrB*	Venkateswaran et al (1998)
V.parahaemolyticus	PCR	*tdh*	Islam et al (2004a)
V.parahaemolyticus	PCR	*tdh, trh*	Robert-Pillot et al (2004)
V.parahaemolyticus	PCR	*orf8*	Myres et al (2003)
V.parahaemolyticus	PCR	*pR72H*	Shi et al (2002)
V.parahaemolyticus	PCR	*gyrB, tl*	Vongxay et al (2006)
V.parahaemolyticus	PCR	*toxR*	Dileep et al (2003)
V.parahaemolyticus	PCR	*toxR, gyrB, tlh pR72H.*	Croci et al (2007)
V.parahaemolyticus	PCR	*tl* gene	Rekha et al (2008)
V.parahaemolyticus		*tdh*	Karunasagar et al (1996)
V.vulnificus	PCR	*gyrB*	Kumar et al (2006)
V.vulnificus	PCR	*vvhA*	Parvathi et al (2005)
V.vulnificus	PCR	*Cytotoxin-Hemolysin mRNA*	Saux et al (2002)
V.vulnificus	PCR	cytotoxin-hemolysin gene	Aono et al (1997)
V.vulnificus	PCR	hemolysin /cytolysin gene	Coleman et al (1996)
V.vulnificus	Nested PCR	23S rRNA	Arias et al (1995)
V.vulnificus	Multiplex PCR	*viuB,vvh*	Panicker et al (2004b)

V.alginolyticus	PCR	species-specific PCR primers	Liu et al (2004)
V.alginolyticus	PCR	species-specific PCR primers	Kasai et al (1998)
V.alginolyticus	PCR	*gyrB*	Chen et al (2002)
V.harveyi	PCR	*16S rDNA* sequences	Oakey et al (2003)
V.harveyi	PCR	*luxA*	Ramaiah et al (2000)
V.harveyi	PCR	*toxR*	Pang et al (2006)

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*

Target Organisms	Target gene / sequence	Reference
V.cholerae, *V.parahaemolyticus*, *V.vulnificus*, *V.mimicus*.	*hsp60* for *V. vulnificus*, *sodB* for *V. cholerae* and *V. mimicus* *flaE* sequence of *V. parahaemolyticus*	Tarr et al (2007)
V.vulnificus , *V.parahaemolyticus*, *V.cholerae*	*vvhA* gene *pR72H* DNA *16S-23S* rRNA intergenic spacer region (ISR)	Hervio-Heath et al (2003)
V.vulnificus *V.cholerae* *V.parahaemolyticus*	*vvh*, *viuB* for *V. vulnificus*, *ompU*, *toxR*, *tcpI*, *hlyA* for *V. cholerae*, *tlh*, *tdh*, *trh*, open reading frame 8 for *V. parahaemolyticus*	Panicker et al (2004)
Salmonella enterica serotype *Typhimurium*, *V.vulnificus*, *V.cholerae*	*hns*, *spvB*, *vvh*, *ctx*, *tl*	Lee et al (2003)

V.parahaemolyticus		
Aeromonas hydrophila *Shigella flexneri,* *Yersinia enterocolitca* *Salmonella typhimurium* *V. cholerae* *V. parahaemolyticus*	*aerolysin (aero) gene ,* *ipaH. ail gene, ipaB. epsM,* *16S-23S rDNA*	Kong et al (2002)
Escherichia coli, *Salmonella typhimurium,* *V.vulnificus.* *V.cholerae,* *V.parahaemolyticus*	*uidA, cth, invA, ctx, tl*	Brasher et al (1998)
V.cholerae and *V.mimicus*	*ctxA, zot, ace, tcpA, ompU,* *toxR*	Singh et al (2002)
V. cholerae *V.parahaemolyticus* *V.alginolyticus*	*collagenase gene*	DiPinto et al (2005)

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 end-point 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.

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. Fluoresence 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 fluorescene 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 mnethods were developed and used foor the detetion of pathogenic vibrios. A few of the methods used for *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*

Organism	Real Time PCR method	Reference
V.cholerae	SYBR Green	Gubala (2006)
V.cholerae	TaqMan PCR	Lyon (2001)
V.cholerae	Molecular beacon	Fykse et al (2007)
V.cholerae	Molecular-Beacon	Gubala and Proll (2006)
V.cholerae		Fedio et al (2007)
V.parahaemolyticus	SYBR Green	Blackstone et al (2003)
V.vulnificus	SYBR Green	Panicker et al (2004a)
V.alginolyticus	SYBR Green	Zhou et al (2007)

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 suceptible 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 (H_2O_2-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 Kangawa-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-celluar 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, H_2O_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 kilogray 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 luminous 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 10^9cfu/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 *V.cholerae* exposed to chlorine, a chlorine level of 4ppm could effect complete destruction of a *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 *V.cholerae* within 10 min.

Hydrogen peroxide: Srisapoom et al (1999) tested the effectiveness of hydrogen peroxide in controlling *V.alginolyticus*, *V.harveyi*, *V.parahaemolyticus* and *V.vulnificus* in Mueller Hinton Broth with 1.5% NaCl and found that *V. harveyi* could be eliminated at 1.2 ppm after 6 hrs of exposure while it took 3 hrs at 7.48 ppm for *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 acitivity 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 *Acinetobacter*, *Bacillus*, *Campylobacter*, *Clostridium*, *E.coli* O157:H7, *Listeria monocytogenes*, *Pseudomonas*, *Salmonella*, *Staphylococcus*, *Vibrio spp* and *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. *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 *Listeria monocytogenes*, the dissociated molecules protectes the organism. Mata et al (1994) tested the killing effect of lime juice (ceviche) on *V.cholerae* O1 El Tor and the effect was evident within 5

min 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 inbitory 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 Carniel, 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 anlysed 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 Momem, 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* O1 El Tor isolates can only be differentiated into four clonal groups on the basis of MEE (Wachsmuth *et al.*, 1993).

Genotyping of *V.cholerae* O1, 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 *BglI* cleavage of whole-cell DNA to study a collection of 89 *V.cholerae* O1 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 targetting 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

(Woods *et al.*, 1993). 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 syntheses 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 interconvertable 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*,

V.alginolyticus, *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 diarroheal 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 populatuions. 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 A_1 and A_2. 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 conataining 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).

CTX? 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 CTX? 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.

CHAPTER · 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Hatchery Samples

Samples of water (n=7) and post-larvae (n=7) were obtained from seven *Penaeus monodon* hatcheries located in Visakhapatnam district, Andhra Pradesh State, on the East Coast of India. Post-larvae were brought to the laboratory in live condition in water, in oxygen filled polythene bags.

3.1.2. Aquaculture samples

Samples of water (n=5), sediment (n=5) and shrimp (n=5) were obtained from five *Penaeus monodon* aquaculture farms located in West Godavari district, Andhra Pradesh state, on the East Coast of India. The area of the farms ranged from 1 acre to 2.5 acres. The post-larvae stocking rate varied from 5 to 7 individuals / m^2. Water samples were collected in sterile bottles and shrimp were caught by cast net and collected in sterile plastic bags. Sediment samples were collected in sterile bottles. Water, sediment and shrimp samples were brought to the laboratory under iced condition.

3.1.3. Bacteriological Media and cultures

The microbiological examination of water, sediment, post-larvae and shrimp samples was carried out using standard media (Oxoid, Difco, Himedia brands). For the isolation, identification and characterization of Vibrios, standard culture media described in Bacteriological Analytical Manual (USFDA-BAM, 2001) were used either by reconstituting dehydrated media or by compounding using ingredients, procured from Difco, Oxoid, HiMedia, Merck, Qualigens, SRL etc. Compositions of media / diluents/ reagents / indicators used in this study were as described in Bacteriological Analytical Manual (USFDA-BAM, 2001), Atlas, (2004); Surendran et al (2006); Himedia Manual, (1998), Difco Manual (online, 11th edn) is given as Annexure I.

The bacterial type cultures used in this study were procured from the respective Type Culture Collections. These are the following:-

Vibrio	Type Culture Number
V.cholerae	MTCC 3906
V.vulnificus	MTCC 1145
V.alginolyticus	ATCC 17749
V.parahaemolyticus	ATCC 17802

3.1.4. Chemicals / preservatives used for special studies

1. Sodium citrate (Merck, India)
2. Potassium sorbate (Loba Chemie, India)
3. Potassium chloride (Sigma Aldrich, USA)
4. Sodium tri poly phosphate (Loba Chemie, India)

3.1.5. Polymerase Chain Reaction chemicals

3.1.5.1. Chemicals for DNA extraction

1. TE buffer (10mM Tris, 1mM EDTA, pH 8)
2. Sodium dodecyl Sulphate (SDS)
3. Proteinase K (20mg/ml),
4. Sodium Acetate (3M, pH 5.2)
5. Phenol
6. Chloroform
7. Isoamyl alcohol
8. Isopropanol
9. Ethanol (70%)
10. Milli Q Water

The chemicals were from Sigma-Aldrich, USA / Amresco, USA / Bangalore Genei, India/ Imperial Life Sciences, India

3.1.5.2. PCR Components

	Component	Concentration
1.	PCR Buffer (Tris buffer / Ammonium Buffer) (Tris –HCl, pH 8.3, 100mM; $MgCl_2$ 15mM; KCl 500mM; Gelatin 0.1%) Or (750mM Tris-HCl, pH 8.5, 200mM $(NH_4)_2SO_4$, 15mM $MgCl_2$, 1% Tween 20)	10X
2.	Deoxynucleoside triphosphates (dNTPs) dATP, dCTP, dGTP, dTTP	200mM
3.	Taq DNA polymerase	5U/µl
4.	Oligonucleotide primers	Sequences given in Tables 3.1.5.4., 3.1.5.5., 3.1.5.6.
5.	Water (RNA, DNA free) / MiiliQ Water	

Components were from Fermentas life sciences, USA/ Bangalore Genei, India / Biogene, USA / Sigma-Aldrich, USA.

3.1.5.3. Agarose gel electrophoresis components

Component	Concentration
Agarose	
Ethidium bromide	10mg/ml
Gel loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% (w/v) sucrose in water)	6X
TAE (Tris base 242g, glacial acetic acid 57.1ml, ethylene diamine tetraacteate, 100ml (0.5M, pH 8)	50x
DNA molecular weight markers	100bp ladder (Bangalore Genei; Fermentas Gene Ruler DNA Ladder Plus (100bp to 3000bp)
TE buffer (Tris 10mM, EDTA 1mM,pH8)	

Components were from Fermentas life sciences, USA/ Bangalore Genei,India / Biogene, USA / Sigma-Aldrich, USA.

3.1.5.4. Oligonucleotide primers used in the detection of pathogenic Vibrios

Details of the primers used in the detection of pathogenic Vibrios by PCR

Organism	Primer*	Sequence	Target	Amplicon Size	Reference
V.cholerae	F	5'-AAG ACC TCA ACT GGC GGT A – 3'	*sodB*	248bp	Tarr *et al.*, 2007
	R	5'-GAA GTG TTA GTG ATC GCC AGA GT – 3'			
V cholerae ctx	F	5'-TGA AAT AAA GCA GTC AGG TG – 3'	*ctxAB*	777bp	Bacteriological Analytical Manual, 2001
	R	5'-GGT ATT CTG CAC ACA AAT CAG– 3'			
V cholerae O1	F	5'-GTT TCA CTG AAC AGA TGG G-3'	O1-rfb	192bp	Hoshino et al., 1998
	R	5'-GGT CAT CTG TAA GTA CAA C- 3'			
V.cholerae O139	F	5'- AGC CTC TTT ATT ACG GGT GG-3'	O139-rfb	449bp	Hoshino et al., 1998
	R	5'-GTC AAA CCC GAT CGT AAA GG-3'			
V. vulnificus	F	5'-GTC TTA AAG CGG TTG CTG – 3'	*hsp*	410bp	Tarr *et al.*, 2007
	R	5'-CGC TTC AAG TGC TGG TAG AAG– 3'			
V. parahaemolyticus	F	5'-GCA GCT GAT CAA AAC GTT GAG T – 3'	*flaE*	897bp	Tarr *et al.*, 2007
	R	5'-ATT ATC GAT CGT GCC ACT CAC– 3'			
Vibrio genus	F	5'-CGG TGA AAT GCG TAG AGA T– 3'	*16S rRNA*	663bp	Tarr *et al.*, 2007
	R	5'-TTA CTA GCG ATT CCG AGT TC– 3'			
V.alginolyticus	F	5'-ATT GAG AAC CCG ACA GAA GCG AAG– 3'	*gyrB*	340bp	Zhou *et al* ., 2007
	R	5'-CCT AAT GCG GTG ATC AGT GTT ACT– 3'			

*All primers were procured from Integrated DNA Technologies (IDT), USA

3.1.5.5. Primers for PCR typing of *V.cholerae*

Details of the primers used for PCR typing of *V.cholerae*

	Primer*	Sequence	Reference
ERIC-PCR	ERIC1R ERIC2	5'-ATG TAA GCT CCT GGG GAT TCA C-3' 5'-AAG TAA GTG ACT GGG GTG AGC G-3'	Wong and Lin., 2001
RS-PCR	L1 G1	5'-CAA GGC ATC CAC CGT-3' 5'-GAA GTC GTA ACA AGG	Wong and Lin., 2001
REP-PCR	REP-1D REP-2D	5'-NNN RCG YCG NCA TCM GGC-3' 5'-RCG YCT TAT CMG GCC TAC-3' Where M IS A or C, R is A or G, Y is C or T and N is any nucleotide	Wong and Lin., 2001

*All primers were procured from Integrated DNA Technologies (IDT), USA

3.1.5.6. Primers for Real Time PCR for *V.cholerae*

Details of the primers used in Real Time PCR for *V.cholerae*

	Primer*	Sequence	Reference
V.cholerae	F R	5'-AAG ACC TCA ACT GGC GGT A – 3' 5'-GAA GTG TTA GTG ATC GCC AGA GT – 3'	Tarr *et al.*, 2007

*All primers were procured from Integrated DNA Technologies (IDT), USA

3.1.5.7. Real time PCR kit - Composition of SYBR green jump start Taq ready mix for quantitative PCR (Sigma)

Tris HCl, pH 8.3	20mM
KCl	100mM
$MgCl_2$	7mM
dNTP, each	0.4 mM
Taq DNA polymerase	0.05 unit/ul
Jumpstart Taq antibody	
SYBR Green I	
Stabilizers	
Internal reference dye	100x dye

3.1.6. Equipment and software

In addition to the standard equipment (Microscope, laminar flow chamber, autoclaves, incubators, refrigerators, hot air oven, centrifuges, Stomacher blender, vortex mixer, water baths, dry bath, flake ice machine, electronic balances, etc.), the following special equipment were used in this study

1. UV-Visible Spectrophotometer, (Cary 100 Bio, Varian, Version 9.0, USA)
2. Thermal Cycler (MJ research, Minicycler, USA)
3. DNA Engine Gradient cycler, (MJ Research, USA) PTC-200 with chromo 4, continuous fluorescence detector.
4. Alpha Imager Gel Documentation system, (Alpha Innotech Corporation, USA)
5. Gel Compar II, Version 4.0, 2005, (Applied Maths, Belgium) software for comparative analysis of electrophoretic patterns.

3.2. Methods

3.2.1. Estimation of abiotic factors in water samples from hatcheries and aquaculture farms.

The abiotic parameters of the water samples i.e., pH, temperature, dissolved oxygen (DO) and salinity were determined. pH and temperature were measured immediately after collection of samples, using GripH meter (Systronics) and digital probe thermometer (Merck). The water collected in DO bottles was fixed immediately. Dissolved Oxygen was estimated by Azide modification of Winkler's Iodometric Method and salinity was determined by direct titration of water with $AgNO_3$ using K_2CrO_4 as the indicator (Trivedy and Goel, 1986).

3.2.2. Bacteriological analysis

The hatchery and aquaculture farm samples were analysed for quantitative and qualitative bacteriological parameters (USFDA-BAM 2001; Bergey's manual of Systematic Bacteriology, 2005; Surendran *et al.*, 2006).

3.2.2.1. Quantitative analysis

The hatchery and aquaculture farm samples were analysed for total bacterial loads, *E.coli* levels and total Vibrio loads, as described below.

3.2.2.1.1. Preparation of sample

10g of the sediment sample was aseptically added to 90ml of sterile normal saline (0.85% NaCl) in a erlen meyer flask and mixed thoroughly to obtain 10^{-1} dilution. 10g of shrimp muscle was aseptically cut and transferred into a sterile stomacher bag and blended with 90ml of sterile normal saline in a Stomacher circulator (Stomacher 400, Seaward, UK) at 230 rpm for 60 sec to obtain 10^{-1} dilution. 10^{-2} dilution was prepared by transferring 1ml from 10^{-1} dilution to 9ml of sterile normal saline and mixed well using vortex mixer. Further dilutions were prepared by serial decimal dilution process. Water samples were used as such or after making appropriate dilutions. For post-larvae samples,

the 10^{-1} dilution was prepared by homogenizing 1g of post-larvae in 9ml of sterile normal saline.

3.2.2.1.2. Total Plate Counts (TPC) / Aerobic Plate Count (APC)

Tryptone glucose agar (TGA) was used to estimate the total plate count. One ml each of the appropriate dilution was pipetted into sterile labeled petri plates, in duplicate. To each plate, about 15 to 18 ml of molten and cooled (40-45°C) TGA was added, mixed and allowed to set. The plates were incubated in inverted position at 36±1°C for 48 hours. The plates were counted using Quebec colony counter and TPC calculated using the relation

TPC (cfu/g or cfu/ml) = Average Count X dilution factor

3.2.2.1.3. *E.coli*

0.5 ml each of the appropriate sample dilution was pipetted onto sterile pre-dried plates of Tergitol-7 (T7) agar, in duplicate and spreaded uniformly on the surface with sterile bent glass rods. The plates were incubated in inverted position at 36±1°C for 18-24 hours. Flat, non-mucoid lemon yellow colonies, occasionally with rust brown centre were counted as presumptive *E.coli*. The presumptive colonies were confirmed on Eosin methylene blue (EMB) agar and by IMVC tests (indole, methyl red, Voges-Proskauer and citrate tests). On EMB, *E.coli* colonies show a greenish metallic sheen by reflected light and appear as purple colonies with dark centres in transmitted light. The IMVC reaction for *E.coli* is '+ + - -'or in the case of *E.coli* biotype 2 it is '- + - -'.

E.coli (cfu/g or cfu/ml) = Average Count X dilution factor X 2

3.2.2.1.4. Total Vibrio Counts (TVC)

0.5 ml each of the appropriate sample dilution was pipetted into sterile pre-dried petri plates containing TCBS agar, in duplicate and distributed over the surface with sterile bent glass rods. The plates were incubated in inverted position at 36±1°C for 18-24 hours. The total of yellow and green colonies for each dilution was recorded as presumptive Vibrio count.

Total Vibrio count (cfu/g or cfu/ml) = Average Count X dilution factor X 2

All the colonies on TCBS agar plate were transferred to T_1N_1 broth and incubated at 37°C for 18-24h. The growth from each T_1N_1 tube is streaked on a TCBS agar plate by the streak dilution technique to obtain pure colonies. One well separated pure colony from each plate was streaked on TSA slants for further confirmation, identification and storage purpose.

Confirmation of Vibrios was done by Gram's staining, nitrate test, oxidase test and H&L glucose O/F test. Those cultures which were Gram –ve, reduce nitrate, oxidase +ve and H&L glucose fermentative were confirmed as Vibrios. Cultures which were Gram –ve and H&L glucose fermentative but could not reduce nitate and oxidase –ve were further tested to rule out *V.metschnikovii* and *V.gazogenes*.

3.2.2.2. Qualitative analysis of Vibrios

The cultures confirmed as Vibrios (as per 3.2.2.1.4) were identified to the species level initially by using the scheme of Alsina and Blanch (1994) and Noguerola and Blanch (2008). All the pathogenic Vibrios were confirmed as per the biochemical reactions described in Bergey's manual of Systematic Bacteriology (2005).

3.2.2.2.1. Identification of pathogenic *Vibrio spp.*

As per Bergeys manual of Systematic Bacteriology (2005) twelve species occur in human clinical specimens; 11 of these are apparently pathogenic for human causing diarrohea or extraintestinal infections. *V.cholerae, V.parahaemolyticus, V.vulnificus V.alginolyticus, V.mimicus, V.cincinnatiensis, V.damsela, V.fluvialis, V.furnissii, V.harveyi (V.carchariae), V.hollisae* and *V.metschnikovii*.

3.2.2.2.2. Scheme of Alsina and Blanch (1994)

This scheme is used for initial identification of *Vibrio spp.* A main key was defined on the basis of the results of three tests viz., arginine dihydrolase (A), lysine decarboxylase (L) and ornithine decarboxylase (O). Eight possible clusters are produced of which two (ALO = + - + and - - +) does not include any species. Every cluster of the main key leads to another key which is the second step of identification (Fig. 3.1a and

Fig. 3.1b). Some species can be present in more than one cluster. 28 tests were used to define the system for initial identification of *Vibrio spp.* These tests were: arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, growth at 0%, 6% and 8% NaCl, growth at 4°C, 35°C and 40°C, a-ketoglutarate, amygdalin, resistance to ampicillin 10μg, arabinose-acid, citrate, d-glucosamine, gelatinase, indole, l-arabinose, luminescence, mannitol acid, NO_2, resistance to O/129 10μg, ONPG, oxidase, salicin sucrose , urease and Voges Proskauer test.

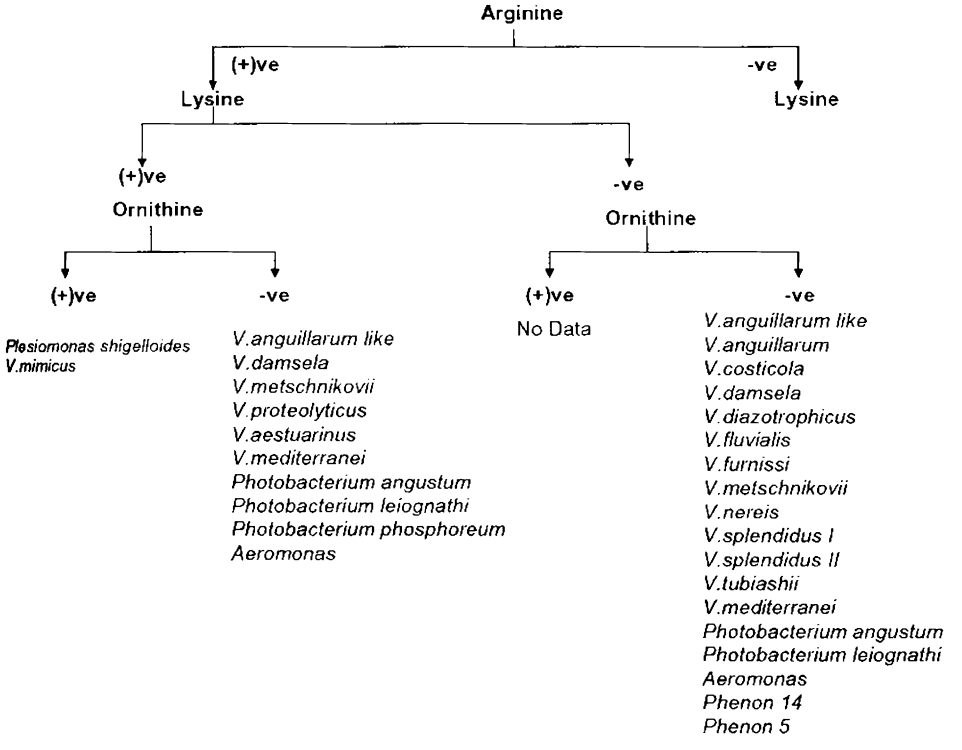


Fig. 3.1a. Scheme of Alsina and Blanch (1994) for identification of *Vibrio species*

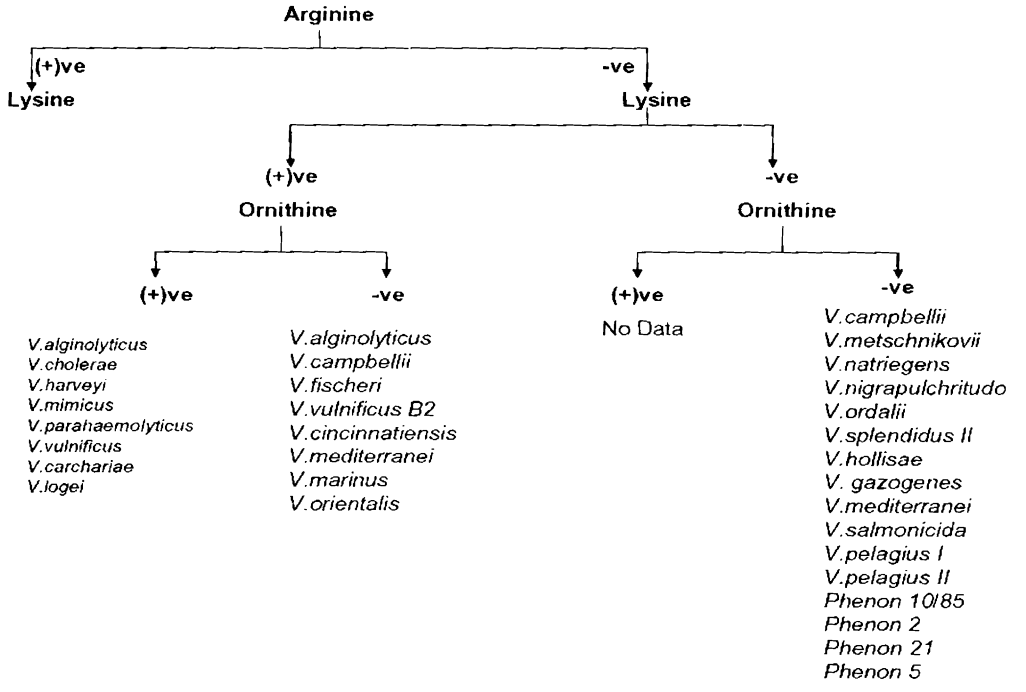


Fig. 3.1b. Scheme of Alsina and Blanch (1994) for identification of *Vibrio species*

3.2.2.2.3. Noguerola and Blanch (2008) scheme

Additional schemes (Noguerola and Blanch 2008) were used to identify pathogenic Vibrios. This new scheme constitutes 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). 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. No more than 14 are needed for even the most complicated identifications

The final set of tests used to identify the new keys were as follows: arginine dihydrolase (Moller; Difco), lysine decarboxylase (Moller), ornithine decarboxylase

(Moller), growth at 0%, 6%, 8% and 10% NaCl, growth at 4°C, 35°C and 40°C, a-ketoglutarate, amygdalin, resistance to ampicillin 10 µg, arabinose-acid, citrate, d-glucosamine cs, d-mannose,gelatinase, glucose gas, indole, l-arabinose cs, luminescence, mannitol acid, melibiose, NO_2, resistance to O /12910 µg, ONPG, oxidase, sucrose acid, urease and the Voges Proskauer test. An initial key was defined based on the results of three tests: arginine dihydrolase (A), lysine decarboxylase (L) and ornithine decarboxylase (O). This key was used as a starting point for any classification process. Accordingly, eight possible clusters (figures) were produced. The primary key defined by arginine dihydrolase, lysine decarboxylase, and ornithine decarboxylase (A / L / O) is depicted in Fig, 3.2. Every cluster leads to another figure i.e. the next identification key. The pathogenic vibrios identified by the remaining figures (Figure 2 to 8 in the original reference) are given in Table 3.2.2.2.3.

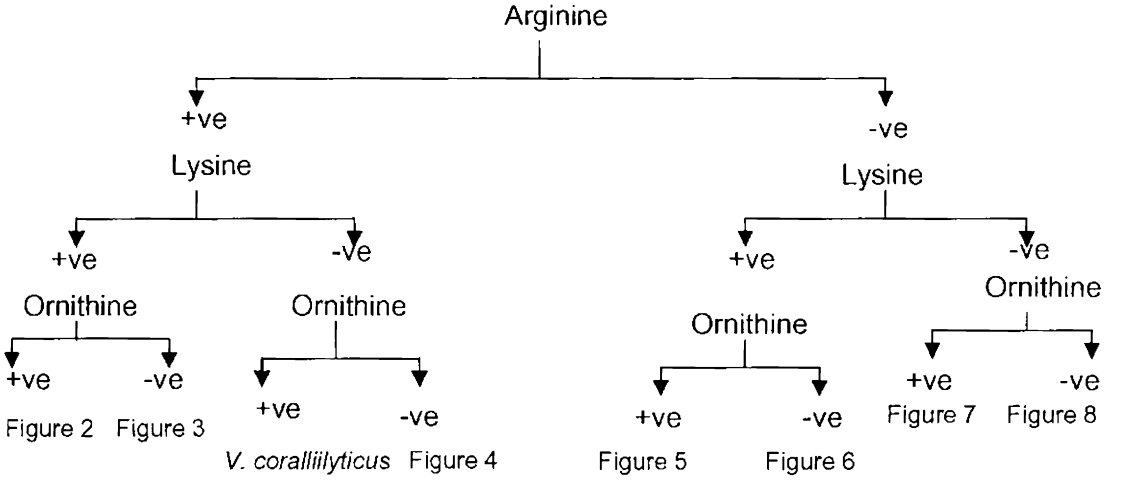


Fig 3.2. Initial key of the Noguerola and Blanch (2008) scheme for identification of *Vibrio* species

Table 3.2.2.2.3. Details of the remaining clusters (figures) of Noguerola and Blanch (2008) scheme for identification of Vibrio species.

Figure Number (Cluster)	Arginine dihydrolase (A), Lysine decarboxylase (L) and Ornithine decarboxylase (O). Reactions	Pathogenic Vibrio spp that the specific figure (cluster) identifies
Cluster 2	A+ / L+ / O+	*V.mimicus*
Cluster 3	A+ / L+ / O-	*V.metschnikovii*
Cluster 4	A+ / L- / O-	*V.metschnikovii, V.fluvialis, V.furnisii*
Cluster 5	A- / L+ / O+	*V.parahaemolyticus, V.vulnificus B1 V.vulnificus B3, V.harveyi, V.alginolyticus, , V.mimicus, V.cholerae,*
Cluster 6	A- / L+ / O-	*V.cincinnatiensis, V.vulnificus B3, V.vulnificus B2 V.metschnikovii, V.alginolyticus*
Cluster 7	A- / L- / O+	Nil
Cluster 8	A- / L- / O-	*V.metschnikovii*

3.2.2.2.4. Biochemical reactions of pathogenic Vibrios

The *Vibrio spp* identified as per the above mentioned scheme were confirmed by carrying out the biochemical reactions (Table 3.2.2.2.4.) as described in Bergey's manual of Systematic Bacteriology (2005) for the different *Vibrio spp.*

Table 3.2.2.2.4. List of biochemical tests for *Vibrio spp* mentioned in the Bergey's manual of Systematic Bacteriology

Sl No.	Biochemical test/ tests
1	Oxidase
2	nitrate reduced to nitrite
3	indole production
4	Voges-Proskauer
5	lysine decarboxylase
6	arginine dihydrolase
7	ornithine decarboxylase
8	Motility

9	acid from D-glucose
10	gas from D-glucose
11	fermentation of adonitol, L-arabinose, D-arabitol, cellobiose, dulcitol, erythritol, D-galactose, D-galactouronate, myo-inositol, lactose, maltose, D-mannitol, D-mannose, melibiose, a-methyl glucoside, raffinose, L-rhamnose, salicin, L-sorbitol, sucrose, trehalose and D-xylose
12	growth in 0% to 12% NaCl
13	Swarming

3.2.2.2.5. Biochemical tests for identification of pathogenic Vibrios isolated from shrimp hatcheries and aquaculture farms

The biochemical tests were performed as per standard methods (MacFaddin, 1980, Collins and Lyne, 1985; USFDA-BAM. 2001; Surendran *et al.*, 2006). The pathogenic Vibrios on TSA slants (*V.cholerae*) or TSA with 3% NaCl slants (*V.parahaemolyticus*, *V.vulnificus*, *V.alginolyticus* and *V.harveyi*) were used for the biochemical tests. The media for the tests involving *V.parahaemolyticus*, *V.vulnificus*, *V.alginolyticus* and *V.harveyi* were supplemented with NaCl to attain a final concentration of 3% (w/v).

3.2.2.2.5.1. Grams staining

Smears prepared from young culture (16-24h old) were stained with crystal violet and Gram's iodine, destained with ethyl alcohol and counterstained with safranine and the slides were observed by microscope under oil-immersion objective (100X).

3.2.2.2.5.2. Motility

Young cultures of pathogenic vibrios grown in T_1N_0 broth (*V. cholerae*) or T_1N_3 broth (*V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus* and *V. harveyi*) for 16-18h at 37°C were tested for motility by hanging drop technique.

3.2.2.2.5.3. Cytochrome Oxidase test

The test for the presence of the intracellular oxidase enzyme was performed by using filter papers pre-moistened with Kovac's cytochrome oxidase reagent. Using a

sterile platinum loop, the Vibrio cultures were smeared on the test paper. Development of dark purple or blue colour within a few seconds indicated a positive test.

3.2.2.2.5.4. Nitrate reduction test

The ability of the microorganism to reduce nitrate to nitrite was tested using nitrate reduction test. Equal quantities of solution A (0.5% α- Naphthylamine in dil. HCl) and solution B (0.8% Sulphanilic Acid in dil. HCl) were mixed and 1 ml. of this mixture was added to 1 ml of the culture grown in nitrate broth (37°C, 24h) . A development of red colour indicated positive reaction. If no red colour develops, then a pinch of Zinc dust was added and immediately observed for colour development. If red colour is observed now, then the culture was negative for nitrate reduction. If red colour doesn't appear even after addition of Zinc dust, the organism was considered as positive for Nitrate reduction because the nitrate has been reduced to Nitrite first and then to Nitrogen or Ammonia. The red colour is produced only in the presence of Nitrite.

3.2.2.2.5.5. Hugh and Leifson's oxidative / fermentative metabolism (H&L O/F) test

H&L O/F medium was used to test the ability of the organism to utilize glucose oxidatively or fermentatively or inert. The tubes containing H&L O/F medium were stab inoculated leaving 1cm gap from the bottom of the tube and incubated at 37°C for 24h. The reaction was noted as fermentative when the entire media turned yellow. Tubes that showed fermentation were observed for gas production. Oxidative reaction was characterized by yellow colour only in the upper portion of the media. Organisms that were neither oxidative nor fermentative produced either slight alkalinity at the surface or were inert. Simultaneously, another set of H&L O/F medium tubes were stab inoculated, overlaid with sterile liquid paraffin and incubated at 37°C for 24h. Appearance of positive colour in these tubes was taken as positive for fermentation.

3.2.2.2.5.6. Urease test

The ability of Vibrios to produce the constitutive enzyme, urease was tested by streaking the pathogenic Vibrios on Christensen's Urea agar slants and incubated at 37°C for 24h. Deep pink colour of the slant indicated a positive reaction.

3.2.2.2.5.7. Indole test

Indole test was used to test the ability of the organism to produce tryptophanases which split indole from tryptophan. To a tube of 48 h old Vibrio culture grown in

Tryptone broth (37°C, 48h), add 0.2ml of Kovac's indole reagent. Development of a pink or red colour at the surface of the media indicated a positive test for Indole.

3.2.2.2.5.8. Voges-Proskauer (VP) test

VP test was carried out to determine the ability of the organism to produce the neutral end product, acetyl methyl carbinol from glucose fermentation.To 1 ml of 48h old bacterial culture grown in MRVP medium (37°C, 48h), 0.6ml of solution A (5% α-naphthol) and 0.2ml solution B (40% KOH) were added. After shaking well, a small crystal of creatinine was added and allowed to stand up to 4 hrs. Development of eosin pink colour indicated a positive VP test.

3.2.2.2.5.9. Methyl Red (MR) test

The ability of the pathogenic Vibrios to produce and maintain stable acid end products from glucose fermentation and to overcome the buffering capacity of the system was tested by adding a few drops of methyl red indicator to 48h culture grown in MRVP broth (37°C). Development of red colour denotes a positive reaction and yellow colour was considered as negative result.

3.2.2.2.5.10. Citrate utilization test

The presence of citritase (citrate oxaloacetate-lyase) or citrate demolase in pathogenic vibrios which makes the organism capable to utilize citrate as the sole source of carbon was tested by streaking pathogenic Vibrios on Simmon's citrate agar slants and incubated at 37°C, 48h. The slants which changed colur from green to blue were considered as positive.

3.2.2.2.5.11. Triple Sugar Iron (TSI) and Kligler Iron Agar (KIA) reactions

TSI and KIA reactions were performed by stabbing the butt and streaking on the slant of TSI and KIA, respectively with pathogenic Vibrio culture and incubated at 37°C for 24h. The acidic (yellow) or alkaline (pink) reactions both in the butt and on the slant were recorded.

3.2.2.2.5.12. Sugar fermentation tests

Pathogenic Vibrios were inoculated to sugar fermentation broth with different sugars (0.5% w/v) viz., D-glucose, L-arabinose, D-arabitol, cellobiose, dulcitol, erythritol, fructose, D-galactose, D-galactouronate, myo-inositol, lactose, maltose, D-mannitol, D-mannose, melibiose, a-methyl glucoside, raffinose, L-rhamnose, salicin, L-

sorbitol, sucrose, trehalose and D-xylose to test the ability of the organism to utilize the sugars and produce acid. The sugar tubes after inoculation were incubated at 37°C for 24h. The change in colour of the medium from red to yellow (phenol red indicator system) or from purple to yellow (bromocresol purple indicator system) was considered as positive result. Gas production was noted in the inverted Durham's tube.

3.2.2.2.5.13. Amino Acid decarboxylase / dihydrolase test

The ability of pathogenic Vibrios to decarboxylase lysine, ornithine, histidine and to dihydrolase arginine was studied by inoculating the pathogenic Vibrio cultures to decarboxylase broth tubes containing the respective amino acids. Uninoculated tubes containing the amino acid served as controls. All the tubes were overlaid with sterile liquid paraffin and incubated at 37°C for 4 days. Purple colour with turbidity indicated positive reaction. Yellow colour or non-turbid tubes were considered negative.

3.2.2.2.5.14. Sensitivity to O/129 vibriostat (2,4,-diamino-6,7-diisopropyl pteridine)

The disk diffusion method was followed. The surface of pre-dried Mueller-Hinton Agar plate was swabbed with pathogenic Vibrio culture. Disks impregnated with 10µg or 150µg of vibriostat O/129 (Oxoid) were placed at separate places on the swabbed area. The plates were incubated at 37°C for 24h. A clear zone of inhibition around the disk was recorded as sensitive. The results were noted separately for 10µg and 150µg disks for each pathogenic Vibrio culture.

3.2.2.2.5.15. Salt tolerance test

The ability of each pathogenic Vibrio to tolerate and grow at different salt (NaCl) concentrations was tested by inoculating the pathogenic Vibrio cultures to T_1N_0, T_1N_3, T_1N_6, T_1N_8 and $T_1 N_{10}$ media that contain 0%, 3%, 6%, 8% and 10% NaCl, respectively. The tubes were incubated at 37°C for 2 to 7 days and observed for visible turbidity. The result was recorded for each culture at all the different salt concentrations.

3.2.3. Slide agglutination tests for identifying *V. cholerae* O1 and *V.cholerae* O139 and *V. cholerae* Non O1 and Non O139

V. cholerae isolates were initially tested with polyvalent somatic O antiserum (Difco *Vibrio cholerae* antiserum Poly [Hikojima, Inaba, Ogawa], Becton, Dickinson and Company, Maryland, USA) and the isolates that gave positive agglutination reaction

were classified as *V. cholerae* O1. Cultures that gave negative reaction with Polyvalent somatic O antiserum were further tested for agglutination using *V. cholerae* O139 antiserum (*V .cholerae* antiserum O139 Bengal, Denka Seiken Co Ltd, Tokyo, Japan) and those *V. cholerae* cultures that gave positive agglutination reaction were classified as *V. cholerae* O139. *V. cholerae* cultures that gave negative reaction both with polyvalent somatic O antiserum and O139 antiserum were classified as *V. cholerae* Non O1 and Non O139.

3.2.4. Biochemical characterization of Vibrios isolated from shrimp hatcheries and farms

3.2.4.1. Utilization of sugars

Pathogenic Vibrios were inoculated to sugar fermentation broth with different sugars (0.5% w/v) viz., D-glucose, aesculin, L-arabinose, D-arabitol, cellobiose, dulcitol, erythritol, fructose, D-galactose, D-galactouronate, glycogen, myo-inositol, lactose, maltose, D-mannitol, D-mannose, melibiose, a-methyl glucoside, raffinose, ribose, L-rhamnose, salicin, L-sorbitol, sorbose, sucrose, trehalose and D-xylose to test the ability of the organism to utilize the sugars and produce acid.

Sugar fermentation tests were also carried out in autoclavable 96 well flat bottom plastic plates having a capacity of 1.2ml per well. The 96 well plates were sterilized by autoclaving g at 121°C for 15 minutes. Pre-sterilized fermentation broth containing a specific sugars as noted above were added in the wells (900µl) column wise (1 to 12) and the pathogenic Vibrio cultures were inoculated (100µl of 24h culture) row wise (A to H) using micropipettes. The plates were covered with sterile lids and incubated at 37°C for 24 h. The change in colour was noted and the results were recorded.

3.2.4.1.1. Utilization of pentoses

The ability of pathogenic Vibrios to utilize pentoses viz., arabinose, deoxyribose, ribose and xylose was studied by inoculating the pathogenic Vibrio cultures to sugar fermentation broth containing the specific pentose and the result noted after incubating the tubes at 37°C for 24h (as described in 3.2.2.2.5.12.).

3.2.4.1.2. Utilization of hexoses

The ability of pathogenic Vibrios to utilize hexoses viz., galactose, glucose, fructose, mannose and mannitol was studied by inoculating the pathogenic Vibrio cultures to sugar fermentation broth containing the specific hexose and the result noted after incubating the tubes at 37°C for 24h (as described in 3.2.2.2.5.12.).

3.2.4.1.3. Utilization of disaccharides

The ability of pathogenic Vibrios to utilize disaccharides viz., cellobiose, lactose, maltose and sucrose was studied by inoculating the pathogenic Vibrio cultures to sugar fermentation broth containing the specific disaccharide and the result noted after incubating the tubes at 37°C for 24h (as described in 3.2.2.2.5.12.).

3.2.4.1.4. Utilization of sugar derivatives

The ability of pathogenic Vibrios to utilize sugar derivatives viz., aesculin (6,7-Dihydoxycoumarin) and salicin (2-hydroxymethyl-phenyl-ß-D-glucopyranoside) was studied by inoculating the pathogenic Vibrio cultures to sugar fermentation broth containing the specific sugar derivative and the result noted after incubating the tubes at 37°C for 24h (as described in 3.2.2.2.5.12.).

3.2.4.1.5. Utilization of Glycogen

The ability of pathogenic Vibrios to utilize glycogen was studied by inoculating the pathogenic Vibrio cultures to sugar fermentation broth containing glycogen instead of sugar and the result noted after incubating the tubes at 37°C for 24h.

3.2.4.2. Utilization of amino acids

The ability of pathogenic Vibrios to decarboxylase lysine, ornithine and histidine and to dihydrolase arginine was studied by inoculating the pathogenic Vibrio cultures to decarboxylase broth tubes containing the respective amino acids (as per 3.2.2.2.5.13.).

3.2.4.3. Enzyme activities of pathogenic Vibrios isolated from shrimp hatcheries and farms

3.2.4.3.1. Determination of Amylolytic activity

The pathogenic Vibrio culture (grown in T_1N_1 broth, incubated at 37°C/24h) was streaked / spotted on pre-dried starch agar. After incubation for 24-48h, a few crystals for

iodine were placed in the bottom lid of the inverted plate and the lid was slightly warmed. The iodine sublimes and produces blue colour in presence of starch. Starch hydrolysis was indicated by a clear zone surrounding the growth and blue colour in the rest of the media. The diameter of the zone of clearance was measured with scale and recorded to allow for comparison.

Whenever plastic petri plates were used, the starch agar plates were flooded with Gram's iodine solution. Starch hydrolysis was indicated by a clear zone surrounding the growth and blue colour in the rest of the media. The diameter of the zone of clearance was measured with scale and recorded.

3.2.4.3.2. Determination of proteolytic activity (Harrigan and Mc Cance, 1976)

Proteolytic activity of pathogenic vibrios was determined using different proteins viz., gelatin, casein, fish protein and shrimp protein.

3.2.4.3.2.1. Gelatin liquefaction

The gelatin medium was inoculated with the Vibrio culture, incubated for up to 4 days. The tubes were then kept in melting ice along with an un-inoculated control. After 20 min the tubes were observed for liquefaction of gelatin. Gelatin in the control tube should remain as hard gel whereas tubes positive for gelatin liquefaction will be in the liquid state.

3.2.4.3.2.2. Gelatin hydrolysis in agar medium

The Vibrio culture (grown in T_1N_1 broth, incubated at 37°C/24h) was spotted on pre-dried gelatin agar plates. After incubation for 48h, the plates are flooded with mercuric chloride solution. Gelatinolytic activity is indicated by a clear zone surrounding the growth and turbid appearance of the rest of the media. The diameter of the zone of clearance was measured with scale and recorded to allow for comparison.

3.2.4.3.2.3. Proteolytic activity against fish protein

The Vibrio culture (grown in T_1N_1 broth, incubated at 37°C/24h) was spotted on pre-dried fish protein agar plates. After incubation for 48h, the plates are flooded with mercuric chloride solution. Fish protein proteolytic activity is indicated by a clear zone surrounding the growth and turbid appearance of the rest of the media. The diameter of the zone of clearance was measured with scale and recorded to allow for comparison.

3.2.4.3.2.4. Proteolytic activity of against shrimp protein

The Vibrio culture (grown in T_1N_1 broth, incubated at 37°C/24h) was spotted on pre-dried shrimp protein agar plates. After incubation for 48h, the plates were flooded with mercuric chloride solution. Shrimp protein proteolytic activity is indicated by a clear zone surrounding the growth and turbid appearance of the rest of the media. The diameter of the zone of clearance was measured with scale and recorded to allow for comparison.

3.2.4.3.3. Determination of DNAse activity (Barry *et al.*, 1973)

The Vibrio culture (grown in T_1N_1 broth, incubated at 37°C/24h) was spotted on pre-dried DNA test plates. After incubation for 48h, the plates are flooded with 1N HCl. DNAse activity was indicated by a clear zone surrounding the growth and turbid appearance of the rest of the media. The diameter of the zone of clearance was measured with scale and recorded to allow for comparison.

3.2.4.3.4. Determination of lipolytic activity (Collins and Lyne, 1985)

Lipolytic activities of pathogenic Vibrios based on their ability to hydrolyse tributyrin (neutral lipid) and phospholipids (in egg yolk) were determined.

3.2.4.3.4.1. Tributyrin hydrolysis by pathogenic Vibrios

The pathogenic Vibrio culture (grown in T_1N_1 broth, incubated at 37°C/24h) was spotted on pre-dried tributyrin agar plates and incubated for 48h. Lipolytic activity was indicated by a clear zone surrounding the bacterial growth and turbid appearance of the rest of the media. The diameter of the zone of clearance was measured with scale and recorded to allow for comparison.

3.2.4.3.4.2. Phospholipase activity of pathogenic Vibrios

The pathogenic Vibrio culture (grown in T_1N_1 broth, incubated at 37°C/24h) was spotted on pre-dried egg yolk agar plates and incubated for 48h. Lipolytic activity was indicated by a clear zone surrounding the bacterial growth and turbid appearance of the

test of the media. The diameter of the zone of clearance was measured with scale and recorded to allow for comparison.

3.2.4.3.5. Determination of phosphatase activity (Collins and Lyne, 1985)

The pathogenic Vibrio culture (grown in T_1N_1 broth, incubated at 37°C/24h) was spotted on pre-dried phenolphthalein phosphate agar and incubated for 24h. After incubation the plates were exposed to ammonia vapour for a few minutes. Red or pink colour of the culture growth indicated free phenolphthalein released by the phosphatase activity of the bacteria and hence a positive reaction.

3.2.5. Studies on growth kinetics of pathogenic Vibrios isolated from shrimp hatcheries and farms

Growth kinetics of pathogenic Vibrios was studied by inoculating *V.cholerae* in T_1N_0 medium and *V.alginolyticus*, *V.parahaemolyticus*, *V.vulnificus* in T_1N_3 medium. T_1N_0 medium and T_1N_3 medium were prepared and distributed in 10ml quantities in test tubes.

V.cholerae culture isolated from pond water was grown in 10ml of T_1N_0 broth and incubated at 37°C for 24h. The cells harvested by centrifugation were washed in sterile normal saline (0.85% NaCl) and re-suspended in sterile normal saline to a known opacity. The suspension was diluted with normal saline to a known opacity and the number of cells/ml of the dilution was determined by drop plate method. The number of viable cells was enumerated by the drop plate method of Miles et al (1938) in the following manner. 10µl was withdrawn aseptically from the culture dilution and dropped from a height of 2cm on pre-dried TSA plates and TCBS agar plates. The plates were incubated at 37°C for 24 h. The number of colonies were counted and recorded. Similarly the growth kinetics of *V.cholerae ctx* was carried out using a *ctx* positive *V.cholerae* isolate obtained from pond water.

The growth kinetics of *V.parahaemolyticus* isolate, *V.vulnificus* isolate and *V.alginolyticus* isolate obtained from hatchery water, were grown separately in 10ml of T_1N_3 broth and incubated at 37°C for 24h. The cells were harvested by centrifugation, washed in sterile seawater and re-suspended in sterile seawater to a known opacity. The

suspension was diluted with normal saline to a known opacity and the number of cells/ml of the dilution was determined by the drop plate method.

100µl of the dilution of the suspended cells was used as inoculums for studies on growth kinetics of the specific pathogenic Vibrio species.

3.2.5.1. Effect of temperature on the growth of Vibrios isolated from shrimp hatcheries and farms

The effect of temperature on the growth of pathogenic Vibrios was tested at different temperatures ranging between 0°C and 56°C. A set of tubes containing T_1N_0 medium was inoculated with known number of *V.cholerae* cells (grown in T_1N_0 medium, 37°C/24h). Another set of tubes containing T_1N_3 medium were inoculated with known number of *V.alginolyticus cells* (grown in T_1N3 medium, 37°C/24h). Similarly, sets of tubes containing T_1N_3 medium were used for *V.parahaemolyticus*, *V.vulnificus* and *V.harveyi*. All tubes were incubated in duplicate at different temperatures viz., 0°C, 8°C, 22°C, 37°C, 42°C and 56°C. The tubes were incubated for a period of 5 days.

Qualitative and quantitative assessment of growth was carried out at regular intervals. Qualitative assessment was done by visual turbidity checking. Quantitative assessment was carried out by checking the optical density (OD) in a spectrophotometer at 630nm and also by enumerating the number of viable cells by the drop plate method of Miles et al (1938). Drop plate method is done in the following manner. 10µl was withdrawn aseptically from each culture tube and dropped from a height of 2cm on pre-dried TSA plates and/or TCBS agar plates. The plates were incubated at 37°C for 24 h. The number of colonies were counted and recorded. For tubes with strong turbid growth, the cultures are serially diluted and the dilutions were used for drop plate analysis.

3.2.5.1.1. Effect of temperature on the utilization of sugars by pathogenic Vibrios

V.cholerae, *V.alginolyticus*, *V.vulnificus*, *V.parahaemolyticus and V.harveyi* cultures were inoculated in sugar fermentation broth supplemented with NaCl (0% NaCl for *V.cholerae*, *V.cholerae ctx* and 3% NaCl for other vibrios) and to which the corresponding sugar was added at 1% level. The tubes were then incubated at different temperatures viz., 4°C, 20°C, 36±1°C, 42°C, 44-45°C and 56°C. Production of acid and gas were noted at the end of incubation period.

3.2.5.1.2. Effect of temperature on the utilization of amino acids by Vibrios

V.cholerae, *V.alginolyticus*, *V.vulnificus*, *V.parahaemolyticus* and *V.harveyi* cultures were inoculated in Moller's decarboxylase broth supplemented with NaCl (0% NaCl for *V.cholerae*, *V.cholerae ctx* and 3% NaCl for other vibrios) and to which the corresponding amino acid was added at 0.5% level. The tubes were then incubated at different temperatures viz., 4°C, 20°C, 36±1°C, 42°C, 44-45°C and 56°C.

3.2.5.2. Effect of pH on the growth of Vibrios isolated from shrimp hatcheries and farms

The effect of pH on the growth of pathogenic Vibrios was tested at different pH ranging between 4 and 12. A set of tubes containing T_1N_0 medium adjusted to 4 different pH viz., 4, 7, 9 and 12 were inoculated with known number of *V.cholerae* cells (grown in T_1N_0 medium, 37°C/24h). Another set of tubes containing T_1N_3 medium adjusted to 4 different pH viz., 4, 7, 9 and 12 were inoculated with known number of *V.alginolyticus cells* (grown in T_1N3 medium, 37°C/24h). Similarly, sets of tubes containing T_1N_3 medium were used for *V.parahaemolyticus*, *V.vulnificus* and *V.harveyi*. All tubes were incubated in duplicate at 37°C for a period of 5 days. Qualitative and quantitative assessment of growth was carried out at regular intervals. Qualitative assessment was done by visual turbidity checking. Quantitative assessment was carried out by checking the optical density (OD) in a spectrophotometer at 630nm. Quantitative assessment was also carried out by enumerating the number of viable cells by the drop plate method of Miles et al (1938).

3.2.5.3. Effect of salt on the growth of Vibrios isolated from shrimp hatcheries and farms

The effect of salt on the growth of pathogenic Vibrios was tested at different salt concentrations ranging between 0% and 15%. Sets of tubes containing T_1N_0 $T_1N_{0.3}$, $T_1N_{0.5}$, T_1N_1, T_1N_2, T_1N_3, T_1N_6, T_1N_8, T_1N_{10}, T_1N_{12} and T_1N_{15} medium (respectively containing 0.3, 0.5, 1, 2, 3, 6, 8, 10, 12 and 15% NaCl in tryptone medium) were inoculated with known number of *V.cholerae* cells (grown in T_1N_0 medium, 37°C/24h) and *V.alginolyticus* *V.parahaemolyticus* and *V.vulnificus* (grown in T_1N3 medium,

37°C/24h) separately. The tubes were incubated at 37°C. Qualitative and quantitative assessment of growth was carried out at regular intervals (3h, 6h, 12h, 24h, 48h, 72h, 6days, 30d, 60d and 90d).

Qualitative assessment was done by visual turbidity checking. Quantitative assessment was carried out by checking the optical density (OD) in a spectrophotometer at 630nm and by enumerating the number of viable cells by the drop plate method of Miles et al (1938).

3.2.5.4. Effect of salt on the enzymatic activities of pathogenic Vibrios isolated from shrimp hatcheries and farms

3.2.5.4.1. Effect of salt on the amylolytic activity of pathogenic Vibrios

Starch agar with different salt concentrations was prepared by adding the specific amount of NaCl (0%, 0.3%, 0.5%, 0.8%, 1%, 3% and 7% w/v) to nutrient agar. The pathogenic Vibrio cultures (grown in T_1N_1 broth, incubated at 37°C/24h) were spotted on pre-dried Starch agar plates. After incubation, the starch agar plates were flooded with Gram's iodine solution. Starch hydrolysis was indicated by a clear zone surrounding the growth and blue colour in the rest of the media. The colony diameter and the diameter of the zone of clearance was measured with scale (in millimeters) and recorded to allow for comparison. The 'Activity Index' was calculated.

The biochemical potential of each culture was determined by calculating the 'Activity Index' which is obtained by dividing the diameter of the zone of clearance by the colony diameter. This ratio helps in uniform comparison of enzymatic activity of pathogenic Vibrios, both within the species and between species.

3.2.5.4.2. Effect of salt on the proteolytic activity of pathogenic Vibrios

Gelatin agar with different salt concentrations was prepared by adding the specific amount of NaCl (0%, 0.3%, 0.5%, 0.8%, 1%, 3% and 7% w/v) to nutrient agar. The pathogenic Vibrio cultures (grown in T_1N_1 broth, incubated at 37°C/24h) were spotted on pre-dried salt gelatin agar plates. After incubation for 48h, the plates are flooded with mercuric chloride solution. Gelatinolytic activity is indicated by a clear zone surrounding the growth and turbid appearance of the rest of the media. The colony diameter and the diameter of the zone of clearance was measured with scale (in millimeters) and recorded

to allow for comparison. The biochemical potential of each culture was determined by calculating the 'Activity Index'.

3.2.5.4.3. Effect of salt on the DNAse activity of pathogenic Vibrios

DNAse agar with different salt concentrations was prepared by adding the specific amount of NaCl (0.5%, 1%, 3% and 7% w/v).The pathogenic Vibrio culture (grown in T_1N_1 broth, incubated at 37°C/24h) was spotted on pre-dried DNAse plates. After incubation for 48h, the plates are flooded with 1N HCl. DNAse activity is indicated by a clear zone surrounding the growth and turbid appearance of the rest of the media. The colony diameter and the diameter of the zone of clearance was measured with scale (in millimeters) and recorded to allow for comparison. The 'Activity Index' was calculated.

3.2.5.5. Effect of salt on the swarming behaviour of *V.alginolyticus*

Migration of bacteria across solid surfaces results in progressive spreading of the bacterial colony, a phenomenon called as swarming. Movement on surfaces or swarming motility is effectively mediated by the lateral flagellar system. Two types of flagella are synthesized by vibrios in different environments. In liquid culture, swimmer cells predominate due to production of single sheathed polar flagellum. When vibrios come in contact with solid surfaces, a series of morphogenetic changes are initiated that result in the conversion of swimmer cells into swarmer cells in some marine species such as *V. alginolyticus*.

The effect of salt on the swarming behavior of *V. alginolyticus* was studied by spotting 10µl of overnight culture of *V. alginolyticus* grown in T_1N_3 on Tryptone Glucose agar (without NaCl) with the corresponding concentration of salt (0% to 25% (w/v) NaCl). The size of the colony and the swarming zone for each NaCl concentration was measured and tabulated.

3.2.5.6. Effect of salt on the utilization of sugars by pathogenic Vibrios

The effect of salt on the utilization of sucrose and mannitol by Vibrios was studied by preparing sugar fermentation broth containing the corresponding sugar at 1% level and to which NaCl was added at different concentrations. Initially the sugar utilization activity was tested at 0%, 3%, 6%, 9% and 12% salt concentrations. Based on the results at these concentrations (0%, 3%, 6%, 9% and 12%) further tests were carried

out for each isolate in media containing intervening and higher salt concentrations (1%, 2%, 4%, 5%, 7%, 8%, 10%, 11%, 15% and 20% NaCl). *V.vulnificus* isolates were tested for their ability to utilize cellobiose at different salt concentrations ranging between 0% and 20% NaCl (w/v).

3.2.5.7. Effect of salt on the utilization of amino acids by pathogenic Vibrios

The effect of salt on the utilization of arginine, lysine and ornithine by Vibrios was studied by preparing Moller's decarboxylase broth containing the corresponding amino acid at 0.5% level and to which NaCl was added at different concentrations. Initially the amino acid utilization activity was tested at 0%, 3%, 6%, 9% and 12% salt concentrations. Based on the results at these concentrations (0%, 3%, 6%, 9% and 12%) further tests were carried out for each isolate in media containing intervening and higher salt concentrations (1%, 2%, 4%, 5%, 7%, 8%, 10%, 11%, 15% and 20% NaCl).

3.2.6. Effect of preservatives / chemicals on the growth of Vibrios isolated from shrimp hatcheries and farms

The effect of potassium chloride, potassium sorbate, sodium citrate and sodium tri polyphosphate on the growth and survivability of pathogenic Vibrios were studied. Muller-Hinton agar containing the chemical/preservative at different concentration (w/v) was used. Filtered sterilized (0.2µ pore size filter) solution of the chemical was added to molten and cooled (40 – 45°C) Mueller-Hinton agar. The volume of the chemical/preservative solution to be added was accounted in the final calculation of Muller-Hinton agar volume.

3.2.6.1. Effect of potassium chloride (KCl) on the growth of pathogenic Vibrios

Serial dilutions of pathogenic Vibrio culture (grown in T_1N_1 broth, incubated at 37°C/24h) were spotted by the drop plate method (Miles *et al.*, 1938) on pre-dried Muller-Hinton agar plates containing KCl at different concentration (1%, 3% and 7%

w/v). The plates were inverted and incubated at 37°C for 24h. The number of colonies at each KCl concentration for the different pathogenic Vibrios were counted and recorded.

3.2.6.2. Effect of potassium sorbate ($C_6H_7O_2K$) on the growth of pathogenic Vibrios

Serial dilutions of pathogenic Vibrio culture (grown in T_1N_1 broth, incubated at 37°C/24h) were spotted by the drop plate method (Miles *et al.*, 1938) on pre-dried Muller-Hinton agar plates containing potassium sorbate at different concentration (1%, 2% and 3% w/v). The plates were inverted and incubated at 37°C for 24h. The number of colonies at each potassium sorbate concentration for the different pathogenic Vibrios were counted and recorded.

3.2.6.3. Effect of sodium citrate ($Na_3C_6H_5O_7$) on the growth of pathogenic Vibrios

Serial dilutions of pathogenic Vibrio culture (grown in T_1N_1 broth, incubated at 37°C/24h) were spotted by the drop plate method (Miles *et al.*, 1938) on pre-dried Muller-Hinton agar plates containing sodium citrate at different concentration (3% and 7% w/v). The plates were inverted and incubated at 37°C for 24h. The number of colonies at each potassium sorbate concentration for the different pathogenic Vibrios were counted and recorded.

3.2.6.4. Effect of sodium tri polyphosphate ($Na_5P_3O_{10}$, STPP) on the growth of pathogenic Vibrios

Serial dilutions of pathogenic Vibrio culture (grown in T_1N_1 broth, incubated at 37°C/24h) were spotted by the drop plate method (Miles *et al.*, 1938) on pre-dried Muller-Hinton agar plates containing STPP at different concentration (3% and 7% w/v). The plates were inverted and incubated at 37°C for 24h. The number of colonies at each STPP concentration for the different pathogenic Vibrios were counted and recorded.

3.2.7. Molecular characterization of pathogenic Vibrios isolated from shrimp hatcheries and farms

Polymerase Chain Reaction (PCR) methods were used for the detection of pathogenic Vibrios. *V.cholerae* (MTCC 3906), *V.vulnificus* (MTCC 1145), *V.alginolyticus* (ATCC 17749) and *V.parahaemolyticus* (ATCC 17802) type cultures were used for standardizing the PCR reactions.

3.2.7.1. PCR for the detection of enterotoxigenic *V.cholerae* (USFDA-BAM 2001)

Cholera toxin production encoded by the *ctxAB* genes is the major factor in the pathogenesis of cholera. A PCR method that selectively amplifies a DNA fragment within the *ctxAB* operon of *V.cholerae* was used. The sequence of cholera toxin PCR primers was mentioned in Table 3.1.5.4. This PCR methods detects only *ctx* positive *V.cholerae*.

Each *V.cholerae* culture was inoculated to APW and incubated at 37°C for 24h. 1ml of APW culture was transferred to 1.5ml microcentrifuge tubes and placed in boiling water for 5 min. This crude lysate was used as template for PCR immediately. The procedure was repeated for all the *V.cholerae* isolated (n = 42) obtained from pond water. The PCR reaction mix contained 10mM Tris-HCl, pH 8.3; 50mM KCl, 1.5mM $MgCl_2$, 200µM each dATP, dCTP, dGTP and dTTP; 0.5µM of each primer, 3% (v/v) APW lysate and 2.5 U *Taq* polymerase per 100µl reaction volume. Depending upon the number of isolates to be tested, sufficient volume of master mix containing all necessary reagents except *Taq* polymerase and template (lysate) was prepared. Taq polymerase was added to the master mix prior to its distribution to 0.2ml PCR tubes (Imperial life sciences, Chandigarh). Template was added at the last to the aliqouted master mix and the PCR tubes were placed in a thermal cycler (Minicycler, MJ research, USA) for amplification. Each PCR cycle consisted of denaturation for 1 min at 94°C, primer annealing for 1 min at 55°C and primer extension for 1 min at 72°C. The cycle was repeated 34 times.

Agarose gel analysis of PCR products was carried out as per Sambrook and Russell (2001). 10µl of PCR product was mixed with 2µl of 6x gel loading buffer, and loaded into sample wells of 2% Agarose (low EE agarose, Imperial, Chandigarh) gel,

containing 1µg/ml ethidium bromide, submerged in 1X TAE. One well was used for loading 100bp DNA ladder (Bangalore Genei, India). After appropriate migration with constant voltage of 5-10 V/cm (Electrophoresis Powerpack, Bangalore Genei) the agarose gel was visualized on a UV-transilluminator (Bangalore Genei). *ctxAB* positive cultures yield an amplicon of 777bp which was ascertained with the help of molecular weight markers. The gel was either photographed with digital camera (Nikon SLR digital) and/or scanned using a gel documentation system (Alpha Imager, Alpha Innotech Corporation, USA).

3.2.7.2. PCR for the detection of *V.cholerae* using species specific primers (Tarr *et al.*, 2007)

This PCR uses species-specific primers that target the house keeping gene *sodB* of *V.cholerae*. As the house keeping genes are invariably present in all isolates, this PCR method helps in the detection of all *V.cholerae* isolates irrespective of their toxigenic status. A multiplex PCR assay targeting species specific regions of *V.cholerae*, *V.mimicus*, *V.vulnificus* and *V.parahaemolyticus* was developed by Tarr et al (2007). In this study, the multiplex PCR conditions were employed in regular PCR targeting individual pathogenic Vibrio species. The sequence of *sodB* primers was mentioned in Table 3.1.5.4.

V.cholerae isolates were grown on TSA plates, by streak plate method. A single colony was aseptically scrapped from the plate's surface and suspended in 200µl of 1X Tris-EDTA, pH 8.0. The suspension was then heated at 95°C for 10 min and centrifuged for 2 min to pellet cellular debris. The crude lysate was used as template for PCR. A typical 20µl reaction contained 1.5µl of crude lysate, 0.2µl of Taq polymerase (2U), 2µl of dNTPs stock (2mM each), 2µl of 10X buffer, 1.2 µl of MgCl2 stock (25mM), 1µl of each *V.cholerae* primer (10µM stock) and the final volume made up to 20µl with ddH_20. The primers were procured from Integrated DNA Technologies, USA and their sequence mentioned in Table 6 (listed under item 3.1.5.4). The thermal cycling profile was as follows: a 15 min soak at 93°C followed by 35 cycles : 92°C for 40sec, 57°C for 1 min, 72°C for 1.5 min; and a final soak at 72°C for 7 min. Agarose gel electrophoresis was carried out as described in 3.2.7.1. *V.cholerae* cultures yield an amplicon of 248bp.

3.2.7.3. Duplex PCR for the simultaneous detection of *V.cholerae* and differentiation of cholera toxin producing *V.cholerae* isolates (*V.cholerae*-duplex PCR)

An experiment was planned as Duplex PCR for detection and confirmation of toxigenic *V.cholerae*. For this, the method of Tarr et al (2007) and USFDA-BAM method (2001) were integrated as described below.

V. cholerae isolates were inoculated in T_1N_1 broth and incubated at 37°C for 24h. 1ml of T_1N_1 culture was transferred to 1.5ml microcentrifuge tubes and centrifuged at 10,000 rpm for 10min. The supernatant was carefully discarded and the cell pellets were resuspended in 100µl of Tris-EDTA (TE) buffer. The microcentrifuge tubes were placed in a dry bath at 95°C for 5 min. This crude lysate was used as template for PCR reaction immediately. A typical 20µl reaction contained 1.2µl of crude lysate, 0.5µM each of PCR species specific primers, 0.5µM each of ctxAB primers and 18µl of master reaction mix containing 10mM Tris-HCl, pH 8.3; 50mM KCl, 1.5mM MgCl2, 200µM each dATP, dCTP, dGTP and dTTP and 1U of *Taq* polymerase.

The thermal cycling profile was as follows: Initial denaturation of template DNA: 93°C for 15 min; 35 amplification cycles wherein each cycle consisted of denaturation at 92°C for 40sec, primer annealing at 57°C for 1 min and primer extension at 72°C for 1.5 min; final extension 72°C for 7 min.

10µl of PCR product was mixed with 2µl of 6x gel loading buffer and loaded into sample wells of 2% Agarose gel (containing 1µg/ml ethidium bromide) submerged in 1X TAE. 100bp DNA ladder (Gene Ruler^{TM}, 100bp DNA ladder plus, Fermentas) was loaded in one well. After appropriate migration with constant voltage of 100 volts for 30-45 min the agarose gel was visualized on a UV-transilluminator. *V.cholerae* cultures yield an amplicon of 248bp. *ctxAB* positive *V.cholerae* cultures yield two amplicons viz., 248bp and 777bp. *ctxAB* negative *V.cholerae* yield only a single amplicon of 248bp. The gel was either photographed with digital camera (Nikon SLR digital) and/or scanned using a gel documentation system (Alpha Imager, Alpha Innotech Corporation, USA).

3.2.7.3.1. End point dilution of *V.cholerae*-duplex PCR

The sensitivity of *V.cholerae*-duplex PCR was determined by making serial 10 fold dilutions of *V.cholerae* culture (grown in T_1N_1, incubated at 37°C for 24h) in normal saline ranging from undiluted (10^6cells/ml), 10^{-1} (10^5 cells/ml), 10^{-2} (10^4 cells/ml), 10^{-3} (10^3 cells/ml), 10^{-4} (10^2 cells/ml) to 10^{-5} (10 cells/ml) and 10^{-6} (1 cell/ml). From each tube, 1 ml was withdrawn for template preparation and subjected to *V.cholerae*-duplex PCR as described in 3.2.7.3.

3.2.7.4. PCR for and *V.cholerae* O1 and *V.cholerae* O139

PCR was performed as per Hoshino et al (1998) using O1F2-1 and O1R2-2 primers (*V.cholerae* O1-rfb specific primers) and O139F2 and O139R2 (O139-rfb specific primers). The sequence of the primers is mentioned in Table 3.1.5.4. *V. cholerae* O1 cultures yield an amplicon 192bp whereas *V. cholerae* O139 cultures yield an amplicon 449bp. *V. cholerae* Non O1 and Non O139 cultures do not yield any of the two amplicons.

3.2.7.5. PCR for the detection of *V.alginolyticus* (Zhou et al., 2007)

A real time PCR assay targeting the house keeping gene *gyrB* of *V.alginolyticus* was developed by Zhou et al (2007). This method was modified to detect *V.alginolyticus* in the present study as described below. The sequence of the *gyrB* primers is mentioned in Table 3.1.5.4.

One-millilitre aliquot of each bacterial culture were centrifuged at 12000g for 10 min and then each supernatant was carefully discarded and the cell pellets were resuspended in 100µl of Tris-EDTA buffer (10mmol l^{-1} of Tris-Cl, 1mmol l^{-1} of NA_2EDTA). Each sample was boiled in an Eppendorf tube for 15 min to release nucleic acid and then the samples were cooled to room temperature and cell debris was pelleted by centrifugation at 3000g for 3 min and an aliquot (2ul) of each resulting supernatant was used as DNA template for PCR. A typical 20µl reaction contained 2µl of crude lysate, 0.16µM of each *V.alginolyticus* primer, 18µl of master reaction mix containing 10mM Tris-HCl, pH 8.3; 50mM KCl, 1.5mM MgCl2, 200µM each dATP, dCTP, dGTP

and dTTP and 1U of *Taq* polymerase. PCR cycle conditions was as follows: Initial denaturation at 95°C for 60 sec; 45 cycles of amplification of the template DNA in which each cycle consisted of denaturation 95°C for 15sec, primer annealing 60°C for 15 sec and primer extension on the template DNA at 72°C for 45 sec. Agarose gel electrophoresis was carried out as described in 3.2.7.1. *V.alginolyticus* cultures yield an amplicon of 340bp.

3.2.7.5.1. End point dilution of *V.alginolyticus* PCR

Serial 10 fold dilutions of *V.alginolyticus* culture (grown in T_1N_1, incubated at 37°C for 24h) were made in normal saline ranging from undiluted from undiluted (9×10^7 cells/ml), 10^{-1} (9×10^6 cells/ml), 10^{-2} (9×10^5 cells/ml), 10^{-3} (9×10^4 cells/ml), 10^{-4} (9×10^3 cells/ml) to 10^{-5} (9×10^2 cells/ml) and 10^{-6} (90 cells/ml). From each tube 1 ml was withdrawn for template preparation and PCR was carried out as elaborated in 3.2.7.5.

3.2.7.6. Duplex PCR for the *V.alginolyticus* using species specific and genus specific primers (*V.alginolyticus*-duplex PCR)

A *V.alginolyticus*-duplex PCR method was developed by utilizing *V.alginolyticus* species specific primers (Zhou et al., 2007) and *Vibrio* genus specific primers (Tarr *et al.*, 2007) This method was designed to detect *Vibrio species and V.alginolyticus*. The sequence of the *gyrB* primers and *Vibrio* genus specific *16S rRNA* primers is mentioned in Table 3.1.5.4.

V.alginolyticus isolates were grown in T_1N_1 and incubated at 37C for 24h. Template DNA was prepared as mentioned in 3.2.7.3. PCR reaction preparation, PCR cycle conditions and agarose gel analysis of PCR products were performed as described earlier (3.2.7.3.). All *V.alginolyticus* cultures yield 2 amplicons of sizes viz., 340bp and 663 bp. Other Vibrio cultures yield only a single amplicon of 663bp size.

3.2.7.7. PCR for detection of *V.vulnificus* (Tarr *et al.*, 2007)

This PCR uses species-specific primers that target the house keeping gene *hsp60* of *V.vulnificus*. As the house keeping genes are invariably present in all isolates this PCR helps in the detection of all *V. vulnificus* isolates. The sequence of the *hsp60* primers is mentioned in Table 3.1.5.4.

V.vulnificus isolates were grown on TSA plates. A single colony was scrapped from the plate's surface and suspended in 200µl of 1X Tris-EDTA, pH 8.0. The suspension was then heated at 95°C for 10 min and centrifuged for 2 min to pellet cellular debris. A typical 20ul reaction contained 1.5µl of crude lysate, 0.2µl of Taq polymerase (2U), 2µl of dNTP stock (2mM each), 2µl of 10X buffer, 1.2 µl of MgCl2 stock (25mM), 0.5µl of each *V.vulnificus* primer (10µM stock) and finally the volume was made up to 20µl with ddH_20. The thermal cycling profile was as follows: a 15 min soak at 93°C followed by 35 cycles : 92°C for 40sec, 57°C for 1 min, 72°C for 1.5 min; and a final soak at 72°C for 7 min. Agarose gel electrophoresis was carried out as described in 3.2.7.1. *V.vulnificus* cultures yield an amplicon of 410bp.

3.2.7.8. PCR for detection of *V.parahaemolyticus* (Tarr *et al.*, 2007)

This PCR uses species-specific primers that target the *flaE* sequence in the flagellin gene of *V.parahaemolyticus*. The sequence of the *flaE* primers is mentioned in Table 3.1.5.4.

V.parahaemolyticus isolates were grown on TSA plates. A single colony was scrapped from the plate's surface and suspended in 200µl of 1X Tris-EDTA, pH 8.0. The suspension was then heated at 95°C for 10 min and centrifuged for 2 min to pellet cellular debris. A typical 20ul reaction contained 1.5µl of crude lysate, 0.2µl of Taq polymerase (2U), 2µl of dNTP stock (2mM each), 2µl of 10X buffer, 1.2 µl of MgCl2 stock (25mM), 2µl of each *V.parahaemolyticus* primer (10µM stock) and finally the volume was made up to 20µl with ddH_20. The thermal cycling profile was as follows: a 15 min soak at 93°C followed by 35 cycles : 92°C for 40sec, 57°C for 1 min, 72°C for 1.5 min; and a final soak at 72°C for 7 min. Agarose gel electrophoresis was carried out as described in 3.2.7.1. *V.parahaemolyticus* cultures yield an amplicon of 897bp.

3.2.7.9. Multiplex PCR for the detection of pathogenic Vibrios viz., *V.cholerae*, *V.cholerae* (*ctx*), *V.alginolyticus*, *V.vulnificus* and *V.parahaemolyticus* (Pathogenic Vibrio-multiplex PCR)

A pathogenic Vibrio-multiplex PCR method was developed by utilizing *V.cholerae* species specific primers (Tarr *et al.*, 2007) and *V.cholerae ctxAB* genes specific primers (USFDA-BAM, 2001), *V.alginolyticus* specific *gyrB* primers (Zhou *et al.*, 2007), *V.vulnificus* specific *hsp60* primers (Tarr *et al.*, 2007) and *V.parahaemolyticus* specific *flaE* primers (Tarr *et al.*, 2007).

V.cholerae, cholera toxin producing *V.cholerae*, *V.vulnificus*, *V.alginolyticus* and *V.parahaemolyticus* were inoculated separately to tubes containing T_1N_1 broth and incubated at 37°C for 24h. 1ml from each T_1N_1 culture was transferred to 1.5ml microcentrifuge tubes and centrifuged at 10,000 rpm for 10min. The supernatant was carefully discarded and the cell pellets were resuspended in 100µl of Tris-EDTA (TE) buffer. The microcentrifuge tubes were placed in a dry bath at 95°C for 5 min. This crude lysate was used as template for PCR reaction immediately.

A typical 20µl reaction contained 1.2µl of crude lysate, 0.5µM each of *V.cholerae* species specific primers, 0.5µM each of *ctxAB* primers, 0.25µM each of *V.vulnificus* primers, 0.16µM each of *V.alginolyticus* primers and 1µM each of *V.parahaemolyticus* primers and 18µl of master reaction mix containing 10mM Tris-HCl, pH 8.3; 50mM KCl, 1.5mM MgCl2, 200µM each dATP, dCTP, dGTP and dTTP and 1U of *Taq* polymerase. The thermal cycling profile was as follows: initial denaturation of template DNA at 93°C for 15 min; 35 amplification cycles wherein each cycle consisted of denaturation at 92°C for 40sec, primer annealing at 57°C for 1 min and primer extension at 72°C for 1.5 min; final extension at 72°C for 7 min.

10µl of PCR product was mixed with 2µl of 6X gels loading buffer and loaded into sample wells of 2% Agarose gel submerged in 1X TAE containing 1µg/ml Ethidium bromide. One well was used for loading 100bp DNA ladder (Bangalore Genei, India). After appropriate migration with constant voltage of 100 volts for 30-45 min the agarose gel was visualized on a UV-transilluminator. Non cholera toxin producing *V.cholerae* cultures yield an amplicon of 248bp; *ctxAB* positive *V.cholerae* cultures yield two amplicons viz., 248bp and 777bp; *V.alginolyticus* cultures yield an amplicon of 340bp;

vulnificus cultures yield an amplicon of 410bp and *V.parahaemolyticus* cultures yield n amplicon of 897bp. The gel was either photographed with digital camera (Nikon SLR igital) and/or scanned using a gel documentation system (Alpha Imager, Alpha Innotech Corporation, USA).

3.2.7.9.1. Multiplex PCR for pathogenic Vibrios using unknown cultures.

Unidentified colonies (yellow) from TCBS agar plate was transferred to T_1N_1 broth and incubated at 37°C for 24h. This culture was used for PCR template preparation. Pathogenic Vibrio-multiplex PCR was performed as described in 3.2.7.9.

3.2.7.10. PCR fingerprinting of *V.cholerae* isolates

Protocol for extraction of genomic DNA from *V. cholerae* isolates was that of Ausubel et al (1987).1.5 ml of *V. cholerae* culture grown in T_1N_1 (37°C/24h) was transferred to microcentrifuge tube and centrifuged at 7500rpm (5850rcf) for 5 min. The supernatant was decanted and the tubes were drained well on blotting paper. The pellet was resuspended in 467µl of TE buffer (10mM Tris-Cl, 1mM EDTA, pH 8.0) by repeated pipetting. 30µl of 10% SDS and 3µl of Proteinase K (20mg/ml) were added, mixed and incubated at 37°C for 1h to lyse the cells. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) mix was added and mixed well by inverting the tube until the phases were completely mixed in order to purify the DNA from proteins and cellular debris. The tubes were centrifuged at 7500rpm for 5 min. The upper aqueous phase was transferred to a new tube and an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) mix was and mixed well. The tubes were centrifuge at 7500 rpm for 2 min. The upper aqueous phase was transferred to a new tube. 1/10 volume of 3M sodium acetate (pH 5.2) and 0.6 volumes of Isopropanol was added and mixed gently until the DNA precipitated. DNA was spooled onto glass rod and washed by dipping the end of rod into 1ml of 70% ethanol for 30 sec. The DNA was resuspended in 50µl of TE buffer and stored at -20°C.

Quantification of extracted DNA was done as follows:- The DNA concentration was measured by diluting 10µl of DNA into 1ml of TE (i.e. 1:100 dilution) and the OD measured at 260nm (UV-Visible Spectrophotometer, Cary 100 Bio, Varian). The purity

of the extracted DNA was examined by measuring the absorbance by at 260nm and 280nm. The ratio of OD 260 to OD 280 was recorded for the extracted DNA. A ratio of 1.8 indicates pure DNA whereas values higher than 1.8 denotes RNA contamination and values lower than 1.8 indicates protein contamination in the extracted DNA.

PCR fingerprinting of *V.cholerae* isolates using RS-PCR, REP-PCR and ERIC-PCR was done as given below:-

The primers used for RS-PCR, REP-PCR and ERIC-PCR were given in Table 3.1.5.5. PCR amplification was performed as per Wong and Lin (2001), with slight modifications. PCR reaction was performed with a buffer (50mM KCl, 1.5mM MgCl2, 10mM Tris-HCl(pH 8.8), 1% Triton-X 100) containing 200µM each dATP, dCTP, dGTP and dTTP, 3 U of Taq polymerase, 50pmol of primers and 100ng of template DNA in a final volume of 50µl. Amplification was performed in a thermal cycler (MJ Research, Minicycler)

PCR cycle conditions for RS-PCR were the following:-

Initial denaturation at 95°C for 7 min;

30 amplification cycles of denaturation at 90°C for 30sec, primer annealing at 55°C for 1 min and primer extension at 70°C for 3 min;

final extension at 70°C for 10 min.

PCR cycle conditions for REP-PCR and ERIC-PCR were:-

Initial denaturation at 95°C for 7 min;

30 amplification cycles of denaturation at 90°C for 30sec, primer annealing at 45°C for 1 min and primer extension at 65°C for 3 min;

final extension at 70°C for 10 min.

Agarose gel analysis of PCR products was done as follows:- 10µl of PCR product was mixed with 2µl of 6X gels loading buffer and loaded into sample wells of 2% Agarose gel submerged in 1X TAE containing 1µg/ml Ethidium bromide. One well was used for loading 100bp DNA ladder (Gene Ruler^{TM}, 100bp DNA ladder plus, Fermentas). After appropriate migration with constant voltage of 75 volts for 60min. The amplified

DNA bands were visualized in a UV transilluminator and scanned using a gel documentation system (Alpha Imager, Alpha Innotech Corporation, USA).

The electrophoretic patterns of the *V.cholerae* isolates obtained using RS-PCR, REP-PCR and ERIC-PCR were analysed using Gel Compar^{R} II software, version 4.0, 2005 (Applied Maths, Belgium). The software was used to construct dendograms, separately for each typing method, based on unweighted pair-group method with arithmetic means (UPGMA) for comparing the similarity among patterns.

3.2.7.11. Real time PCR for *V.cholerae*

Real time PCR for *V.cholerae* was performed using the corresponding sigma kit (SYBR green jump start Taq ready mix for quantitative PCR). Real time PCR was performed in 8 well strips in DNA Engine Gradient Cycler (MJ Research, PTC 200 Peltier thermal cycler) with Chromo 4, continuous fluorescence detector.

The sequences of the species specific primers used in Real Time PCR for *V.cholerae* were given in Table 3.1.5.6. The primers used in regular PCR were utilized for the Real Time PCR.

Real time PCR Reagent preparation:-

The PCR Mix was prepared by adding the following reagents as per the kit manufacturer's instructions.

SYBR Green Ready Mix	12.5µl
Dye (supplied with kit)	0.25µl
Forward Primer (0.1µMl)	0.6µl
Reverse primer (0.1µM)	0.6µl
DNA	10µl
Sterile MilliQ Water	1.05µl

Dedicated micropipettes and sterile filter barrier tips were used in dispensing the Real Time PCR reagents.

Real Time PCR amplification protocol was as given below:-

95°C for 2 min;

35 cycles of 94°C for 30sec, 57°C for 1min and 72°C for 1 min

Incubate 60°C for I min

Melting curve analysis was performed between 65°C to 95°C with the plate being read every 1°C.

Reaction was set up using *V.cholerae* DNA at different concentration (6 standards) viz., 105ng, 10ng, 1ng, 0.1ng, 0.01ng and 0.001ng in wells A1 to F1. An unknown sample was run in well G1.

CHAPTER · 4

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

Microbiological examination of post larvae and water samples from seven *Penaeus monodon* hatcheries located in Visakhapatnam district, Andhra Pradesh on the East coast of India and water, sediment and shrimp samples from five aquaculture farms located in West Godavari district, Andhra Pradesh, on the East Coast of India was carried out.

4.1. Estimation of abiotic factors in water samples from hatcheries and aquaculture farms.

The abiotic parameters of the water samples i.e., pH, temperature, dissolved oxygen (DO) and salinity were estimated. Salinity of shrimp hatchery waters ranged from 8.97 ppt to 28.8 ppt whereas in pond waters the salinity was lower ranging between 0.79 ppt to 2.0 ppt. There was no significant difference in the pH (8.0 – 8.5), dissolved oxygen (5.0-7.0ppm) and temperature (27-29°C) of hatchery and pond waters.

4.2. Bacteriological analysis of hatchery and farm samples

4.2.1. Quantitative analysis

The hatchery and aquaculture farm samples consisting of 7 hatchery water samples, 7 post-larvae samples, 5 pond water samples, 5 pond sediment samples, 5 shrimp head samples and 5 shrimp muscle samples were analysed for total bacterial loads, *E.coli* levels and total Vibrio loads.

4.2.1.1. Total Plate Counts (TPC) / (Aerobic Plate Count APC) of hatchery and farm samples

The total plate counts (TPC) of hatchery water, aquaculture pond water, pond bottom soil samples, post-larvae (PL) and shrimp samples are presented in Table 4.1.

Table 4.1. Total Plate Counts in *Penaeus monodon* hatcheries and aquaculture farms

Source	Mean Total Plate Counts
Hatchery water, cfu/ml	$5.6 \times 10^5 \pm 3890$* (1.6×10^3 to 1.2×10^4)**
Aquaculture Pond water, cfu/ml	$3.5 \times 10^3 \pm 790$ (2.6×10^3 to 4.4×10^3)
Aquaculture Pond Soil, cfu/g	$2.9 \times 10^5 \pm 1.4 \times 10^4$ (2.8×10^5 to 3.04×10^5)
Post Larvae from Hatcheries, cfu/g	$2.2 \times 10^6 \pm 1.9 \times 10^6$ (9.2×10^5 to $4.5\ 10^6$)
Shrimp Head, cfu/g	$4.78 \times 10^5 \pm 3.0 \times 10^4$ (4.4×10^5 to 5.12×10^5)
Shrimp Muscle, cfu/g	$2.7 \times 10^5 \pm 1.95 \times 10^4$ (2.4×10^5 to 2.94×10^5)

* mean ± SD

** value in parentheses indicates range

In aquaculture pond samples the mean TPC of pond sediment ($2.9 \times 10^5 \pm 1.4 \times 10^4$ cfu/g) was 2 logs higher than pond water ($3.5 \times 10^3 \pm 790$ cfu/ml). The TPC of pond sediments ranged from 2.8×10^5 to 3.04×10^5 cfu/g while the TPC of pond water ranged from 2.6×10^3 to 4.4×10^3 cfu/ml in shrimp aquaculture farms. The TPC results obtained in this study were similar to the values obtained by Otta et al (1999). They reported that total bacterial count ranged from 10^3-10^5 cfu /ml in shrimp culture ponds, growing *P.monodon*. Sharmila et al (1996) reported that bacterial loads ranged from1.8×10^3 to 4.5×10^3 cfu/ml in rearing water and from 1.82×10^6 to 4.72×10^6 cfu/g in sediment of *Penaeus indicus* culture ponds. Dalmin et al (2002) observed that sediments had more bacterial load than water and shrimps. Similarly, Li et al (2002) reported that the bacterial counts in sediment were 10 to 20 times higher than those in water column. The higher

bacterial loads in pond sediments obtained in this study could be attributed to the accumulation of organic matter at the pond bottom which stimulated bacterial growth.

Shrimp head had relatively higher bacterial load ($4.78 \times 10^5 \pm 3.0 \times 10^4$ cfu/g) when compared to shrimp muscle ($2.7 \times 10^5 \pm 1.95 \times 10^4$ cfu/g). The TPC of shrimp head ranged from 4.4×10^5 to 5.12×10^5 cfu/g and in shrimp muscle the TPC values ranged between 2.4×10^5 to 2.94×10^5 cfu/g.

In shrimp hatchery samples, the post-larvae ($2.2 \times 10^6 \pm 1.9 \times 10^6$ cfu/g) had higher bacterial load than water ($5.6 \times 10^3 \pm 3890$ cfu/ml). The TPC value of post-larvae ranged between 9.2×10^5 and $4.5\ 10^6$ cfu/g whereas the TPC of hatchery waters ranged from1.6×10^3 to 1.2×10^4 cfu/ml. The TPC of hatchery waters obtained in this study were lower than the values reported by Otta et al (2001), which might be due to the better water management systems adopted by the shrimp hatcheries. Otta et al (2001) reported that the total bacterial counts ranged from and 10^4 to 10^6/ml in larval tanks of *P. monodon*, hatcheries in India. Abraham and Palaniappan (2004) noticed a gradual but significant increase in the mean total viable counts (TVC) from eggs (log 4.92 ± 0.16/g) to post-larvae (log 7.00 ± 0.55 /g). In early stages, the total bacterial (cfu) number was maintained at low level but in mid stages, cfu number increased suddenly to a peak 10^6 cfu/g which appeared on stages of Zoea II and Mysis (Liu *et al.*, 1994). In this study post-larvae samples were used for analysis had a mean bacterial load of $2.2 \times 10^6 \pm 1.9 \times 10^6$ cfu/g and the TPC values were also in the similar range of 10^6 cfu/g as reported by other workers.

4.2.1.2. *Escherichia coli* (*E.coli*) in *P. monodon* hatcheries and aquaculture farms

The presence of *E.coli* indicates faecal contamination. The result with respect to *E.coli* in shrimp aquaculture samples is given in Table 4.2. The mean *E.coli* counts were higher in aquaculture pond sediment (204±133 cfu/g) and pond water (124±88 cfu/ml). Relatively lower *E.coli* counts were obtained from shrimp samples (12±11 to 16±16.7 cfu/g). The presence of *E.coli* in aquaculture environment might have been from the source water. *E.coli* was not detected in hatchery waters and post-larvae.

Table 4.2. *E.coli* counts in *P. monodon* hatchery and aquaculture samples

Source	Mean *E.coli* counts
Hatchery water, cfu/ml	Not detected
Aquaculture Pond water, cfu/ml	123.6 ± 87.8* (48 to 260)**
Aquaculture Pond Soil, cfu/g	204 ± 132.96 (140 to 440)
Post Larvae from Hatcheries, cfu/g	Not detected
Shrimp Head, cfu/g	16 ± 16.7 (0 to 40)
Shrimp Muscle, cfu/g	12 ± 10.95 (0 to 20)

* mean ± SD

** value in parentheses indicates range

E.coli was not detected in hatchery water and post-larvae samples. Higher mean *E.coli* counts were obtained from pond sediment (204± 132.9) and pond water 123.6 ± 87.8) samples. The *E.coli* counts ranged from 140 to 440 cfu/g in pond sediment and from 48 to 260 cfu/ml in pond water samples. *E.coli* was detected in shrimp in three shrimp head and three shrimp muscle samples but the counts were lower. *E.coli* in shrimp head samples ranged between 0 and 40 cfu/g and in shrimp muscle it ranged between 0 and 20 cfu/g.

4.2.1.3. Total Vibrio Counts (TVC) in *P. monodon* hatcheries and aquaculture farms

TCBS agar of Kobayashi et al (1963) is widely used for detection and isolation of all the enteropathogenic Vibrios. TCBS agar was used in this study also (Fig. 4.1). The data

on the total vibrios from *P. monodon* hatcheries and aquaculture farms are presented in Table 4.3.

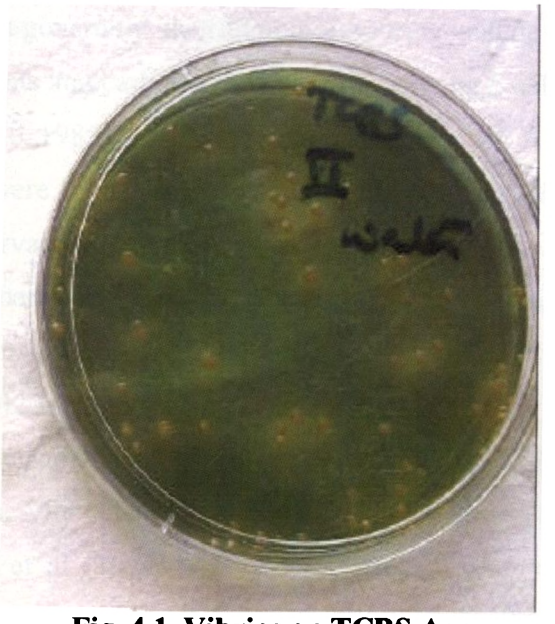


Fig. 4.1. Vibrios on TCBS Agar

Table 4.3. Mean Vibrio loads in *P. monodon* hatcheries and Aquaculture farms

Source	Total Vibrio Load
Hatchery water, cfu/ml	$2.4 \times 10^3 \pm 2.2 \times 10^3$ * (6.6×10^2 to 2.8×10^3)**
Aquaculture Pond water, cfu/ml	$1.5 \times 10^2 \pm 42$ (80 to 2×10^2)
Aquaculture Pond soil, cfu/g	$1.5 \times 10^3 \pm 6.4 \times 10^2$ (8×102 to 2.2×10^3)
Post larvae from hatcheries, cfu/g	$2.1 \times 10^5 \pm 1.1 \times 10^5$ (7.6×10^4 to 3.68×10^5)
Shrimp Head, cfu/g	$3.5 \times 10^4 \pm 2.2 \times 10^4$ (1.72×10^4 to 7.02×10^4)
Shrimp muscle, cfu/g	$1.4 \times 10^4 \pm 1.42 \times 10^4$ (1.84×10^3 to 3.6×10^4)

* mean ± SD

** value in parentheses indicates range

of ERIC-PCR techniques to environmental samples may aid in understanding the molecular ecology of the cholera agent and related enteric pathogens in the environment.

2.9.2. REP-PCR (Repetitive Extragenic Palindromes)

Another repetitive element from *V.cholerae* is discovered from the nucleotide sequence of a 6.3-kb *Bam*HI fragment of the chromosome (Barker *et al.*, 1994). This region contains nine copies of a 124-bp direct repeat that are shown by Southern hybridization to occur at least 60 to 100 times in the *V.cholerae* O1 chromosome. The authors named these repeats VCR for *V.cholerae* repetitive DNA sequence. The REP-PCR method is based on the presence of 38-bp REPs in *Enterobacteriaceae* and other bacteria and has been applied to many species (Stern *et al.*, 1984; Rodriguez *et al.*, 1995; Marshall *et al.*, 1999; Stubbs *et al.*, 1999).

Shangkuan et al (1997) studied the diversity of DNA sequences among *V.cholerae* O1 and non-O1 isolates detected by whole-cell repetitive element sequence based polymerase chain reaction (rep-PCR) and noticed that the PCR fingerprinting profiles of toxigenic O1 isolates were not only homogenous with primers from ERIC sequences but also allowed the differentiation from non-toxigenic O1 and non O1 strains. The results indicated that rep-PCR can be used to identify and differentiate different toxigenic O1, non-toxigenic O1 and non-O1 *V.cholerae* isolates. Highly polymorphic fingerprints were obtained from non-toxigenic El Tor strains. Six types from non-toxigenic El Tor strains were isolated from seafood and an environmental source in Taiwan. Sixteen different rep-PCR types were present from 24 non-O1 *V.cholerae* strains isolated from human and seafood from Taiwan. Four of the 24 strains (16.7%) belonged to one type which was the most common type. *V.cholerae* non-O1 strains were diverse. Since non O1 isolates represented *V.cholerae* O2 through O138, a diverse set of fingerprint types would expectantly result from the strains used. The capability to generate simple and reproducible genomic fingerprints which vary within and between *V.cholerae* serogroups and biotypes indicates that rep-PCR may have applications in epidemiological analysis and in examination of genotypic diversity of *V.cholerae* strains. A substantial similarity among the rep-PCR fingerprints of many toxigenic *V.cholerae* O1 isolates suggest that limited genotypic diversity exists within that toxigenic serogroup.

The factors that govern the distribution of Vibrios include human, animal or plant hosts, inorganic nutrients and carbon availability, temperature, salinity, dissolved oxygen (Simidu and Tsukamoto, 1985).

Vibrio loads were higher in *P. monodon* hatchery samples than in aquaculture pond samples. Post-larvae had maximum loads of Vibrios ($2.1 \times 10^5 \pm 1.1 \times 10^5$ cfu/g) with counts ranging between $2.1 \times 10^5 \pm 1.1 \times 10^5$ cfu/g. Shrimp head portion had relatively higher counts of Vibrios ($3.5 \times 10^4 \pm 2.2 \times 10^4$ cfu/g) than shrimp muscle portion ($1.4 \times 10^4 \pm 1.42 \times 10^4$cfu/g).

Hatchery water had higher Vibrio loads (2400± 2200 cfu/ml) than pond water (150 ± 42 cfu/ml). The vibrio loads in pond water were within the range of previously reported values. Otta et al (1999) studied the bacterial flora associated with shrimp culture ponds growing *P. monodon* in India and reported that Vibrio count ranged from 10^1-10^4 cfu/ml. The higher counts of vibrios in shrimp hatchery water samples were reported earlier. Otta et al (2001) observed that in the larval tanks, the proportion of Vibrio species ranged from 50% to 73%, as compared to 31% in raw sea water and that a mixed bacterial flora was observed in hatchery water but in larval tanks, the flora in the larvae was predominantly made up of Vibrio species. Liu et al (1994) observed that in giant tiger prawn (*Penaeus monodon*) hatchery, at prior stages the major bacterial flora were Gram positives but after Zoea III stage, the Gram negative bacteria become the main flora, of which the *Vibrio* (68%) were dominant species.

In the present study the mean TVC of pond sediment (5 x 103 cfu/g) was 10 times higher than the mean TVC of pond water (1.5 x 102 cfu/ml) which was similar to previous studies. Li et al (2002) reported that the number of Vibrio in sediment surface were 10 to 20 times higher than those in water column. Su et al (1994) observed that vibrio numbers in mud was 5 to 160 times higher than that in pond water. 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.

In the present study a negative correlation was observed between total vibrio counts and *E.coli* (r = -0.54) in the shrimp culture system which is in accordance with previous reports that state that the counts of vibrios were either negatively correlated or showed no correlation with counts of indicator bacteria (*Escherichia coli*, *Enterococci*,

fecal coliforms, and total coliforms) (Koh *et al.*, 1994). *V. cholerae* presence is not correlated with the commonly used coliforms as fecal indicators (Hood and Ness, 1982; Kaper *et al.*, 1979). Surendran et al (2000) studied the comparative microbial ecology of fresh water and brackish water prawn farms and observed indicator bacteria like total coliforms and faecal coliforms were high in number in the fresh water farm. A poor correlation between the level of faecal indicator organisms and the incidence of Vibrios indicate that these bacteria are a part of the natural microflora of the shrimp culture environment.

The relative occurrence of sucrose fermenting and non-fermenting vibrios in different samples is given in Table 4.4. The same is presented in graphical form in Fig 4.2 as well, for ease of comparison. It can be seen from the table that sucrose non-fermenting vibrios were higher in shrimp head portion (59%) and hatchery waters (49%) whereas more than 90% of the vibrios in post-larvae, pond water, pond sediment and shrimp muscle portions were sucrose fermenters (Fig 4.2).

Sucrose is a disaccharide and *Vibrio species* differ in their ability to ferment this sugar. The sucrose fermenters appear as yellow colonies on TCBS agar and sucrose non-fermenters appear as green colonies. According to the Bergey's manual of Systematic Bacteriology (2005) the percentage of isolates within each *Vibrio species* that can ferment sucrose were as follows: *V. cholerae* (100%), *V. alginolyticus* (100%), *V. cincinnatiensis* (100%) *V. anguillarum* (100%), *V. metschnikovii* (100%), *V. fluvialis* (100%), *V. furnissi* (100%), *V. harveyi* (83%), *V. vulnificus* (20%), *V. damsela* (20%), *V. hollisae* (0%), *V. mimicus* (0%), *V. parahaemolyticus* (0%), *V. splendidus* biogroup 2 (0%), *V. fischeri* (0%), *V. penaeicida* (0%) and *V. proteolyticus* (0%).

Table 4.4. Relative occurrence of sucrose fermenting and non-fermenting Vibrios in *P.monodon* hatchery and aquaculture samples

Source	Sucrose Fermenting Vibrios	Sucrose non-fermenting Vibrios
Hatchery water (n=7)	49% 1152 ± 953 cfu/ml* (297 to 3080 cfu/ml)**	51% 1248 ± 1230 cfu/ml (363 ± 3920 cfu/g)
Aquaculture Pond water (n=5)	97% 144 ± 39 cfu/ml (79 to 182 cfu/ml)	3% 5 ± 7.4 cfu/ml (0 to 18 cfu/ml)
Aquaculture Pond Soil (n=5)	100% $1.5 \times 10^3 \pm 639$ (800 to 2200 cfu/g)	0%
Post Larvae from Hatcheries (n=7)	97% $2 \times 10^5 \pm 1.1 \times 10^5$ cfu/g (7.3×10^4 to 3.6×10^5 cfu/g)	3% $7.4 \times 10^3 \pm 5.9 \times 10^3$ cfu/g (8.8×10^2 to 1.7×10^4 cfu/g)
Shrimp head (n=5)	41% $1.5 \times 10^4 \pm 8.5 \times 10^3$ cfu/g (6.5×10^3 to 2.7×10^4 cfu/g)	59% $2.1 \times 10^4 \pm 1.3 \times 10^4$ cfu/g (1.1×10^4 to 4.3×10^4 cfu/g)
Shrimp Muscle (n=5)	90% $1.3 \times 10^4 \pm 1.4 \times 10^4$ cfu/g (1.7×10^3 to 3×10^4 cfu/g)	10% $1.5 \times 10^3 \pm 2.4 \times 10^3$ 1.1×10^2 to 5.8×10^3 cfu/g

* mean value ± SD

** value in parentheses indicates range

In the present study a higher percentage of sucrose non-fermenters were observed in the shrimp hatchery waters (51%) but not in shrimp post larvae (3%). Generally, lower counts of sucrose non-fermenters indicate healthy post-larvae because most of the shrimp pathogenic vibrios such as *V. vulnificus*, *V. parahaemolyticus*, *V. splendidus*, *V. fischeri*, *V. penaeicida*, *V. proteolyticus* are sucrose non-fermenters. 17% of the strains of *V.harveyi* are sucrose non-fermenters. The higher proportion of sucrose non-fermenters in

the head of shrimp obtained in this study (Fig. 4.2) were in accordance with earlier studies. Gomez-Gil et al (1998) reported that the *Vibrio spp.* isolated from the digestive tract of a population of healthy juvenile *L.vannamei* consisted of both sucrose and non-sucrose fermenters whereas the haemolymph contained only non-sucrose fermenters. Vibrio loads in haemolymph of diseased *P. monodon* shrimps varied from 0.7 x 10^2 to 5.8 x 10^5 cfu/ml (Jayasree *et al.*, 2001) and 5.6 x10^2 -1.1 x 10^4 cfu/ml (Janakiram *et al.*, 2001).

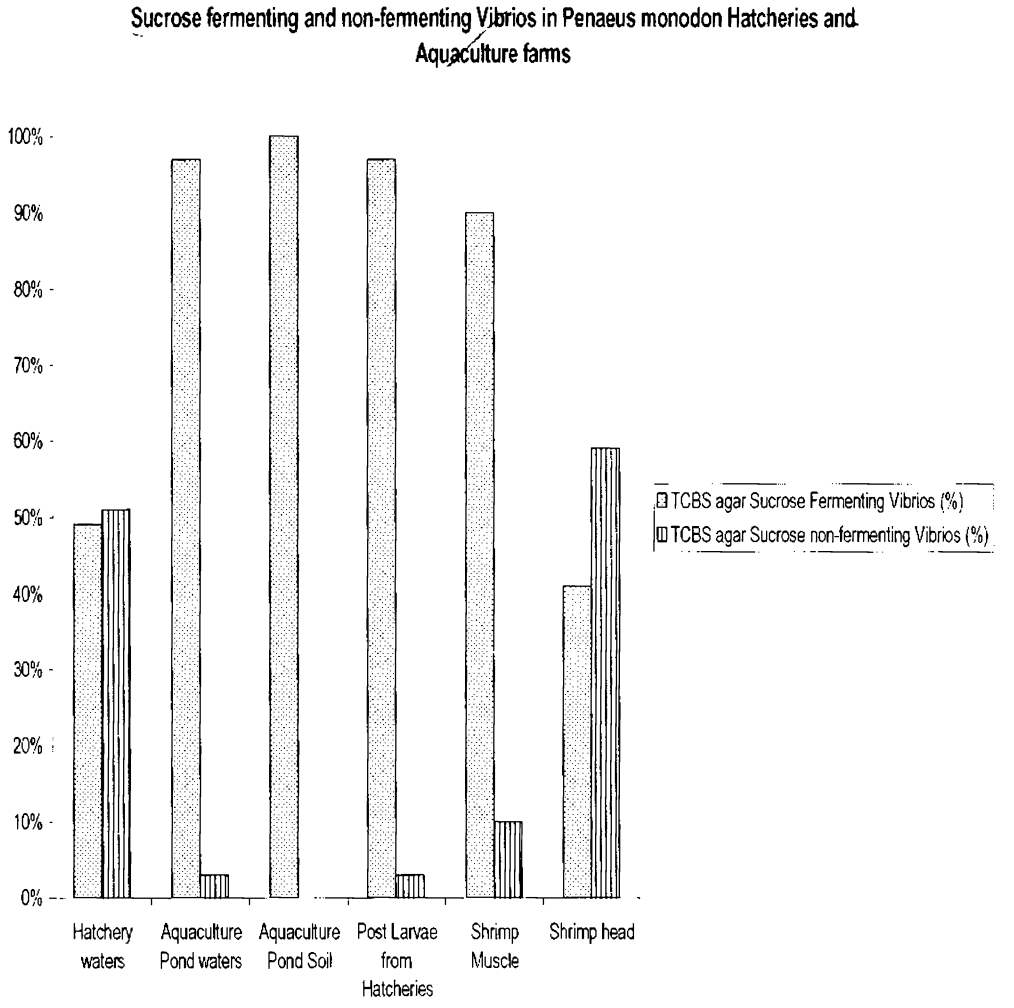


Fig. 4.2. Sucrose fermenting and Non-fermenting Vibrios in hatchery and aquaculture samples

4.2.2. Qualitative analysis of Vibrios isolated from shrimp hatcheries and aquaculture farms.

The vibrio cultures isolated from the aquaculture and hatchery samples were qualitatively investigated as described in material and methods (3.2.2.2.). The cultures confirmed as Vibrios (3.2.2.1.4.) were identified to the species level initially by using the schemes of Alsina and Blanch (1994) (3.2.2.2.2.) and Noguerola and Blanch (2008) (3.2.2.2.3.). Finally, all the pathogenic Vibrios were confirmed as per the biochemical reactions described in Bergey's manual of Systematic Bacteriology (2005).

4.2.2.1. Identification of pathogenic *Vibrio spp.* isolated from shrimp hatcheries and aquaculture farms.

Bergey's manual of Systematic Bacteriology (2005) lists 44 species under the genus Vibrio of which 12 are pathogenic to humans viz., *V.cholerae*, *V.vulnificus*, *V.parahaemolyticus*, *V.furnissi*, *V.metschnikovii*, *V.cincinnatiensis*, *V.alginolyticus* , *V.mimicus*, *V.fluvialis*, *V.hollisae*, *V.damsela and V.harveyi*. Vibrios considered pathogenic to shrimps include *V.harveyi*, *V.alginolyticus*, *V.parahaemolyticus*, *V.vulnificus*, *V.proteolyticus*, *V.fischeri*, *V.anguillarum* and *V.splendidus*. Vibrios related to post harvest shrimp quality are mainly *V.cholerae*, *V.parahaemolyticus* and *V.vulnificus*.

4.2.2.1.1. Incidence of pathogenic Vibrios in shrimp hatcheries

A total of 210 Vibrio cultures isolated and purified from the water (105 Vibrio cultures) and post-larvae samples (105 Vibrio cultures), were screened for the presence of pathogenic *Vibrio spp* based on their biochemical reactions and the results given in Table 4.5.

Table 4.5. Incidence of pathogenic Vibrios in *P.monodon* hatcheries

Pathogenic *Vibrio spp.*	Incidence of pathogenic Vibrios	
	Number of Cultures	Percentage *
V.alginolyticus	51	24.3%
V.vulnificus	19	9.1%
V.parahaemolyticus	18	8.6%
V.harveyi	8	3.8%
V.cholerae	0	0%
Other *Vibrio spp.*	114	54.2%

* Values as percentage of the total Vibrios isolated from shrimp hatcheries (210 isolates)

The incidence of total pathogenic Vibrios in hatchery samples was 45.8%, of the total 210 vibrio cultures studied. *V.alginolyticus* (24.3%) was the most common pathogenic *Vibrio spp* detected in hatchery samples followed by *V. vulnificus* (9.1%), *V. parahaemolyticus* (8.6%) and *V. harveyi* (3.8%). The pathogenic Vibrios were most commonly encountered in the water samples (34.76%) than the post-larvae samples (10.95%). The relative incidence of pathogenic *Vibrio spp* in hatchery water and post-larvae samples is given in Table 4.6.

Table 4.6. The incidence of pathogenic Vibrios in hatchery water and post-larvae

Pathogenic *Vibrio spp.*	Hatchery water	Post-larvae
V.alginolyticus (n=51)	17%* (n=36)**	7.1% (n=15)
V.vulnificus (n=19)	6.7% (n=14)	2.4% (n=5)
V.parahaemolyticus (n=18)	7.1% (n=15)	1.4% (n=3)
V.harveyi (n=8)	3.8% (n=8)	0% (n=0)

* Values as percentage of the total Vibrios isolated from shrimp hatcheries (210 isolates)

** Value in parentheses indicates number of positive cultures

V. alginolyticus was the most dominant pathogenic *Vibrio spp* in hatchery water (17%) and post-larvae samples (7.1%). *V. parahaemolyticus* (7.1%) was slightly higher than *V. vulnificus* (6.7%) in hatchery water samples, whereas *V. vulnificus* (2.4%) was slightly higher than *V. parahaemolyticus* (1.4%) in post-larvae. *V.harveyi* was detected in hatchery waters (3.85%) but not in post-larvae.

The luminous vibrios *V.harveyi*, *V.fischeri* and *V.splendidus* are important shrimp pathogens. The primary source of *V.harveyi* in a shrimp hatchery was the faecal matter from brood stock, possibly at the time of spawning. (Abraham and Palaniappan, 2004). The mean luminous bacterial counts showed an increase from eggs (log 2.00/g) to mysis (log 5.34 ± 0.93 /g) and decreased thereafter (log 4.54 ±1.22/g post-larvae). Vibrio-like-bacteria in Artemia nauplii was found to be the possible source of these pathogenic bacteria (*V.harveyi*) in the hatchery environments (Vaseeharan and Ramasamy, 2003). Jayaprakash et al (2006) studied the Vibrios associated with *M.rosenbergii* larvae 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. *V.alginolyticus* and *V.harveyi* were isolated from shrimp-farm water, sediment, shrimp larvae and hatchery water samples (Kumar *et al.*, 2007).

The lower incidence of *V.harveyi* in hatchery samples in the present study can be attributed to better hatchery water quality management and the size of the post-larvae samples used for analysis. The post-larvae size was above PL-15 and healthy and had attained the marketable size for shrimp culture.

4.2.2.1.2. Incidence of pathogenic Vibrios in aquaculture farms

A total of 250 Vibrio cultures isolated and purified from the farm water (75 Vibrio cultures), farm sediment (75 Vibrio cultures) and farmed shrimp (100 Vibrio cultures) were screened for the presence of pathogenic Vibrio spp based on their biochemical reactions.

The relative incidence of total pathogenic *Vibrio spp* in pond sediment, pond water and shrimp samples is given in Table 4.7. The pathogenic Vibrios were most commonly encountered in pond sediment (21.6%) followed by pond water (20%) and shrimp (13.6%). Within the shrimp, the incidence of pathogenic Vibrios was relatively higher in the shrimp head portion (8.4%) than in the shrimp muscle (5.2%) portion.

Table 4.7. The incidence of total pathogenic Vibrios in pond water, sediment and Shrimp

Source	Total Pathogenic Vibrios
Pond Water	20%
Pond Sediment	21.6%
Shrimp (whole)	13.6%
Shrimp head	8.4%
Shrimp muscle	5.2%

The incidence of total pathogenic Vibrios in aquaculture samples is given in Table 4.8. The pathogenic vibrios formed 55.2% of the total 250 vibrio cultures studied. *V.alginolyticus* (38.4%) was the most common pathogenic *Vibrio spp* detected in aquaculture samples followed by *V. cholerae* (16.8%). Other pathogenic *Vibrio spp* that were detected in hatchery samples viz., *V.parahaemolyticus, V.vulnificus* and *V.harveyi* were not detected in aquaculture samples.

Table 4.8. Incidence of pathogenic *Vibrio spp* in aquaculture farms

Pathogenic Vibrio spp.	Percentage Incidence
V.alginolyticus (n=96)**	38.4%*
V.cholerae (n=42)	16.8%
V.vulnificus (n=0)	0%
V.parahaemolyticus (n=0)	0%
V.harveyi (n=0)	0%
Other non-pathogenic group of *Vibrio spp.* (n=112)	44.8%

* Values as percentage of the total Vibrios isolated from shrimp aquaculture farms (250 isolates)

** Value in parentheses indicates number of positive cultures

The relative distribution of *V.alginolyticus* and *V.cholerae* within the black tiger shrimp aquaculture system is presented in Table 4.9. *V.alginolyticus* was the most predominant pathogenic *Vibrio spp* in pond sediment (16.4%), pond water (12.4%) and shrimp samples (9.6%). *V.cholerae* incidence was higher in pond water (7.6%) than in pond sediment (5.2%) and shrimp (4%). Shrimp head portion had relatively higher incidence of *V.alginolyticus* (6%) and *V.cholerae* (3.6%) when compared to shrimp muscle where the incidence levels were 2.4% and 1.6%, respectively.

Table 4.9. The distribution of pathogenic *Vibrios spp* in pond water, sediment and shrimp

Source	*V.alginolyticus*	*V.cholerae*
Pond Water	12.4% (n=31)	7.6% (n=19)
Pond Sediment	16.4% (n=41)	5.2% (n=13)
Shrimp (whole)	9.6% (n=24)	4.0% (n=10)
Shrimp head	6% (n=15)	2.4% (n=6)
Shrimp muscle	3.6% (n=9)	1.6% (n=4)

The distribution of *V.alginolyticus* in the shrimp aquaculture system is represented as Fig. 4.3. A total of 96 isolates of *V.alginolyticus* were obtained from aquaculture samples of which 42.7% were isolated from culture pond sediment, 32.3% from pond water, 15.6% from shrimp head portion and 9.4% from shrimp muscle (Table 4.9). It indicated that farm sediment had a higher presence of *V.alginolyticus* compared with their presence in farm water. But the incidence of *V.alginolyticus* had been relatively very low in the farmed shrimp. In the farmed shrimp itself, the head region had a higher percentage of *V.alginolyticus* (15.6%) than the shrimp muscle (9.4%).

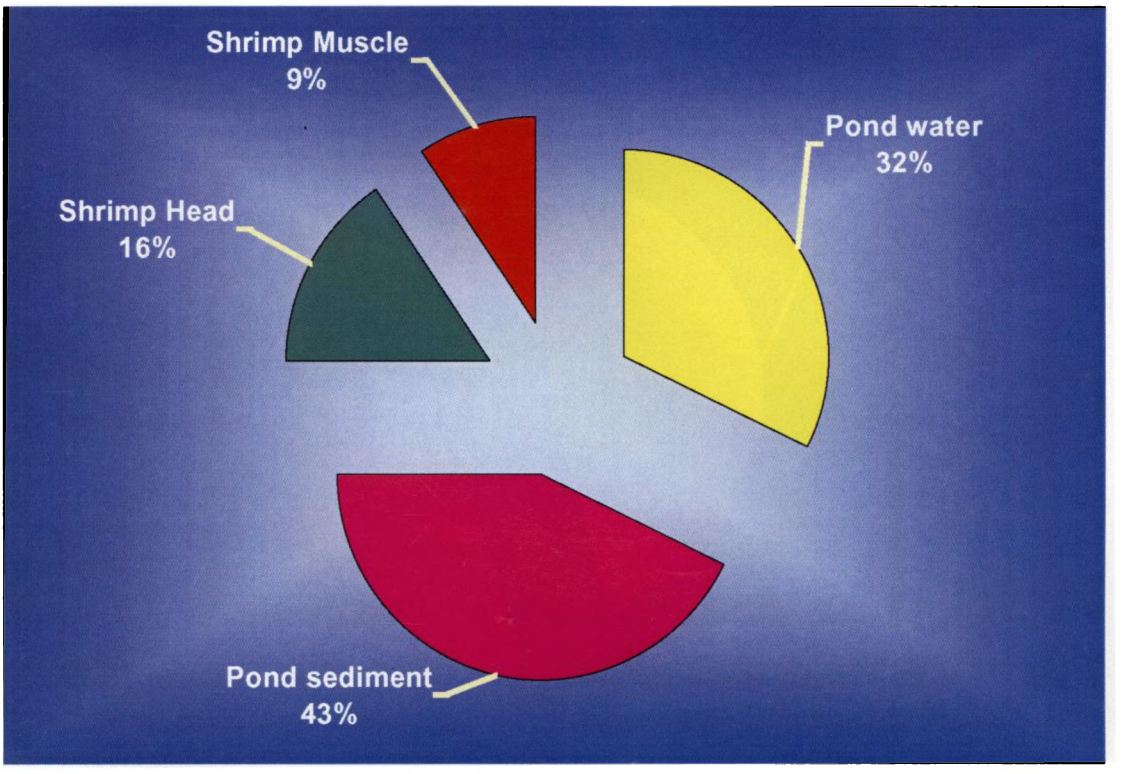


Fig. 4.3. Distribution of *V.alginolyticus* in *P.monodon* Aquaculture farms

The distribution of *V.cholerae* in *P.monodon* aquaculture system is represented as Fig. 4.4. A total of 42 isolates of *V.cholerae* were obtained from aquaculture samples. Of these, 45.2% were isolated from pond water, 31% from pond sediment, 14.3% from shrimp head portion and 9.5% from shrimp muscle. It indicated that shrimp farm water had a higher presence of *V.cholerae* compared with their presence in farm sediment. But the incidence of *V.cholerae* had been relatively very low in the farmed shrimp. In the farmed shrimp itself, the head region had a higher percentage of *V.cholerae* (14.3%) than the shrimp muscle (9.5%).

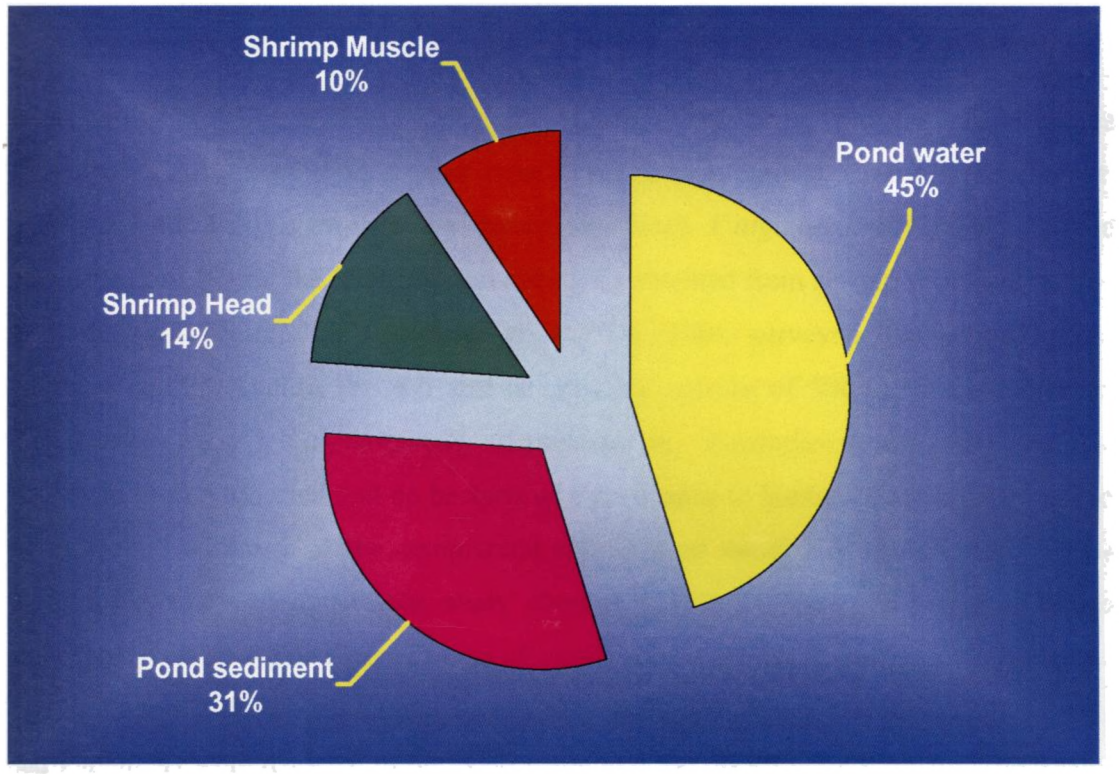


Fig. 4.4. Distribution of *V.cholerae* in *P.monodon* Aquaculture farms

The incidence of total pathogenic Vibrios in aquaculture samples was 55.2% which was higher than that of hatchery samples (45.8%) but the diversity of Vibrios was higher in hatchery environment than in aquaculture farms. Species wise, *V. alginolyticus* was the most commonly encountered pathogenic *Vibrio species* in hatchery (24.3%) as well as farm (38.4%) samples. *V.parahaemolyticus*, *V.vulnificus* and *V.harveyi* were found only in hatchery samples and while they were totally absent in the farm samples. Similarly *V.cholerae* was not detected in hatchery samples but it was found in significant numbers in farm samples. The pathogenic Vibrio species isolated in this study from shrimp farms were similar to the previously reported results.

Vibrio species were the largest group in all the *P.monodon* ponds in India (Otta *et al.*, 1999). *V.alginolyticus* accounted for 5.2-25% of the flora in various farms. O1 serotype *V.cholerae* was absent and Non O1 serotype *V.cholerae* and *V.vulnificus* were present in a few farms. Bhaskar and Setty (1994; 1998) noticed that *V.alginolyticus* was the most common *Vibrio species* (57% incidence) in the farming phase of tiger shrimp

(*P.monodon*). *V.cholerae*, *V.parahaemolyticus* and *V.vulnificus* were the other species encountered. All these *Vibrio species* were found to be present in shrimp and sediment, pond water and feed (clam meat and formulated feed). *V.alginolyticus* (28.8%) was the dominant flora among the Vibrio isolates (n=278) obtained from shrimp farming systems of Tamil Nadu (Felix, 2000). Jayasree et al (2001; 2006) surveyed *P.monodon* culture ponds of coastal Andhra Pradesh and isolated six species of Vibrios viz., *V.harveyi*, *V.parahaemolyticus*, *V.alginolyticus*, *V.anguillarum*, *V.vulnificus*, and *V.splendidus*. Raghavan (2003) reported that no bacteria of significance to human health were found to be associated with any of the commercial shrimp 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*, *E.coli* and *Staphylococcus aureus*. 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 (Ni *et al.*, 1995). Dalsgaard et al (1995) isolated 143 *V. cholerae* non O1 isolates from shrimp farms in Thailand.

V.alginolyticus (81.48%), *V.parahaemolyticus* (14.8%) and *V.cholerae* non O:1 (3.7%) were isolated from widely consumed fresh seafood products (Baffone et al., 2000). Total presumptive *V.parahaemolyticus* count was above the limit in 71% of the samples comprising of shellfish, finfish, and cephalopods collected from various fish markets in and around Cochin (Rekha *et al.*, 2008).

The interaction of virus and *V.alginolyticus* in the earlier stage of virus disease of *P.chinensis* showed that insidious infection of vibrio is advantageous to the infection of virus (Ding *et al.*, 2000). White shrimp *Litopenaeus vannamei* transferred from 25 ppt to low salinity levels (5 and 15 ppt) had reduced immune ability and decreased resistance against *V. alginolyticus* infection (Wang and Chen, 2005). The present study reports that *V.alginolyticus* was the most commonly encountered pathogenic *Vibrio species* in hatchery (24.3%) as well as farm (38.4%) samples. Recently, the shrimp farmers have begun culturing *L.vannamei* in India. The role of *V.alginolyticus* as a predisposing factor for viral disease assumes greater significance as it is the most predominant vibrio in the shrimp culture system.

4.2.3. Slide agglutination tests for identification of *V.cholerae* O1 and *V.cholerae* O139 and *V.cholerae* Non O1 and Non O139

The 42 *V.cholerae* isolates obtained from shrimp aquaculture system (19 isolates from pond water; 13 isolates from pond sediment, 6 isolates from shrimp head and 4 isolates from shrimp muscle) were initially tested with *V.cholerae* polyvalent somatic O antiserum. All the isolates gave negative reaction with polyvalent somatic O antiserum and were grouped as *V.cholerae* Non O1. These Non O1 cultures were further tested for agglutination using *V.cholerae* O139 antiserum. All the 42 *V.cholerae* cultures gave negative agglutination reaction with *V.cholerae* O139 antiserum.

All the 42 *V.cholerae* isolates obtained from shrimp aquaculture pond water, sediment, shrimp head and muscle culture system were grouped as *V.cholerae* Non O1 and Non O139 as they yielded negative agglutination results with polyvalent somatic O antiserum and O139 antiserum. This observation concurs with the earlier reports of Non O1 and Non O139 *V.cholerae* incidence in shrimp and environment. *V.cholerae* non O1 strains are far more frequently isolated from the environmental sources than O1 strains and appear to constitute part of the microflora of prawns (Nair *et al.*, 1991) and oysters (Baine *et al.*, 1974; Blake *et al.*, 1977; McIntyre *et al.*, 1979; Salamaso *et al.*, 1980). Non O1/non O139 *V.cholerae* was isolated from shrimp farms in Thailand (Dalsgraad *et al.*, 1995), squid and cuttle fish from India (Bhaskar *et al.*, 1998); cooked frozen shrimp products originating from aquaculture produced shrimp (Dalsgaard *et al.*, 1996).

4.2.4. Biochemical characterization of Vibrios isolated from aquaculture farms and hatcheries.

Four isolates of each pathogenic Vibrio isolated from aquaculture farm and / or shrimp hatchery samples were selected for the biochemical studies. The details of the cultures were given in Table 4.10.

Table 4.10. Pathogenic Vibrio cultures isolated from aquaculture farms and hatcheries used for biochemical characterization studies

Organism	Name	Source
V.cholerae	VC1	Pond Water
	VC2	Pond Sediment
	VC3	Shrimp Head
	VC4	Shrimp Muscle
V.cholerae ctx	VC ctx1	Pond Water
	VC ctx2	Pond Water
	VCctx3	Pond Water
	VCctx4	MTCC 3906
V.alginolyticus	VA1	Hatchery Water
	VA2	Post larvae
	VA3	Pond Water
	VA4	Shrimp Muscle
V parahaemolyticus	VP1	Hatchery Water
	VP2	Post larvae
	VP3	Hatchery Water
	VP4	Post larvae
V.harveyi	VH1	Hatchery Water
	VH2	Hatchery Water
	VH3	Hatchery Water
	VH4	Hatchery Water
V.vulnificus	VV1	Hatchery Water
	VV2	Post larvae
	VV3	Hatchery Water
	VV4	Post larvae

4.2.4.1. Biochemical reactions of pathogenic Vibrios isolated from shrimp hatcheries and farms used for biochemical studies

The *Vibrio spp* identified as per the above mentioned scheme (Noguerola and Blanch 2008) were confirmed by carrying out the biochemical reactions given in Bergey's manual of Systematic Bacteriology (2005) for the different *Vibrio spp* and as per the procedures described in Materials and Methods section.

The strains were purified and stocks were grown on T1N1 agar (1% Tryptone, 1% NaCl, 0.5% agar) for identification (Venkateswaran *et al.*, 1989). Janda et al (1988) suggested that media commonly used for identification of members of the *Enterobacteriaceae* can be used for Vibrios, if the NaCl concentration is increased to 1% (wt/vol)).

4.2.4.1.1. Biochemical reactions of *V.cholerae* cultures isolated from aquaculture farms

Table 4.11. Biochemical reactions of *V.cholerae* isolated from aquaculture farms

Test /reaction	VC1	VC2	VC3	VC4	VC ctx1	VC ctx2	VC ctx3	VC MTCC 3906	Bergey's Manual*
Grams stain	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Oxidase (Kovacs)	+	+	+	+	+	+	+	+	100%
Nitrate reduced to nitrite	+	+	+	+	+	+	+	+	100%
Motility	+	+	+	+	+	+	+	+	100%
H&L O/F test	FANG**	FANG	FANG	FANG	FANG	FANG	FANG	FANG	
Swarming	-	-	-	-	-	-	-	-	0%

Indole	+	+	+	+	+	+	+	+	100%
Voges-Proskauer	+	+	+	+	+	+	+	+	100%
Growth in 0% NaCl	+	+	+	+	+	+	+	+	100%
Growth in 3% NaCl	+	+	+	+	+	+	+	+	100%
Growth in 6% NaCl	+	+	-	+	-	-	-	-	20%
Growth in 8% NaCl	-	-	-	-	-	-	-	-	0%
Growth in 10% NaCl	-	-	-	-	-	-	-	-	0%
Growth in 12% NaCl	-	-	-	-	-	-	-	-	0%
Lysine decarboxylase	+	+	+	+	+	+	+	+	100%
Arginine dihydrolase	-	-	-	-	-	-	-	-	0%
Ornithine decarboxylase	+	+	+	+	+	+	+	+	100%
Aesculin	-	-	-	-	-	-	-	-	0%
Adonitol	-	-	-	-	-	-	-	-	0%
L-Arabinose	-	-	-	-	-	-	-	-	0%
Cellobiose	-	-	-	-	-	-	-	-	0%
Dextrose (Acid)	+	+	+	+	+	+	+	+	100%
Dextrose (Gas)	-	-	-	-	-	-	-	-	0%
Dulcitol	-	-	-	-	-	-	-	-	0%
Fructose	+	+	+	+	+	+	+	+	
D-Galactose	+	+	+	+	+	+	+	+	60%

Myo-Inositol	-	-	-	-	-	-	-	-	**0%**
Lactose	-	-	-	-	-	-	-	-	0%
Maltose	+	+	+	+	+	+	+	+	100%
D-mannitol	+	+	+	+	+	+	+	+	100%
D-Mannose	+	+	+	+	+	+	+	+	80%
Ribose	-	-	-	-	-	-	-	-	
L-Rhamnose	-	-	-	-	-	-	-	-	0%
Salicin	-	-	-	-	-	-	-	-	0%
L-Sorbitol	-	-	-	-	-	-	-	-	0%
Sorbose	-	-	-	-	-	-	-	-	
Sucrose	+	+	+	+	+	+	+	+	100%
Trehalose	+	+	+	+	+	+	+	+	80%
D-Xylose	-	-	-	-	-	-	-	-	0%
Glycogen	+	+	+	+	+	+	+	+	

VC- *V.cholerae*, VC ctx - choleratoxigenic *V.cholerae*)

* Information from Bergey's manual of Systematic Bacteriology (2005); percentage of isolates that gave positive reaction.

**FANG – Fermentative with acid, but no gas.

The isolates identified as *V. cholerae* as per the scheme of Noguerola and Blanch (2008) were found to be *V.cholerae* as they yielded the *V.cholerae* specific results (Table 4.11) mentioned in Bergey's manual of Systematic Bacteriology (2005).

4.2.4.1.2. Biochemical reactions of *V.vulnificus* isolated from shrimp hatcheries

Table 4.12. Biochemical reactions of *V.vulnificus* isolated from shrimp hatcheries

Test /reaction	VV1	VV2	VV3	VV4	VV MTCC 1145	Bergey's Manual*
Grams stain	-ve	-ve	-ve	-ve	-ve	-ve
Oxidase (Kovacs)	+	+	+	+	+	100%
Nitrate reduced to nitrite	+	+	+	+	+	100%
Motility	+	+	+	+	+	100%
H&L O/F test	FANG	FANG	FANG	FANG	FANG	
Swarming	-	-	-	-	-	0%
Indole	+	+	+	+	+	100%
Voges-Proskauer	+	+	+	+	+	100%
Growth in 0% NaCl	-	-	-	-	-	0%
Growth in 3% NaCl	+	+	+	+	+	100%
Growth in 6% NaCl	+	+	+	+	+	100%
Growth in 8% NaCl	-	-	-	-	-	0%
Growth in 10% NaCl	-	-	-	-	-	0%
Growth in 12% NaCl	-	-	-	-	-	0%
Lysine decarboxylase	+	+	+	+	+	100%
Arginine dihydrolase	-	-	-	-	-	10%
Ornithine decarboxylase	+	+	+	+	+	100%
Aesculin	-	-	-	-	-	40%
Adonitol	-	-	-	-	-	0%
L-Arabinose	-	-	-	-	-	0%
Cellobiose	+	+	+	+	+	100%

Dextrose (Acid)	+	+	+	+	+	100%
Dextrose (Gas)	-	-	-	-	-	0%
Dulcitol	-	-	-	-	-	0%
Fructose	+	+	+	+	+	
D-Galactose	+	+	+	+	+	100%
myo-Inositol	-	-	-	-	-	0%
Lactose	-	-	-	-	-	0%
Maltose	+	+	+	+	+	100%
D-mannitol	+	+	+	+	+	80%
D-Mannose	+	+	+	+	+	100%
Ribose	-	-	-	-	-	
L-Rhamnose	-	-	-	-	-	0%
Salicin	-	-	-	-	-	0%
L-Sorbitol	-	-	-	-	-	0%
Sorbose	-	-	-	-	-	
Sucrose	-	-	-	-	-	20%
Trehalose	+	+	+	+	+	100%
D-Xylose	-	-	-	-	-	0%
Glycogen	+	+	+	+	+	

VV- *V.vulnificus*

* Information from Bergey's manual of Systematic Bacteriology (2005); percentage of isolates that gave positive reaction

The isolates identified as *V.vulnificus* as per the scheme of Noguerola and Blanch (2008) were found to be *V. vulnificus* as they yielded the *V. vulnificus* specific results (Table 4.12) mentioned in Bergey's manual of Systematic Bacteriology (2005).

4.2.4.1.3. Biochemical reactions of *V.alginolyticus* isolated from shrimp hatcheries and aquaculture farms

Table 4.13. Biochemical reactions of *V.alginolyticus* isolated from shrimp hatcheries and aquaculture farms

Test /reaction	VA1	VA2	VA3	VA4	VA ATCC 17749	Bergey's Manual*
Grams stain	-ve	-ve	-ve	-ve	-ve	-ve
Oxidase (Kovacs)	+	+	+	+	+	100%
Nitrate reduced to nitrite	+	+	+	+	+	100%
Motility	+	+	+	+	+	100%
H&L O/F test	FANG	FANG	FANG	FANG	FANG	
Swarming	+	+	+	+	+	100%
Indole	+	+	+	+	+	80%
Voges-Proskauer	+	+	+	+	+	80%
Growth in 0% NaCl	-	-	-	-	-	0%
Growth in 3% NaCl	+	+	+	+	+	100%
Growth in 6% NaCl	+	+	+	+	+	100%
Growth in 8% NaCl	+	+	+	+	+	100%
Growth in 10% NaCl	+	+	+	+	+	100%
Growth in 12% NaCl	+	+	+	+	+	100%
Lysine decarboxylase	+	+	+	+	+	100%
Arginine dihydrolase	-	-	-	-	-	10%
Ornithine decarboxylase	+	+	+	+	+	60%
Aesculin	-	-	-	-	-	3%
Adonitol	-	-	-	-	-	0%
L-Arabinose	-	-	-	-	-	0%
Cellobiose	-	-	-	-	-	

Dextrose (Acid)	+	+	+	+	+	100%
Dextrose (Gas)	-	-	-	-	-	0%
Dulcitol	-	-	-	-	-	0%
Fructose	+	+	+	+	+	
D-Galactose	-	-	-	-	-	60%
myo-Inositol	-	-	-	-	-	0%
Lactose	-	-	-	-	-	0%
Maltose	+	+	+	+	+	100%
D-mannitol	+	+	+	+	+	100%
D-Mannose	+	+	+	+	+	100%
Ribose	-	-	-	-	-	
L-Rhamnose	-	-	-	-	-	0%
Salicin	-	-	-	-	-	0%
L-Sorbitol	-	-	-	-	-	0%
Sorbose	-	-	-	-	-	
Sucrose	+	+	+	+	+	100%
Trehalose	+	+	+	+	+	80%
D-Xylose	-	-	-	-	-	0%
Glycogen	+	+	+	+	+	

VA- *V.alginolyticus*

* Information from Bergey's manual of Systematic Bacteriology (2005); percentage of isolates that gave positive reaction

The isolates identified as *V.alginolyticus* as per the scheme of Noguerola and Blanch (2008) were found to be *V.alginolyticus* as they yielded the *V.alginolyticus* specific results (Table 4.13) mentioned in Bergey's manual of Systematic Bacteriology (2005).

4.2.4.1.4. Biochemical reactions of *V.parahaemolyticus* isolated from shrimp hatcheries

Table 4.14. Biochemical reactions of *V.parahaemolyticus* isolated from shrimp hatcheries

Test /reaction	VP1	VP2	VP3	VP4	VP ATCC 17802	Bergey's Manual*
Grams stain	-ve	-ve	-ve	-ve	-ve	-ve
Oxidase (Kovacs)	+	+	+	+	+	100%
Nitrate reduced to nitrite	+	+	+	+	+	100%
Motility	+	+	+	+	+	100%
H&L O/F test	FANG	FANG	FANG	FANG	FANG	
Swarming	+	+	+	+	+	80%
Indole	+	+	+	+	+	100%
Voges-Proskauer	-	-	-	-	-	0%
Growth in 0% NaCl	-	-	-	-	-	0%
Growth in 3% NaCl	+	+	+	+	+	100%
Growth in 6% NaCl	+	+	+	+	+	100%
Growth in 8% NaCl	+	+	+	+	+	100%
Growth in 10% NaCl	+	-	-	-	-	0%
Growth in 12% NaCl	-	-	-	-	-	0%
Lysine decarboxylase	+	+	+	+	+	100%
Arginine dihydrolase	-	-	-	-	-	0%
Ornithine decarboxylase	+	+	+	+	+	60%
Aesculin	-	-	-	-	-	0%
Adonitol	-	-	-	-	-	0%
L-Arabinose	+	+	+	+	+	80%

Cellobiose	-	-	-	-	-	0%
Dextrose (Acid)	+	+	+	+	+	100%
Dextrose (Gas)	-	-	-	-	-	0%
Dulcitol	-	-	-	-	-	0%
Fructose	+	+	+	+	+	
D-Galactose	+	+	+	+	+	60%
myo-Inositol	-	-	-	-	-	0%
Lactose	-	-	-	-	-	0%
Maltose	+	+	+	+	+	100%
D-mannitol	+	+	+	+	+	80%
D-Mannose	+	+	+	+	+	100%
Ribose	-	-	-	-	-	
L-Rhamnose	-	-	-	-	-	0%
Salicin	-	-	-	-	-	0%
L-Sorbitol	-	-	-	-	-	0%
Sorbose	-	-	-	-	-	
Sucrose	-	-	-	-	-	0%
Trehalose	+	+	+	+	+	100%
D-Xylose	-	-	-	-	-	0%
Glycogen	+	+	+	+	+	

VP- *V parahaemolyticus*

* Information from Bergey's manual of Systematic Bacteriology (2005);

percentage of isolates that gave positive reaction

The isolates identified as *V.parahaemolyticus* as per the scheme of Noguerola and Blanch (2008) were found to be *V.parahaemolyticus* as they yielded the *V.parahaemolyticus* specific results (Table 4.14) mentioned in Bergey's manual of Systematic Bacteriology (2005). One of the *V.parahaemolyticus* isolate showed growth even at 10% NaCl concentration. Although Bergey's manual of Systematic Bacteriology (2005) mentions that *V.parahaemolyticus* strains do not grow at 10% NaCl concentration, several studies have reported that *V.parahaemolyticus* strains are capable of growth in the

presence of 10% NaCl (Colwell 1970; Kampelmacher *et al.*, 1972; Gjerde and Boe 1981; Schandevyl *et al.*, 1984; Zen-Yoji *et al.*, 1973).

4.2.4.1.5. Biochemical reactions of *V.harveyi* isolated from shrimp hatcheries.

Table 4.15. Biochemical reactions of *V.harveyi* isolated from shrimp hatcheries

Test /reaction	VH1	VH2	VH3	VH4	Bergey's Manual*
Grams stain	-ve	-ve	-ve	-ve	-ve
Oxidase (Kovacs)	+	+	+	+	100%
Nitrate reduced to nitrite	+	+	+	+	100%
Motility	+	+	+	+	100%
H&L O/F test	FANG	FANG	FANG	FANG	
Swarming	-	-	+	-	33%
Indole	+	+	+	+	100%
Voges-Proskauer	-	-	-	-	0%
Growth in 0% NaCl	-	-	-	-	0%
Growth in 3% NaCl	+	+	+	+	100%
Growth in 6% NaCl	+	+	+	+	100%
Growth in 8% NaCl	+	+	+	+	100%
Growth in 10% NaCl	-	-	-	-	100%
Growth in 12% NaCl	-	-	-	-	100%
Lysine decarboxylase	+	+	+	+	100%
Arginine dihydrolase	-	-	-	-	0%
Ornithine decarboxylase	+	+	+	+	100%
Aesculin	-	-	-	-	0%
Adonitol	-	-	-	-	0%
L-Arabinose	-	-	-	-	0%
Cellobiose	+	+	+	+	100%

Dextrose (Acid)	+	+	+	+	83%
Dextrose (Gas)	-	-	-	-	0%
Dulcitol	-	-	-	-	0%
Fructose	+	+	+	+	
D-Galactose	+	+	+	+	67%
myo-Inositol	-	-	-	-	0%
Lactose	-	-	-	-	0%
Maltose	+	+	+	+	100%
D-mannitol	+	+	+	+	100%
D-Mannose	+	+	+	+	100%
Ribose	-	-	-	-	
L-Rhamnose	-	-	-	-	0%
Salicin	-	-	-	-	0%
L-Sorbitol	-	-	-	-	0%
Sorbose	-	-	-	-	
Sucrose	+	+	+	+	83%
Trehalose	+	+	+	+	100%
D-Xylose	-	-	-	-	0%
Glycogen	+	+	+	+	

VH- *V.harveyi*

* Information from Bergey's manual of Systematic Bacteriology (2005); percentage of isolates that gave positive reaction

The isolates identified as *V.harveyi* as per the scheme of Noguerola and Blanch (2008) were found to be *V.harveyi* as they yielded the *V.harveyi* specific results (Table 4.15) mentioned in Bergey's manual of Systematic Bacteriology (2005).

4.2.4.2. Utilization of sugars by Vibrio cultures isolated from shrimp hatcheries and aquaculture farms

Pathogenic Vibrios were inoculated to sugar fermentation broth in small glass test tubes with different sugars (0.5% w/v) to test the ability of the organism to utilize the sugars and produce acid. Sugar fermentation tests were also carried out in autoclavable 96 well plastic plates having a capacity of 1.2ml per well. The 96 well plates were sterilized by autoclaving at 121°C for 15 minutes and pre-sterilized fermentation broth containing a specific sugars (900µl) were added in the wells column wise and the pathogenic Vibrio cultures were inoculated (100µl of 24h culture) row wise using micropipettes. The change in colour was noted and the results were recorded. The result of sugar fermentation tests obtained using autoclavable 96 well plastic plates (Fig. 4.5) was identical to the conventional glass tube method. The advantages observed while using 96 well plastic plates were - convenience, saving of media and can screen large number of isolates.

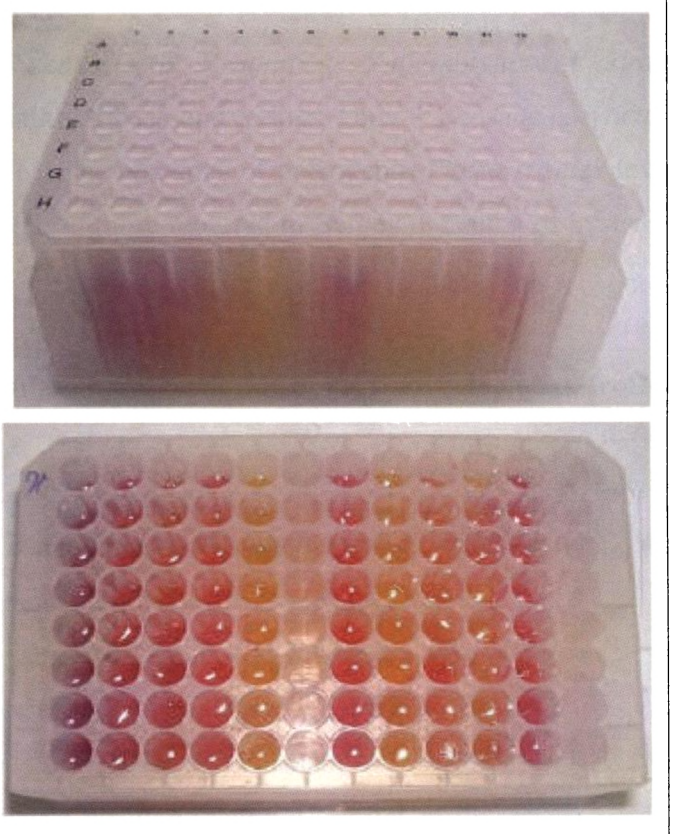


Fig 4.5. Autoclavable 96 well plastic plates for Sugar fermentation tests

4.2.4.2.1. Utilization of pentoses by Vibrio cultures isolated from shrimp culture system

Table 4.16. Utilization of pentoses by pathogenic Vibrios

	L- Arabinose	Ribose	Xylose
V.cholerae (n=4)	-	-	-
V.cholerae ctx (n=3)	-	-	-
V.vulnificus (n=4)	-	-	-
V.parahaemolyticus (n=4)	+	-	-
V.alginolyticus (n=4)	-	-	-
V.harveyi (n=4)	-	-	-

V.cholerae, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* failed to utilize ribose and xylose. Only *V.parahaemolyticus* isolates utilized arabinose (Table 4.16). The reactions of pathogenic *Vibrio species* towards L-arabinose and xylose were similar to those reported in Bergey's manual of Systematic Bacteriology (2005).

4.2.4.2.2. Utilization of hexoses by Vibrio cultures isolated from shrimp culture system

V.cholerae, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* utilized glucose, fructose, mannose and mannitol. Only *V.alginolyticus* isolates were negative for galactose (Table 4.17). The reactions of pathogenic *Vibrio species* towards hexoses were similar to those reported in Bergey's manual of Systematic Bacteriology (2005). In the Bergey's manual, 60% of the *V.alginolyticus* isolates were reported to be galactose positive (Table 4.13) but in this study all the *V.alginolyticus* isolates tested were found to be negative for galactose utilization. Fructose reaction was not mentioned in Bergey's manual of Systematic Bacteriology (2005) and in this study it

was found that all the pathogenic Vibrio *species* isolated from farms and hatcheries utilized fructose.

Table 4.17. Utilization of hexoses by pathogenic Vibrios

	Galactose	D-Glucose	Fructose	D-Mannose	D-Mannitol
V.cholerae	+	+	+	+	+
V.cholerae ctx	+	+	+	+	+
V.vulnificus	+	+	+	+	+
V.parahaemolyticus	+	+	+	+	+
V.alginolyticus	(-)ve	+	+	+	+
V.harveyi	+	+	+	+	+

4.2.4.2.3. Utilization of disaccharides by Vibrio cultures isolated from shrimp culture system

V.cholerae, *V.cholerae ctx*, *V.alginolyticus* and *V.harveyi* utilized sucrose whereas *V.vulnificus* and *V.parahaemolyticus* failed to ferment sucrose. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* utilized maltose and all these pathogenic Vibrios failed to utilize lactose. *V.vulnificus* and *V.harveyi* utilized cellobiose (Table 4.18). The reactions of pathogenic *Vibrio species* towards disaccharides were similar to those reported in Bergey's manual of Systematic Bacteriology (2005). In the Bergey's manual, 85% of the *V.harveyi* isolates were reported to be sucrose positive (Table 4.15) but in this study 100% of *V.harveyi* isolates tested were found to be positive for sucrose utilization.

Table 4.18. Utilization of disaccharides by pathogenic Vibrios

	Cellobiose	Lactose	Maltose	Sucrose
V.cholerae	-	-	+	+
V.cholerae ctx	-	-	+	+
V.vulnificus	+	-	+	-
V.parahaemolyticus	-	-	+	-
V.alginolyticus	-	-	+	+
V.harveyi	+	-	+	+

4.2.4.2.4. Utilization of sugar derivatives by Vibrio cultures isolated from shrimp culture system

V.cholerae, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* failed to utilize aesculin and salicin (Table 4.19).

The reactions of pathogenic *Vibrio species* towards sugar derivatives were similar to those reported in Bergey's manual of Systematic Bacteriology (2005). In the Bergey's manual, 60% of the *V. vulnificus* (Table 4.12) and 3% of *V.alginolyticus* (Table 4.13) *V.alginolyticus* isolates were reported to be aesculin positive but in this study all the *V.vulnificus* and *V.alginolyticus* isolates tested were found to be negative for aesculin utilization.

Table 4.19. Utilization of sugar derivatives by pathogenic Vibrios

	Aesculin	Salicin
V.cholerae	-	-
V.cholerae ctx	-	-
V.vulnificus	-	-
V.parahaemolyticus	-	-
V.alginolyticus	-	-
V.harveyi	-	-

4.2.4.2.5. Utilization of Glycogen by Vibrio cultures isolated from shrimp culture system

V.cholerae, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* utilized glycogen (Table 4.20). Glycogen reaction was not mentioned in Bergey's manual of Systematic Bacteriology (2005) and in this study it was found that 100% of the pathogenic Vibrio *species* isolated from farms and hatcheries utilized glycogen.

Table 4.20. Utilization of Glycogen by pathogenic Vibrios

	Glycogen
V.cholerae	+
V.cholerae ctx	+
V.vulnificus	+
V.parahaemolyticus	+
V.alginolyticus	+
V.harveyi	+

4.2.4.4. Utilization of amino acids by Vibrio cultures isolated from shrimp culture system

Utilization of amino acids by Vibrio cultures isolated from hatcheries / farms were studied using 4 critical amino acids, namely, arginine, lysine, ornithine and histidine and the results are presented in Table 4.21.

V.cholerae, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* showed decarboxylase activity with lysine, ornithine but not with histidine. All these pathogenic *Vibrio spp* were negative for arginine dihydrolase activity (Table 4.20).

The reactions of pathogenic *Vibrio species* towards amino acids were almost similar to those reported in Bergey's manual of Systematic Bacteriology (2005). In the Bergey's manual, 10% of *V.vulnificus and V.alginolyticus* were reported to be positive for arginine dihydrolase activity (Table 4.12 and 4.13) but in this study all the *V.vulnificus and V.alginolyticus* isolates tested were found to be negative for arginine dihydrolase activity. Similarly, in the Bergey's manual, 60% of *V.alginolyticus* and *V. parahaemolyticus* (Table 4.13 & 4.14) were reported to be positive for ornithine decarboxylase activity but in this study all 100% of the *V.alginolyticus* and *V. parahaemolyticus* isolates tested were found to be positive for ornithine decarboxylase activity.

Table 4.21. Utilization of amino acids by pathogenic Vibrios

	Arginine	Lysine	Ornithine	Histidine
V.cholerae (n=4)	-	+	+	-
V.cholerae ctx (n=3)	-	+	+	-
V.vulnificus (n=4)	-	+	+	-
V.parahaemolyticus (n=4)	-	+	+	-
V.alginolyticus (n=4)	-	+	+	-
V.harveyi (n=4)	-	+	+	-

4.2.4.5. Enzyme activities of pathogenic Vibrios isolated from hatcheries and farms

All the *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* were studied for their potential to produce enzymes like Amylase, Gelatinase and other protease, DNAses, Lipases, Phospholipases, Phosphatases etc., and the results are presented in the following Tables.

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.

4.2.4.5.1. Determination of Amylolytic activity by Vibrio cultures isolated from shrimp culture system

Starch agar was employed to determine amylolytic activity (Fig. 4.6). *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, and *V.parahaemolyticus* showed amylolytic activity. *V.harveyi* isolates were found to be negative for amylase activity (Table 4.22). The mean amylolytic activity index was higher in *V.parahaemolyticus* and *V.alginolyticus*. Amylase activity of vibrios was reported in several studies. Saramma et al (1994) reported that the percentage of amylolytic population was greater in the genus Vibrio (82.12%) among the bacteria isolated from water, sediment, fish, prawns and clams. Amylase lacking *V.alginolyticus* strains were reported (Kumar *et al.*, 2007) but in this study all the *V.alginolyticus* cultures tested were found to be positive for amylase activity. Cai and Cheng (2006) observed that 64.3% of Vibrio strains isolated from abalone (*Haliotis diversicolor*) produced amylase while 100% of Vibrio strains isolated from farming water produced amylase.

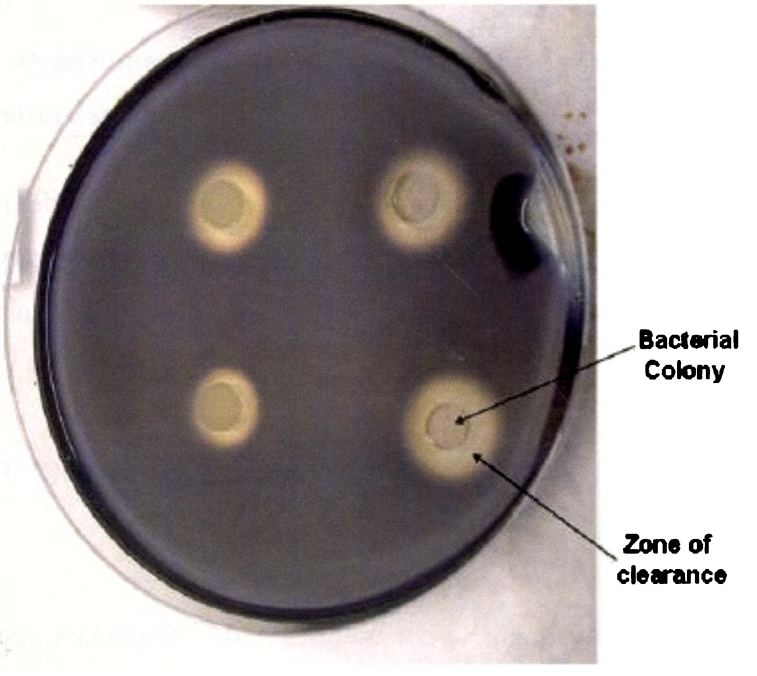


Fig. 4.6. Amylolytic Activity on Starch Agar

Table 4.22. Amylolytic activity of pathogenic Vibrios

	Amylolytic activity*	Activity Index Mean
V.cholerae (n=4)	+	1.30
V.cholerae ctx (n=3)	+	1.24
V.vulnificus (n=4)	+	1.0
V.parahaemolyticus (n=4)	+	2.60
V.alginolyticus (n=4)	+	2.42
V.harveyi (n=4)	-	0

* Starch agar having 0.5% NaCl

4.2.4.5.2. Determination of proteolytic activity by Vibrio cultures isolated from shrimp culture system

Proteinaceous virulence factors, including alkaline proteases, metalloproteases, cysteine proteases and alkaline serine proteases, have been identified as important elements in *Vibrio* pathogenesis (Liu *et al.*, 1996; Sudeesh and Xu, 2001; Aguirre-Guzman *et al.*, 2004).

4.2.4.5.2.1. Gelatin liquefaction by Vibrio cultures isolated from shrimp culture system

V.cholerae, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* were positive for gelatinase activity (Table 4.23) and liquefied gelatin.

Table 4.23. Gelatin liquefaction by pathogenic Vibrios

	Gelatin liquefaction
V.cholerae	+
V.cholerae ctx	+
V.vulnificus	+
V.parahaemolyticus	+
V.alginolyticus	+
V.harveyi	+

4.2.4.5.2.2. Proteolytic activity (gelatin) by Vibrio cultures isolated from shrimp culture system

V.cholerae, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* were positive for gelatinase activity on gelatin agar (Table 4.24, Fig 4.7). This observation was similar to that of Baffone et al (2001) wherein they have reported that the gelatinase activity was observed in 100% of the Vibrio strains (*V.alginolyticus, V. parahaemolyticus, V. cholerae non-01, V. vulnificus*) isolated from sea water. The mean gelatinase activity index was higher in *V.vulnificus, V.cholerae* and *V.cholerae ctx*.

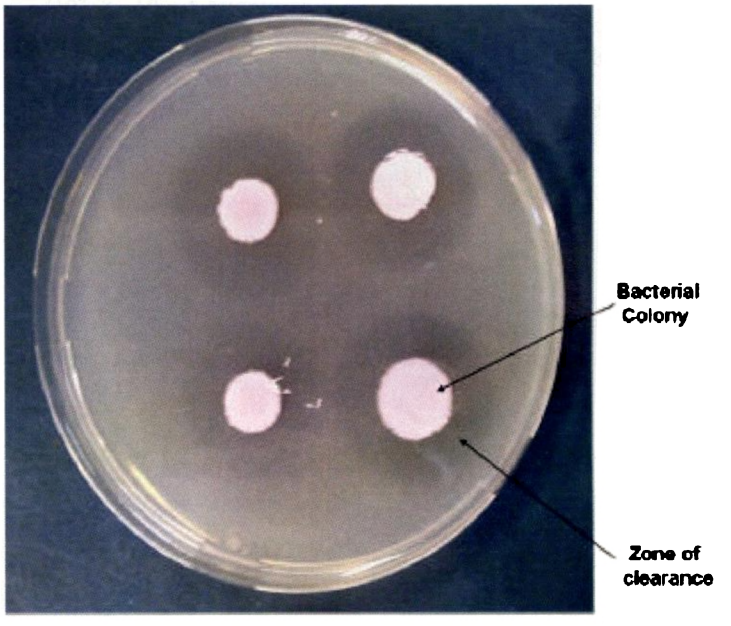


Fig 4.7. Proteolytic activity on Gelatin Agar

Table 4.24. Proteolytic activity (gelatin) by Vibrio cultures isolated from shrimp culture system

	Proteolytic Activity (on Gelatin agar)*	Activity Index Mean
V.cholerae	+	2.02
V.cholerae ctx	+	2.02
V.vulnificus	+	2.39
V.parahaemolyticus	+	1.37
V.alginolyticus	+	1.0
V.harveyi	+	1.33

*Gelatin agar having 0.5% NaCl

4.2.4.5.2.3. Proteolytic activity of pathogenic Vibrio cultures on fish protein

Fish protein agar was employed to determine fish protein proteolytic activity of pathogenic vibrios (Fig. 4.8). 25% of *V.cholerae, 25%* of *V.cholerae ctx*, 50% of *V.parahaemolyticus*, 100% of *V.alginolyticus* and 100% of *V.vulnificus* showed proteolytic activity on fish protein agar (Table 4.25). *V. harveyi* failed to show proteolytic activity on fish protein agar. *V.alginolyticus* and *V.vulnificus* showed higher proteolytic activity index on fish protein.

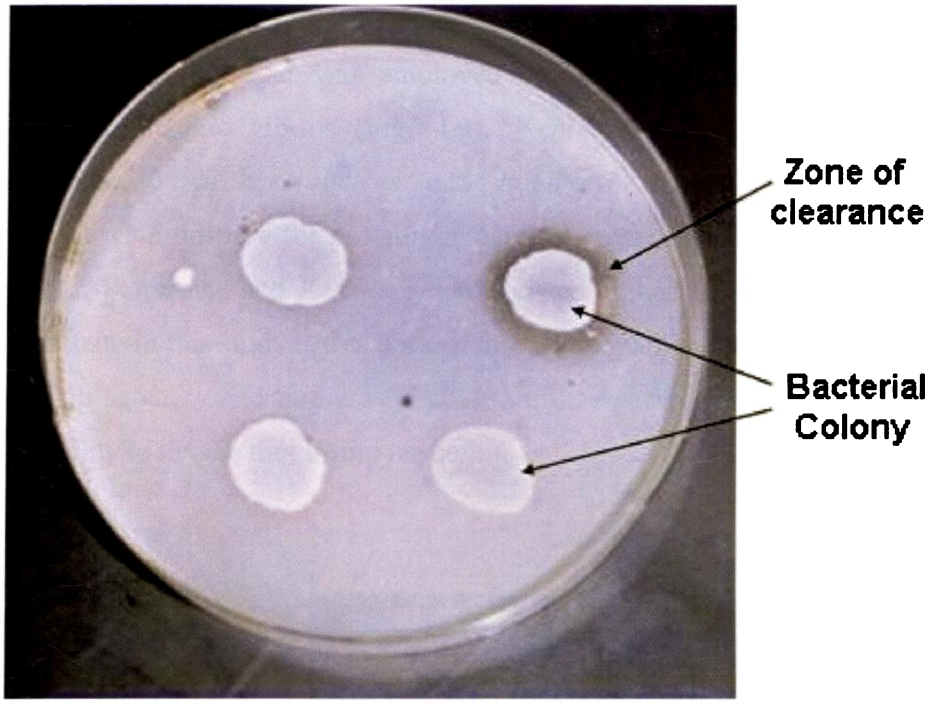


Fig 4.8. Proteolytic Activity of *V.cholerae* on Fish protein agar

Table 4.25. Proteolytic activity of pathogenic Vibrios on fish protein

	Proteolytic Activity (on Fish protein agar)*	**Activity Index Mean**
V.cholerae	+/-**	0.34
V.cholerae ctx	+/-	0.29
V.vulnificus	+	1.37
V.parahaemolyticus	+/-	0.54
V.alginolyticus	+	1.09
V.harveyi	-	0

* Fish protein agar having 0.5% NaCl; ** indicates some isolates were negative

4.2.4.5.2.4. Proteolytic activity of pathogenic Vibrios on shrimp protein

Shrimp protein agar was employed to determine shrimp protein proteolytic activity of pathogenic vibrios (Fig. 4.9). *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* isolated from shrimp culture environment were positive for proteolytic activity on shrimp protein agar (Table 4.26). *V.vulnificus*, *V. harveyi* and *V.cholerae ctx* showed higher proteolytic activity index on shrimp protein. In this study it was noticed that all the pathogenic Vibrio species isolated from shrimp hatchery and farms were able to utilize shrimp protein thereby suggesting their capability to invade the shrimp tissue.

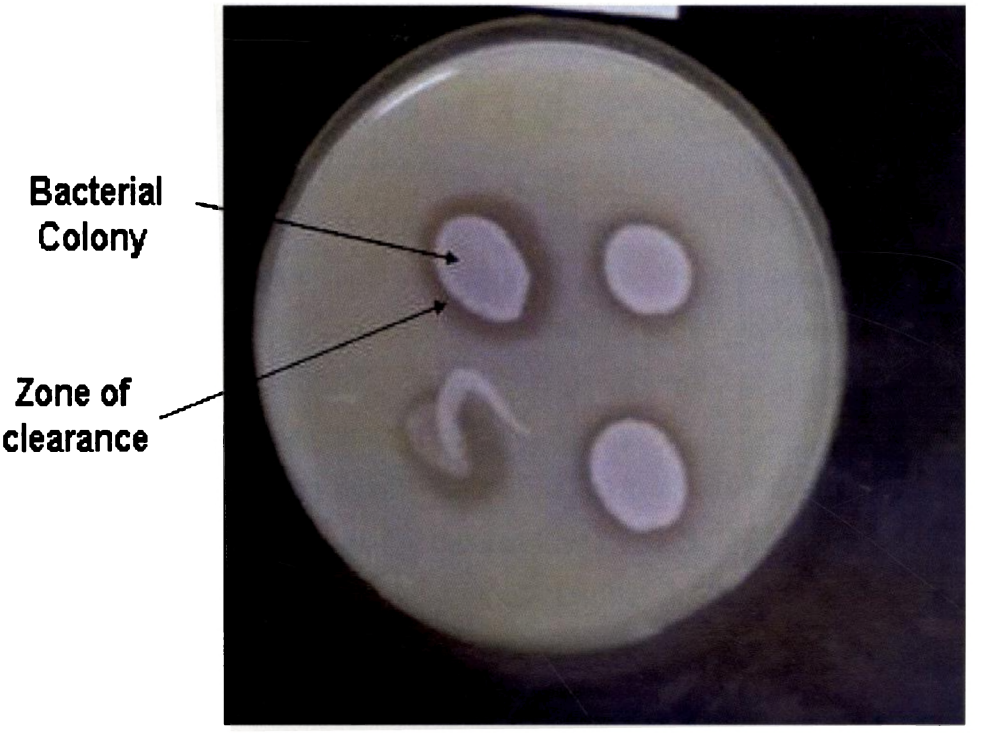


Fig 4.9. Proteolytic Activity on Shrimp protein agar

Table 4.26. Proteolytic activity of pathogenic Vibrios on shrimp protein

	Proteolytic Activity (on Shrimp protein agar)	Activity Index Mean
V.cholerae	+	0.90
V.cholerae ctx	+	1.34
V.vulnificus	+	1.44
V.parahaemolyticus	+	1.2
V.alginolyticus	+	1.23
V.harveyi	+	1.4

* Shrimp protein agar having 0.5% NaCl

The proteolytic activity differed with the protein substrate. 75% of *V.cholerae*, 75% of *V.cholerae ctx*, and 100% of *V.harveyi* isolates failed to show proteolytic activity on fish protein agar whereas all these vibrios showed proteolytic activity on gelatin and shrimp protein. The mean gelatinase activity index was higher in *V.vulnificus, V.cholerae* and *V.cholerae ctx*. *V.alginolyticus* and *V.vulnificus* showed higher proteolytic activity index on fish protein. *V.vulnificus*, *V. harveyi* and *V.cholerae ctx* showed higher proteolytic activity index on shrimp protein (Fig 4.10).

Venugopal and Lewis (1985) reported that proteases from marine bacteria could degrade both casein and fish protein equally. Cai and Cheng (2006) observed that 63.6% of Vibrios isolated from farm water produced protease. Baffone et al (2001) reported that 100% of *V.alginolyticus, V. parahaemolyticus, V. cholerae non-01, V. vulnificus* showed gelatinase activity. Albuminase, caseinase, elastase, collagenase and gelatinase activity were detected in *V.vulnificus* (Huelsmann *et al.*, 2003).

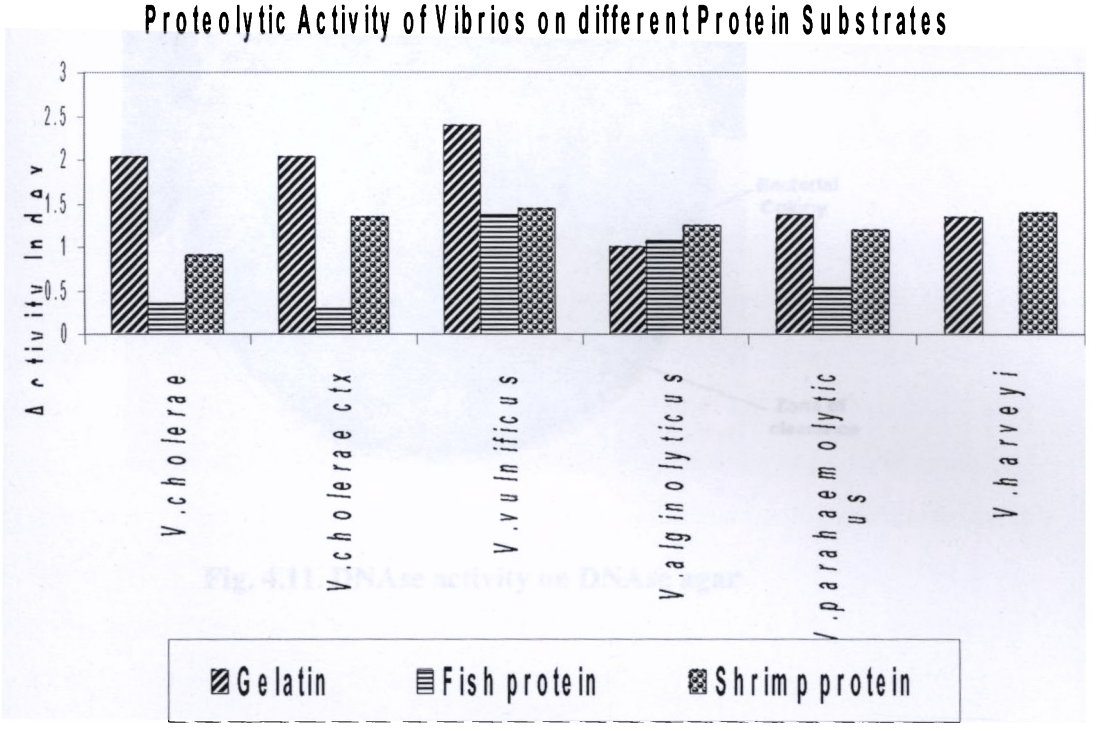


Fig. 10. Proteolytic Activity of pathogenic Vibrios on different protein substrates

4.2.4.5.3. Determination of DNAse activity of Vibrio cultures isolated from shrimp culture system

DNAse activity was determined using DNAse agar (Fig 4.11). All *V.cholerae* and *V.cholerae ctx* isolates were negative for DNAse activity whereas *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* showed DNAse activity (Table 4.27). DNAse activity was greater in *V.alginolyticus*. At lower salt concentration the swarming behaviour of *V.alginolyticus* made it difficult to estimate the activity index. Beleneva and Maslennikova (2005) observed that *Vibrio spp.* isolated from mussel (*Mytilus trossulus*) produced DNAses and RNAses. Bergey's manual of Systematic Bacteriology (2005) reports that 100% isolates of *V.harveyi*, 95% isolates of *V.alginolyticus*, 93% isolates of *V.cholerae*, 92% isolates of *V.parahaemolyticus* and 50% isolates of *V.vulnificus* produce DNAse at 25°C.

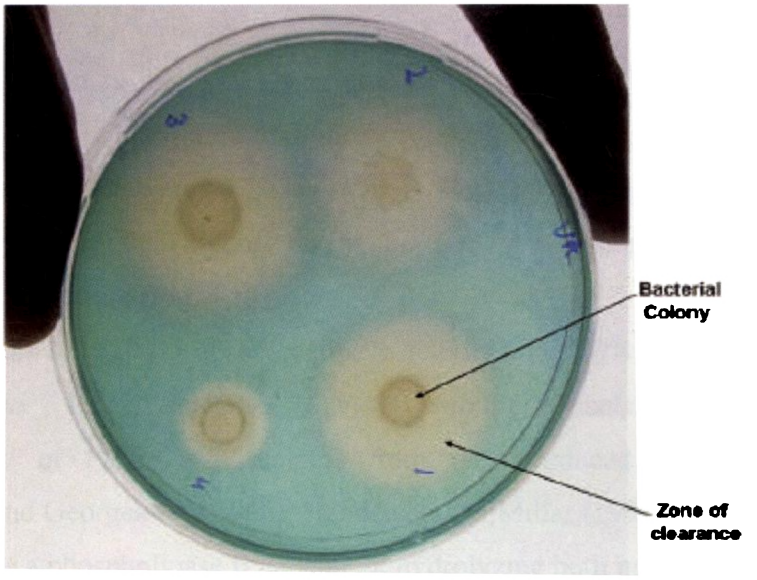


Fig. 4.11. DNAse activity on DNAse agar

Table 4.27. DNAse activity of pathogenic Vibrios

	DNAse activity*	Activity Index Mean
V.cholerae	-	0
V.cholerae ctx	-	0
V.vulnificus	+	0.5
V.parahaemolyticus	+	0.58
V.alginolyticus	+	0.97
V.harveyi	+	0.5

* DNAse agar having 0.5% NaCl

4.2.4.5.4. Determination of lipolytic activity of Vibrio cultures isolated from shrimp culture system

Lipolytic activity of the human pathogenic vibrios depended on the type of lipid. Neutral lipid (tributyrin) was not utilized by *V.alginolyticus* and *V.harveyi* (Table 4.28).

Phospholipid (lecithin in egg yolk) was utilized by all the pathogenic vibrio species (Table 4.29, Fig. 4.12). Maximum lipolytic activity index on phospholipid was given by *V.alginolyticus* (1.7) followed by *V.vulnificus* (1.5) and *V.parahaemolyticus* (1.4). The lipolytic activity index of *V.cholerae* and *V.cholerae ctx* was slightly higher on neutral lipid than on phospholipid (Fig. 4.13).

Baffone *et al.*, (2001) observed lipase activities in 100% of *V.alginolyticus, V. parahaemolyticus , V. cholerae non-01* and *V. vulnificus* isolates obtained from sea water. Majority of *Vibrio spp* isolated from fish produced lipase and protease (Dhevendaran and Georgekutty, 1998). Henderson and Millar (1998) reported that *Vibrio species* produces a phospholipase B capable of hydrolyzing both intact phospholipids and lysophospholipids 100% lecithinase production was reported in *V.parahaemolyticus* (Sudha et al., 2002), *V.vulnificus* (Moreno and Landgraf, 1998) and *V.harveyi* (Liu *et al.*, 1996). 100% lipase production was reported for *V.vulnificus* on Tween-80 medium and 96.9% in egg yolk medium (Moreno and Landgraf, 1998).

Table 4.28. Tributyrin hydrolysis by pathogenic Vibrios

	Lipase activity (On Tributyrin Agar)	**Activity Index Mean**
V.cholerae	+	1.14
V.cholerae ctx	+	1.43
V.vulnificus	+ / -*	0.67
V.parahaemolyticus	+ / -	0.69
V.alginolyticus	-	0
V.harveyi	-	0

*some isolates were negative

Table 4.29. Phospholipase activity of Vibrio cultures isolated from shrimp culture system

	Phospholipase Activity (Lecithinase) (on egg yolk agar)	Activity Index Mean
V.cholerae	+	1.18
V.cholerae ctx	+	1.14
V.vulnificus	+	1.45
V.parahaemolyticus	+	1.38
V.alginolyticus	+	1.67
V.harveyi	+	1.0

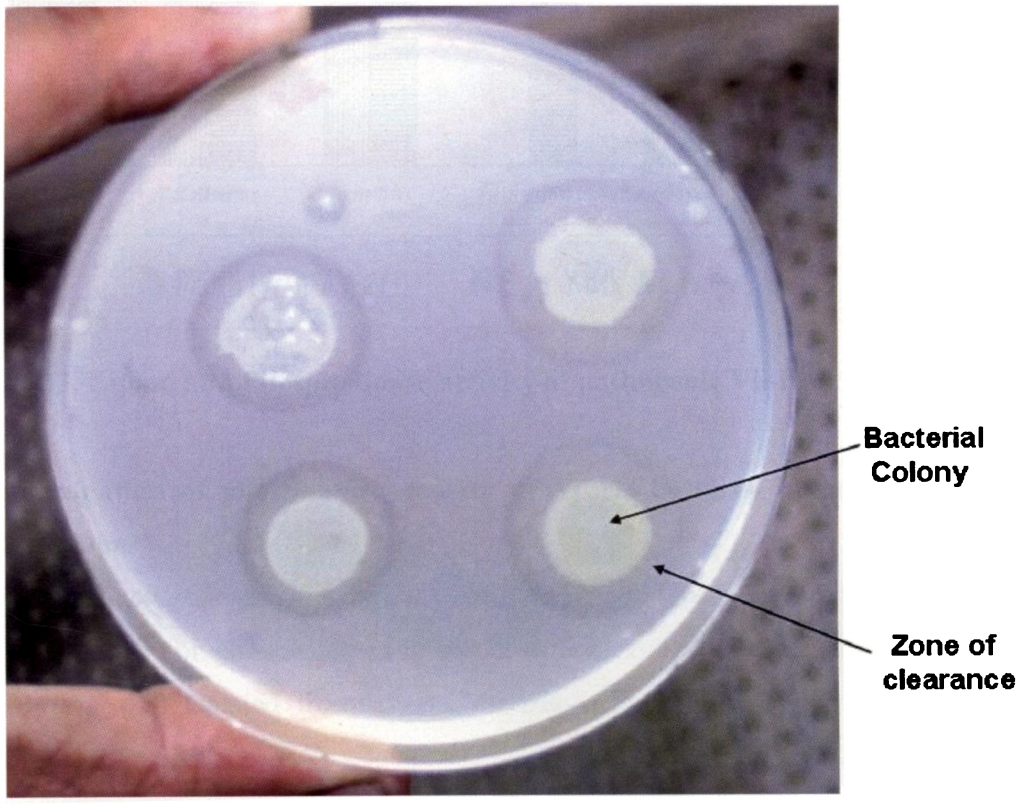


Fig 4.12. Phospholipase activity (lecithinase) on Egg Yolk agar

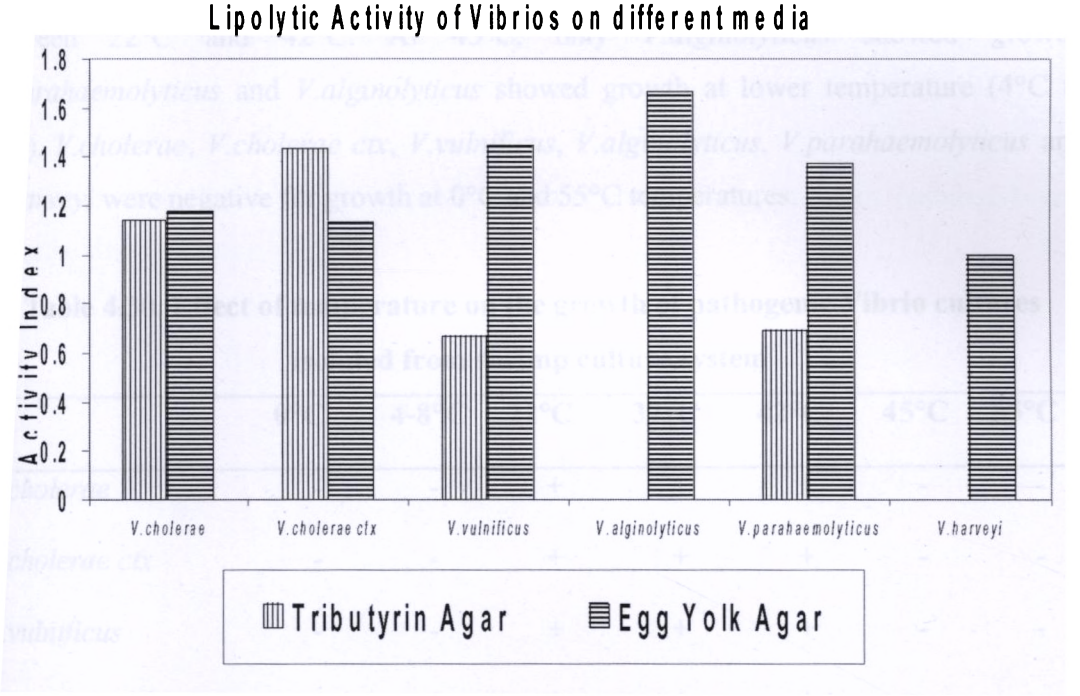


Fig 4.13. Lipase and Phospholipase activity of pathogenic Vibrios

4.2.4.5.5. Determination of phosphatase activity of Vibrio cultures isolated from shrimp culture system

V.cholerae, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* were positive for phosphatase activity. Beleneva and Maslennikova (2005) reported that *Vibrio spp.* isolated from mussel (*Mytilus trossulus*) produced alkaline phosphatases.

4.2.4.6. Growth kinetics of pathogenic Vibrios cultures isolated from shrimp culture system

The growth kinetics studies were carried out as described in the section (3.2.5.) under Materials and Methods.

4.2.4.6.1. Effect of temperature on the growth of pathogenic Vibrio cultures isolated from shrimp culture system

The result of the study on the growth of pathogenic vibrios at different temperature is given in Table 4.30. All the pathogenic vibrios showed good growth between 22°C and 42°C. At 45°C, only *V.alginolyticus* showed growth. *V.parahaemolyticus* and *V.alginolyticus* showed growth at lower temperature (4°C to 8°C). *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* were negative for growth at 0°C and 55°C temperatures.

Table 4.30. Effect of temperature on the growth of pathogenic Vibrio cultures isolated from shrimp culture system

	0°C	4-8°C	22°C	37°C	42°C	45°C	55°C
V.cholerae	-	-	+	+	+	-	-
V.cholerae ctx	-	-	+	+	+	-	-
V.vulnificus	-	-	+	+	+	-	-
V.parahaemolyticus	-	+	+	+	+	-	-
V.alginolyticus	-	+	+	+	+	+	-
V.harveyi	-	-	+	+	+	-	-

The results were similar to the findings of Guzman-Murillo et al (1994) who reported that by incubating TCBS plates at 40-42°C, it was possible to differentiate between pathogenic and non-pathogenic Vibrios. Sudha et al (1998) reported that the optimal temperature for growth of *V.parahaemolyticus*, *V.vulnificus*, *V.alginolyticus*, *V.mimicus* and *V.harveyi* was 37°C and all these species grew slowly at 15°C, but failed to grow at 4°C. Culture-based detection of *V. cholerae* is usually possible above a temperature of approximately 10°C to 15°C (Kirschner *et al.*, 2008). Magalhaes et al (2000) observed that the greatest survival of the *V.parahaemolyticus* was at ambient (28°C) temperature. Kaspar and Tamplin (1993) noticed that *V.vulnificus* survives poorly

below 8.5°C and temperatures outside the range of 13°C to 22°C reduce the survival of *V. vulnificus* in seawater. According to ICMSF (1996) *V. parahaemolyticus* and *V.alginolyticus* exhibit growth at 42°C. *V.parahaemolyticus* can grow between 5°C and 44°C with an optimum temperature of 30°C to 37°C; *V.cholerae* can grow between 10°C to 43°C with an optimum of 30°C to 37°C and *V.vulnificus* can grow between 8°C to 43°C. The results obtained in the present study are similar to these reported values.

4.2.4.6.2. Effect of pH on the growth of pathogenic Vibrio cultures isolated from shrimp culture system

Table 4.31. Effect of pH on the growth of pathogenic Vibrios

	pH 4	pH 7	pH 9	pH 12
V.cholerae	-	+	+	-
V.cholerae ctx	-	+	+	-
V.vulnificus	-	+	+	-
V.parahaemolyticus	-	+	+	-
V.alginolyticus	-	+	+	-
V.harveyi	-	+	+	-

The result of the study on the growth of pathogenic vibrios at different pH shows that these organisms grow at pH between 7 and 9 (Table 4.31). None of the pathogenic vibrios isolated from shrimp culture system showed growth either at pH 4 or pH 12. The results were in accordance with the values previously reported. According to ICMSF (1996) *V.parahaemolyticus* grows at pH between 4.8 and 11 with an optimum pH of 7.6 to 8.6; *V.cholerae* grows at pH between 5.0 and 9.6 with an optimum pH of 7.6 and *V.vulnificus* grows at pH between 5.0 and 10.0 with an optimum pH of 7.8. Vanderzant and Nickelson (1972) reported that *V. parahaemolyticus* was very sensitive to pH values

below 6.0. Lysine Decarboxylase plays a role in the adaptive acid tolerance in V.*parahaemolyticus* (Tanaka *et al.*, 2008)

4.2.4.6.3. Effect of salt on the growth of pathogenic Vibrio cultures isolated from shrimp culture system

Microorganisms require an aqueous environment for performing biochemical reactions. Addition of salt to media reduces water availability and adversely affects enzyme activities thereby impairs growth.

4.2.4.6.3.1. Effect of salt on the growth of *V.cholerae* isolated from shrimp farm

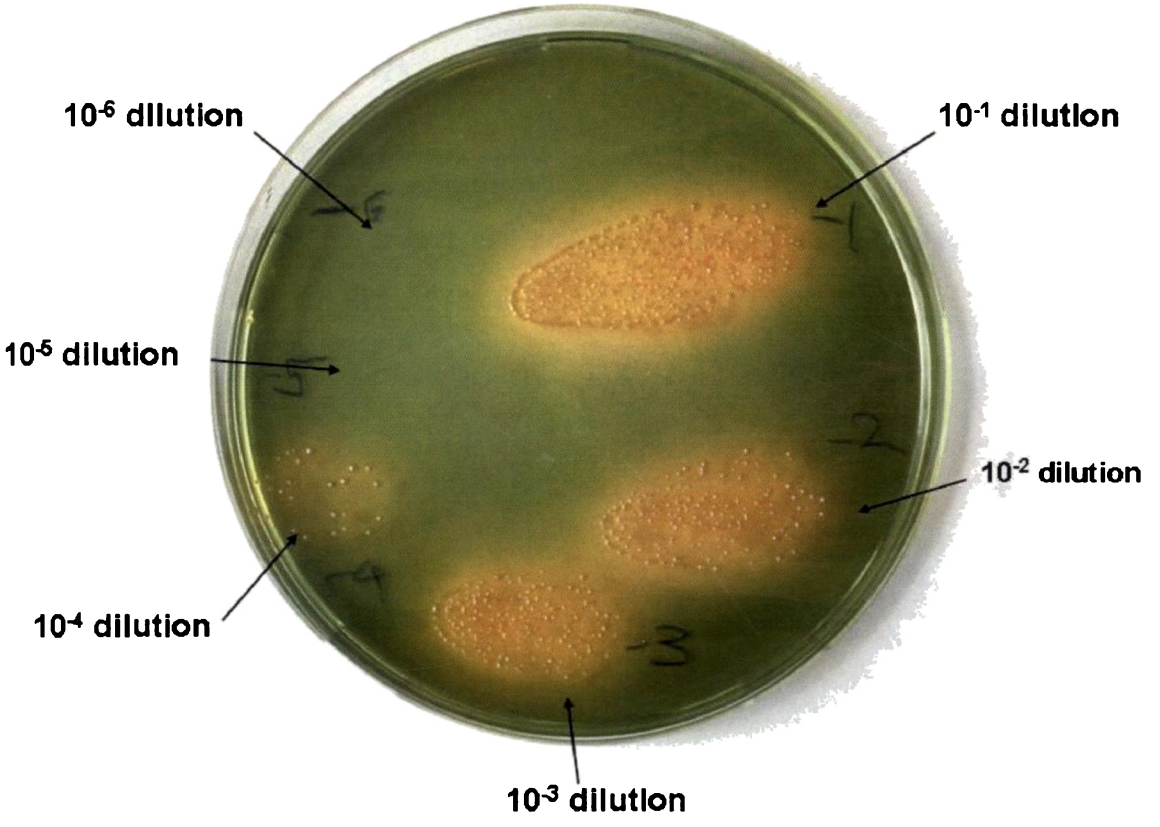


Fig. 4.14. Drop plate method on TCBS Agar for obtaining *V.cholerae* counts

The drop plate method was found to be very effective as it saves time, labour and media. The result on TCBS has the added advantage of differentiating sucrose fermenters from non-fermentors (Fig 4.14). The results of the drop plate method were identical to the conventional plating method.

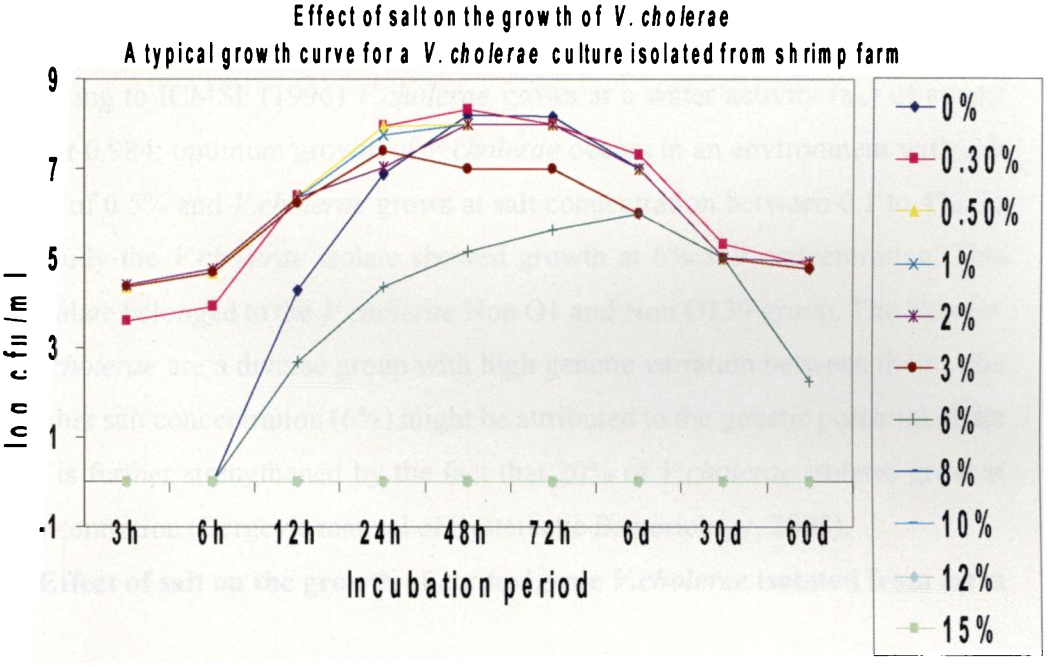


Fig 4.15. Effect of salt on the growth of *V.cholerae* isolated from shrimp farm

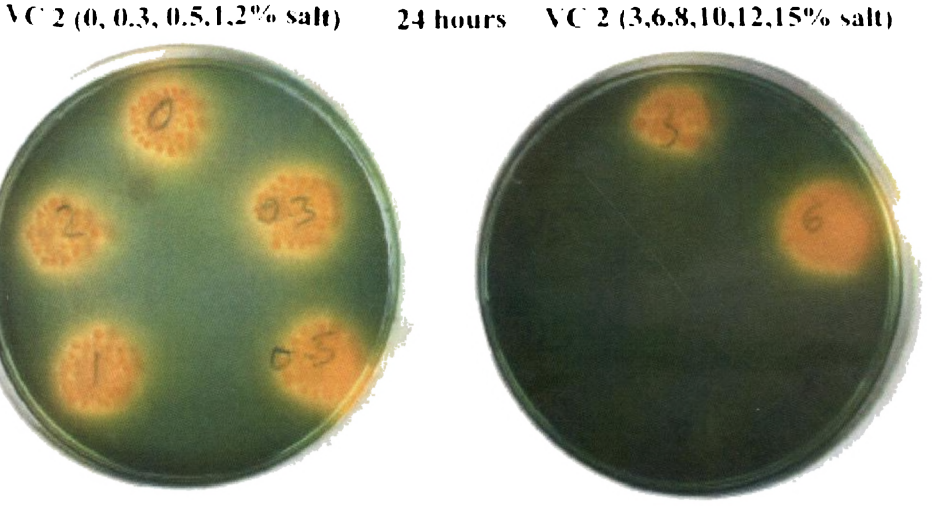


Fig 4.16. Quantitative assessment of effect of salt on the growth of *V.cholerae* by employing the Drop Plate method on TCBS agar

The result of the study on the effect of salt on the growth of *V.cholerae* was depicted in Fig. 4.15 and Fig. 4.16. Maximum growth of *V.cholerae* was observed between 0% and 2% salt concentration (log 8 cfu/ml to 8.3cfu/ml) followed by 3% (log 7cfu/ml) and 6% (log 5.6 cfu/ml) salt concentrations. The peak growth was achieved by 48h at 0% to 2% salt concentrations and by 6th day at 6% salt concentration. No growth was observed above 6% salt concentration.

According to ICMSF (1996) *V.cholerae* grows at a water activity (a_w) of atleast 0.97 but prefer 0.984; optimum growth of *V.cholerae* occurs in an environment with salt concentration of 0.5% and *V.cholerae* grows at salt concentration between 0.1 to 4%. In the present study the *V.cholerae* isolate showed growth at 6% salt concentration. This *V.cholerae* isolate belonged to the *V.cholerae* Non O1 and Non O139 group. The Non O1 and O139 *V.cholerae* are a diverse group with high genetic variation between them. The growth at higher salt concentration (6%) might be attributed to the genetic potential of the isolate. This is further strengthened by the fact that 20% of *V.cholerae* isolates grow at 6% NaCl concentration (Bergey's manual of Systematic Bacteriology, 2005).

4.2.4.6.3.2. Effect of salt on the growth of *ctx* toxigenic *V.cholerae* isolated from farm water

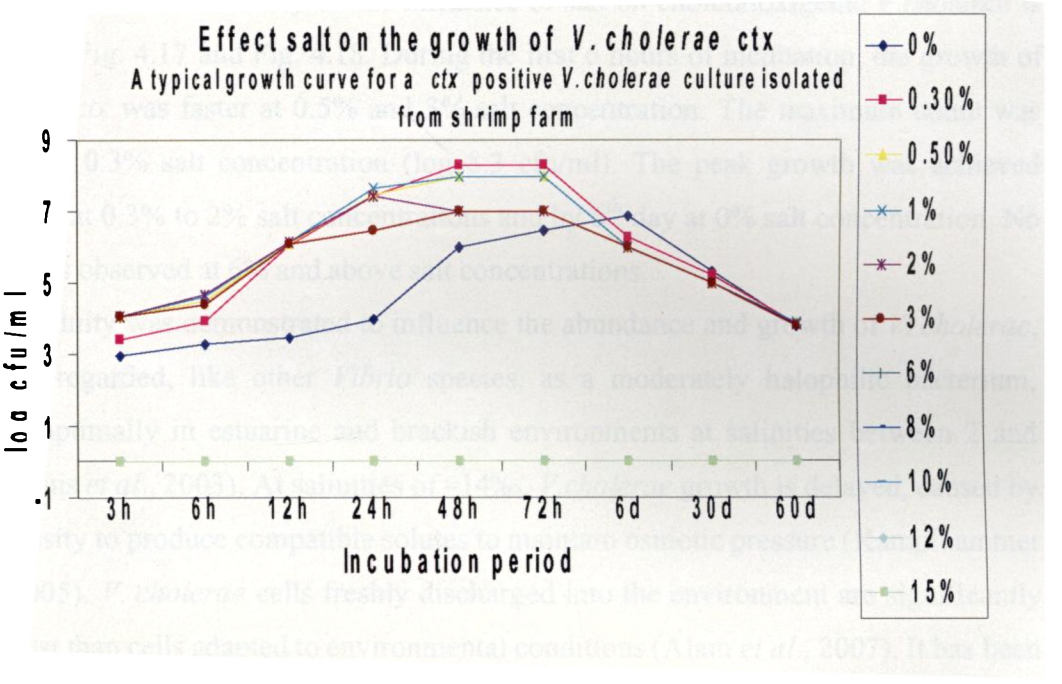


Fig. 4.17. Effect of salt on the growth of *ctx* toxigenic *V.cholerae*

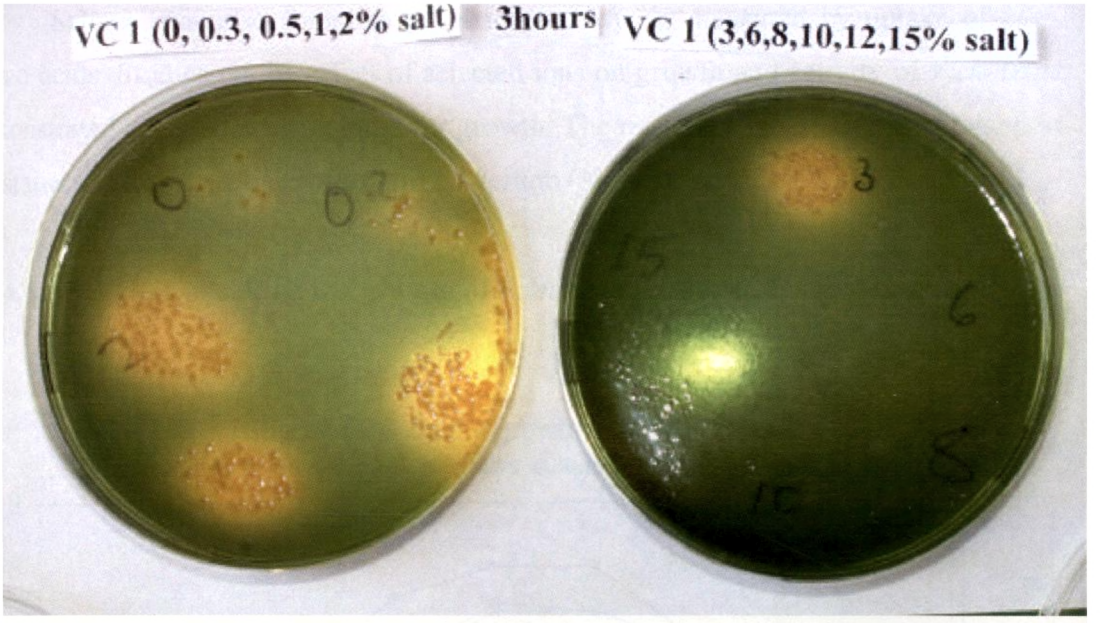


Fig. 4.18. Quantitative assessment of effect of salt on the growth of choleratoxigenic *V.cholerae* by employing the Drop Plate method on TCBS agar

The result of the study on the influence of salt on choleratoxigenic *V.cholerae* is depicted in Fig. 4.17 and Fig. 4.18. During the first 6 hours of incubation, the growth of *V.cholerae ctx* was faster at 0.5% and 3% salt concentration. The maximum count was obtained at 0.3% salt concentration (log 8.3 cfu/ml). The peak growth was achieved within 48h at 0.3% to 2% salt concentrations and by 6^{th} day at 0% salt concentration. No growth was observed at 6% and above salt concentrations.

Salinity was demonstrated to influence the abundance and growth of *V. cholerae*, which is regarded, like other *Vibrio* species, as a moderately halophilic bacterium, thriving optimally in estuarine and brackish environments at salinities between 2 and 14‰ (Louis *et al.*, 2003). At salinities of =14‰, *V.cholerae* growth is delayed, caused by the necessity to produce compatible solutes to maintain osmotic pressure (Kampfhammer *et al.*, 2005). *V. cholerae* cells freshly discharged into the environment are significantly less robust than cells adapted to environmental conditions (Alam *et al.*, 2007). It has been

shown that *V.cholerae* can survive in a starvation medium for as long as 75 days without significant decrease in culturability (Baker *et al.*, 1983).

Salinity was also found to influence activity, as measured by uptake of 14C-amino acids. Studies on the effect of selected ions on growth and activity of *V.cholerae* demonstrated that Na+ was required for growth. The results of this study further support the status of *V.cholerae* as an estuarine bacterium (Singleton *et al.*, 1982)

4.2.4.6.3.3. Effect of salt on the growth of *V.vulnificus* isolated from shrimp hatchery

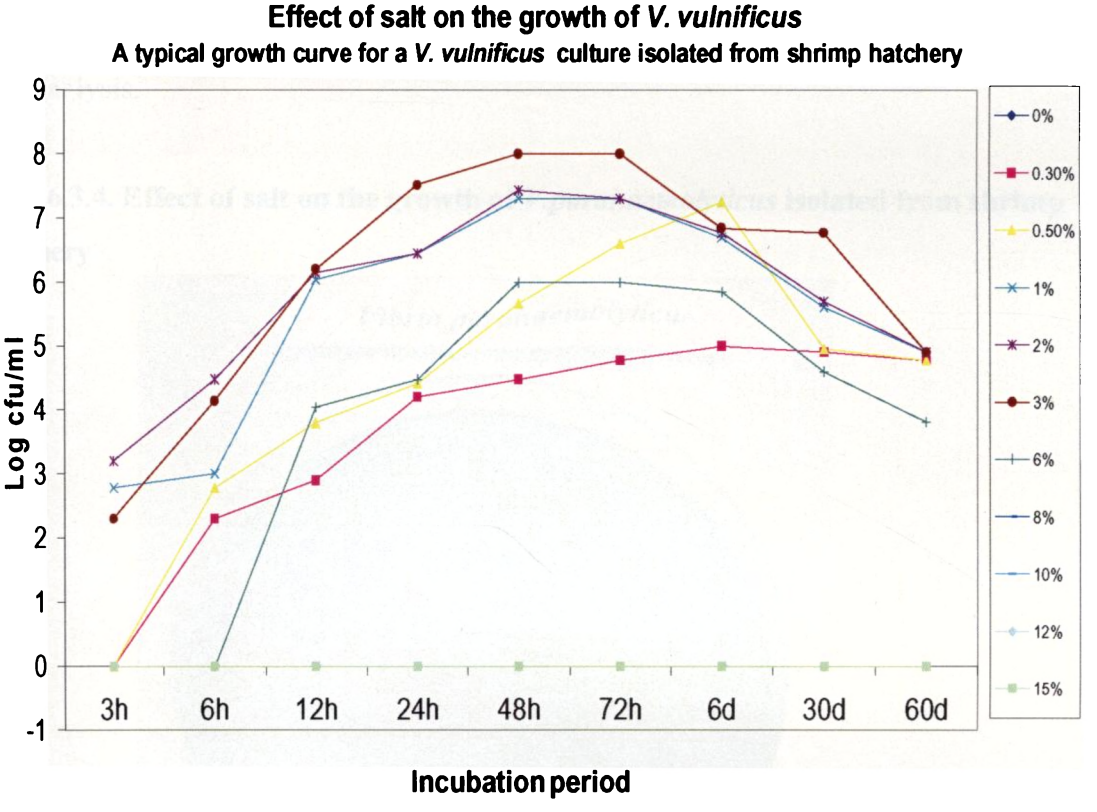


Fig. 4.19. Effect of salt on the growth of *V.vulnificus* isolated from shrimp hatchery

The influence of salt on the growth of *V.vulnificus* is depicted in Fig. 4.19. Maximum growth of *V.vulnificus* was observed at 3% (log 8 cfu/ml) followed by 2% (log 7.43 cfu/ml) and 1% (log 7.3 cfu/ml) salt concentrations. The peak growth was achieved

by 48h at 1% to 6% salt concentrations and by 6th day at 0.3% and 0.5% salt concentration. No growth at 0% and above 8% salt concentration. The results were in accordance with that mentioned in Bergey's manual of Systematic Bacteriology (2005) wherein the growth of *V.vulnificus* was reported at NaCl concentrations between 0.1% and 6%.

V.vulnificus strains from environmental or clinical sources were similar in susceptibility to various stresses (4 and 52 degree C, 0.1 and 10% NaCl, and pH 3.2) (Wong *et al.*, 2005). Although concentrations of *V.vulnificus* were positively correlated with temperature, salinity was a more important factor influencing variability of this organism (Lipp *et al.*, 2001). Marco-Noales et al (1999) observed that in the absence of salts, culturability of *V.vulnificus* dropped to zero in a few days, without evidence of cellular lysis.

4.2.4.6.3.4. Effect of salt on the growth of *V.parahaemolyticus* isolated from shrimp hatchery

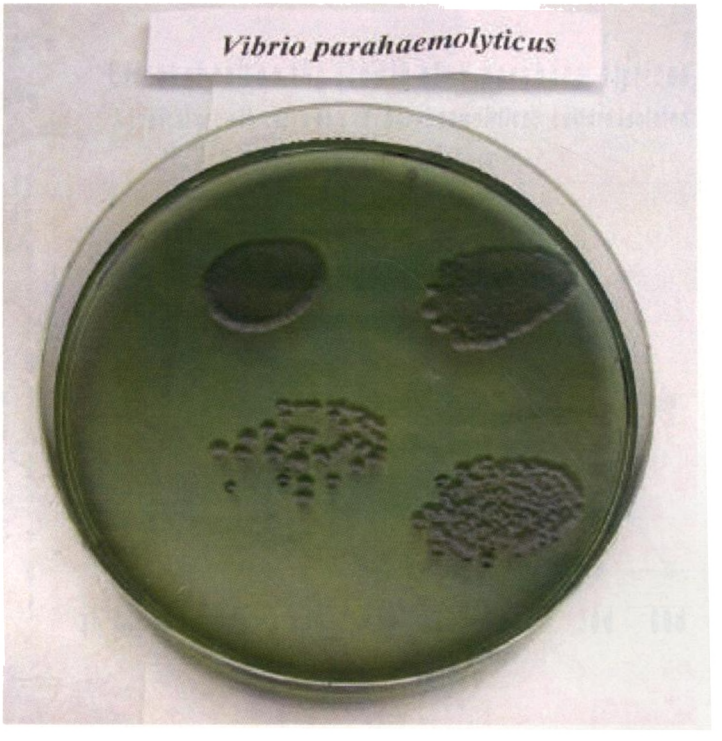


Fig. 4.20. Drop plate method on TCBS Agar for obtaining *V.parahaemolyticus* counts

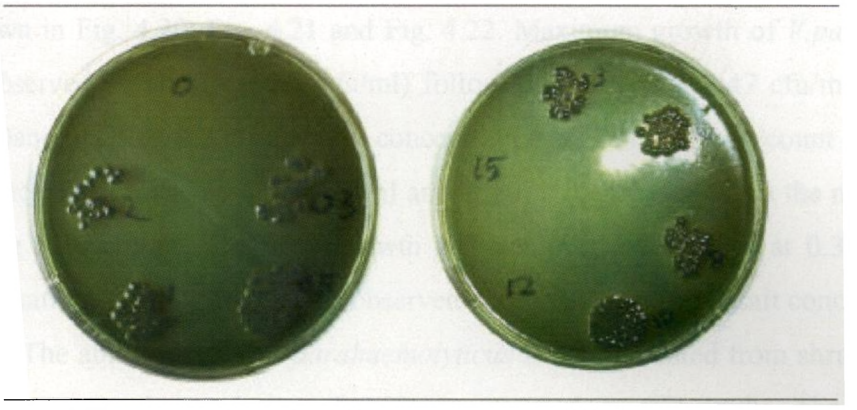


Fig. 4.21. Quantitative assessment of effect of salt on the growth of *V.parahaemolyticus* by employing the Drop Plate method on TCBS agar

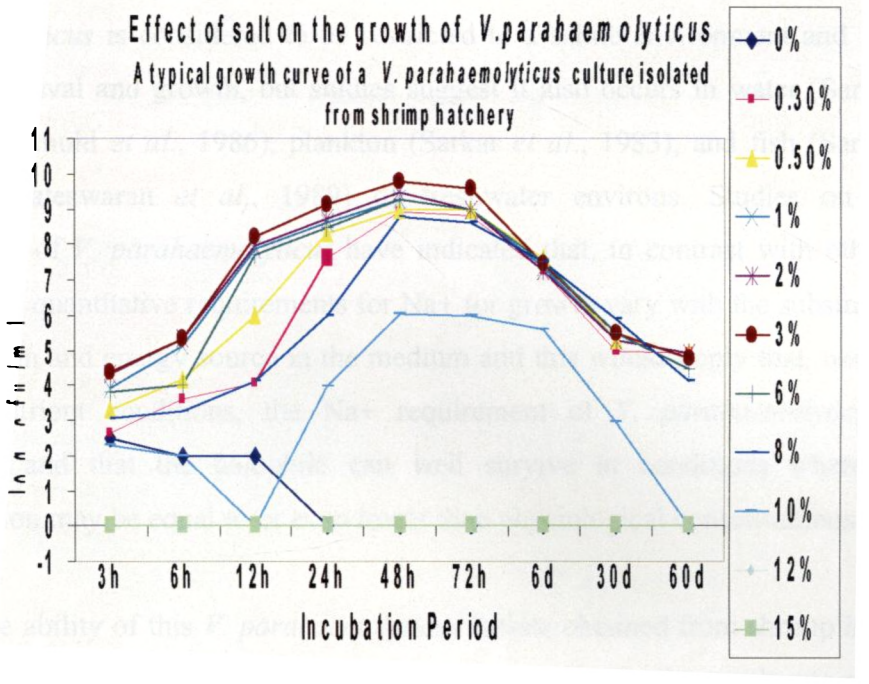


Fig. 4.22. Effect of salt on the growth of *V.parahaemolyticus* isolated from shrimp hatchery

The result of the studies on the effect of salt on the growth of *V.parahaemolyticus* is shown in Fig. 4.20, Fig. 4.21 and Fig. 4.22. Maximum growth of *V.parahaemolyticus* was observed at 3% (log 9.77 cfu/ml) followed by 2% (log 9.47 cfu/ml), 1% (log 9.3 cfu/ml)and 6% (log 9.3 cfu/ml) salt concentrations. The maximum count achieved at 8% salt concentration was log 8.77 cfu/ml and at 10% salt concentration the maximum count was log 6.04 cfu/ml. The peak growth was achieved within 48h at 0.3% to 10% salt concentrations and no growth was observed at 0%, 12% and 15% salt concentration.

The ability of this *V. parahaemolyticus* isolate obtained from shrimp hatchery to grow at a very low salinity of 0.3% is reported in this study. The growth of *V. parahaemolyticus* at lower salinities was reported in few cases. 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) and 80% of the *V.parahaemolyticus* isolates showed growth at 0.4% NaCl concentration. *V. parahaemolyticus* is considered to be restricted to a saline environment and it requires Na+ for survival and growth, but studies suggest it also occurs in water (Sarkar *et al.*, 1985; Bockemuhl *et al.*, 1986), plankton (Sarkar *et al.*, 1983), and fish (Sarkar *et al.*, 1985, Venkateswaran *et al.*, 1989) of freshwater environs. Studies on the Na+ requirement of *V. parahaemolyticus* have indicated that, in contrast with other marine bacteria, the quantitative requirements for Na+ for growth vary with the substrate serving as the carbon and energy source in the medium and this would imply that, under certain specific nutrient conditions, the Na+ requirement of *V. parahaemolyticus* is not mandatory and that the halophile can well survive in conditions where the salt concentration may be equal to or even lower than physiological concentrations (Sarkar et al., 1985).

The ability of this *V. parahaemolyticus* isolate obtained from shrimp hatchery to grow at a higher salinity of 10% is reported in this study. Similar result was reported by other studies. Other studies have reported that *V. parahaemolyticus* is capable of growth in the presence of 10% NaCl (Colwell 1970; Kampelmacher *et al.*, 1972; Gjerde and Boe 1981; Schandevyl *et al.*, 1984). Zen-Yoji *et al.*, (1973) reported that all

V.parahaemolyticus strains tested grew in the presence of 9% NaCl but that only one strain grew in the presence of 11% NaCl.

4.2.4.6.3.5. Effect of salt on the growth of *V.alginolyticus* isolated from shrimp farm

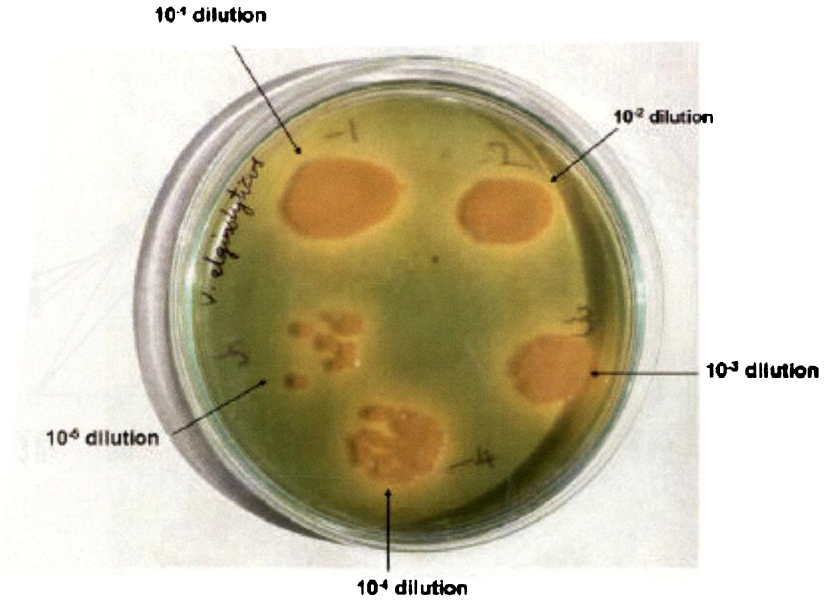


Fig. 4.23. Drop plate method on TCBS Agar for obtaining *V. alginolyticus* counts

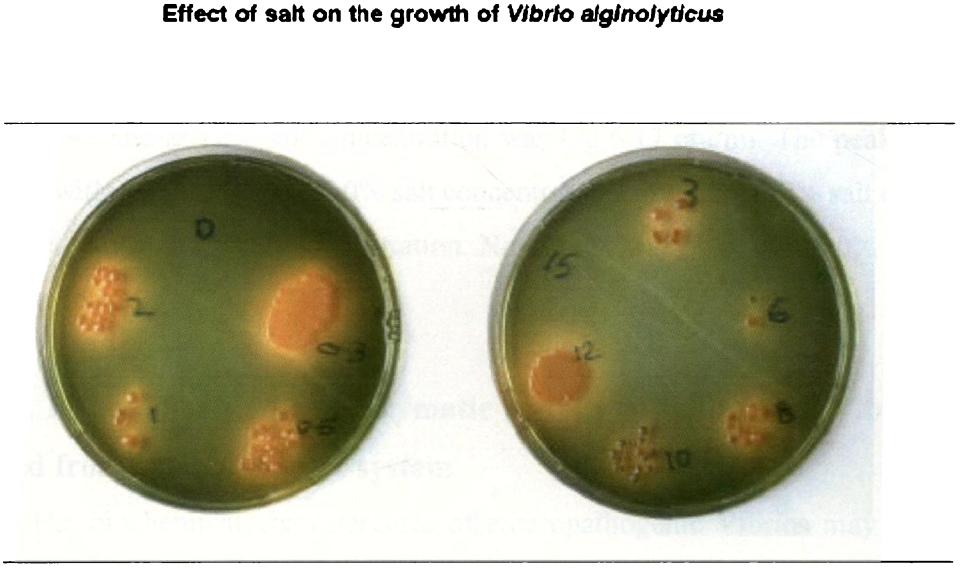


Fig. 4.24. Quantitative assessment of effect of salt on the growth of *V. alginolyticus* by employing the Drop Plate method on TCBS agar

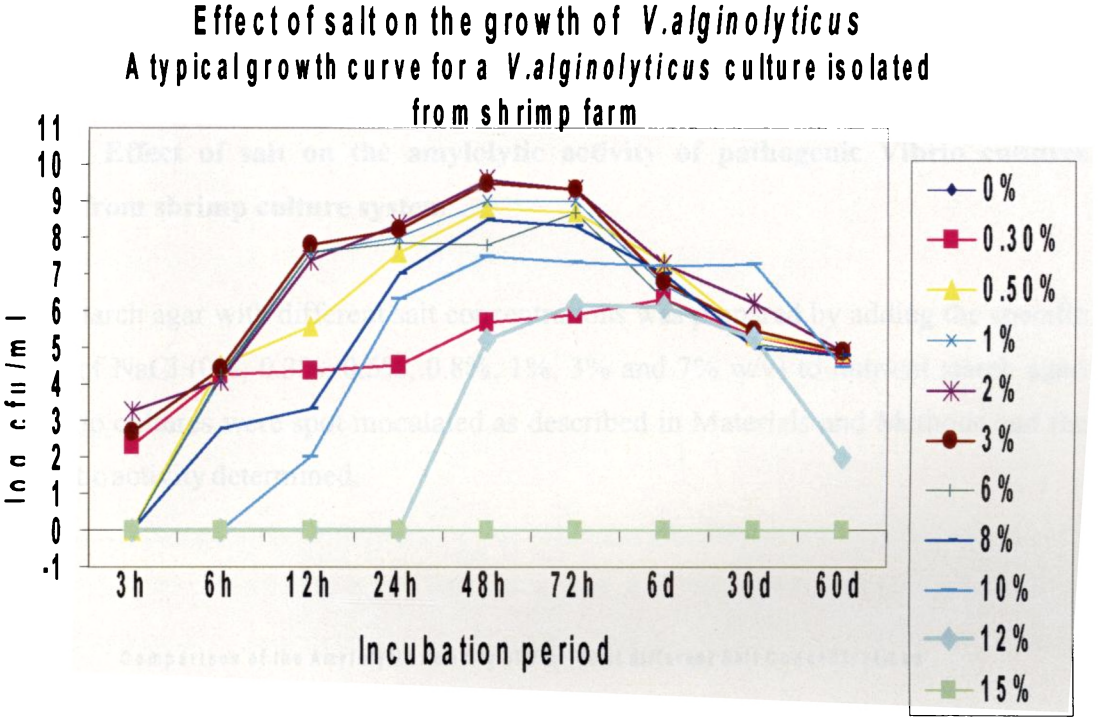


Fig. 4.25. Effect of salt on the growth of *V.alginolyticus* isolated from shrimp farm

The result of the study on the influence of salt on the growth of *V.alginolyticus* is depicted in Fig. 4.23, Fig. 4.24 and Fig. 4.25. Maximum growth was observed at 2% salt concentration (log 9.6 cfu/ml) followed by 3% (log 9.47 cfu/ml) and 0.5% salt concentrations (log 8.78 cfu/ml). The maximum count achieved at 10% salt concentration was log 7.47 and at 12% salt concentration was log 6.17 cfu/ml. The peak growth was achieved within 48h at 0.5% to 10% salt concentrations; by 72h at 12% salt concentration and by 6th day at 0.3% salt concentration. No growth was observed at 0% and 15% salt concentrations.

4.2.4.7. Effect of salt on the enzymatic activity of pathogenic Vibrio cultures isolated from shrimp culture system

The biochemical characteristics of enteropathogenic Vibrios may be markedly affected by small change in NaCl concentration. A wide range of biochemical characteristics may be affected and there is considerable variation from strain to strain. In general the greatest number of biochemical characteristics is expressed at or just below the optimal NaCl concentration for growth. Temperature dependence appears secondary.

Both carbohydrate fermentation and amino acid decarboxylase reactions are inhibited at NaCl concentrations above or below the optimum.

4.2.4.7.1. Effect of salt on the amylolytic activity of pathogenic Vibrio cultures isolated from shrimp culture system

Starch agar with different salt concentrations was prepared by adding the specific amount of NaCl (0%, 0.3%, 0.5%, 0.8%, 1%, 3% and 7% w/v) to nutrient starch agar. The vibrio cultures were spot inoculated as described in Materials and Methods and the amylolytic activity determined.

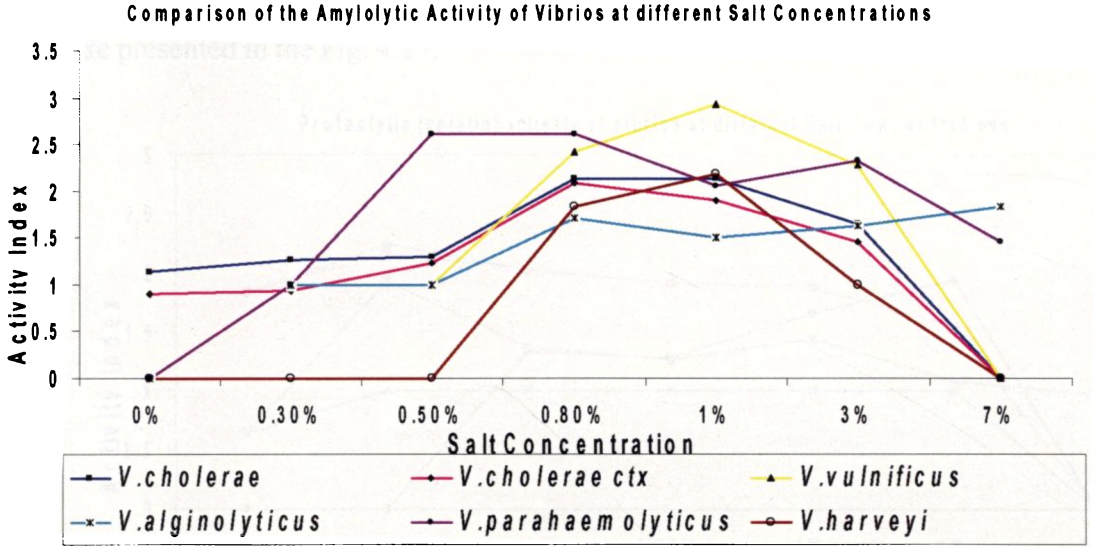


Fig. 4.26. Comparison of amylolytic activity at different salt concentrations of pathogenic Vibrios isolated from shrimp culture system

Amylolytic activity was detected in all the human pathogenic Vibrio species. The amylolytic activity varied with the salt concentration and the results are shown in Fig. 4.26. All isolates showed amylolytic activity at 0.8% salt concentration. At 0% salt concentration, only *V.cholerae* and *V.cholerae ctx* showed amylolytic activity.

Amylolytic activity was maximum at 0.8% salt concentration for *V.cholerae*, *V.cholerae ctx*, *V.parahaemolyticus*; at 0.5% concentration for *V.alginolyticus* and at 1% concentration for *V.vulnificus* and *V. harveyi*. At higher salt concentration (7%) *V.parahaemolyticus* and *V.alginolyticus* only produced amylase. The maximum amylolytic activity index was given by *V.vulnificus* at 1% (2.9) followed by *V.parahaemolyticus* at 0.8% (2.615) and *V.alginolyticus* at 0.5% (2.415).

4.2.4.7.2. Effect of salt on the proteolytic activity of pathogenic Vibrio cultures isolated from shrimp culture system

Gelatin agar with different salt concentrations was prepared by adding the specific amount of NaCl (0%, 0.3%, 0.5%, 0.8%, 1%, 3% and 7% w/v) to nutrient gelatin agar. Gelatinolytic activity was determined as described under Materials and Methods. Typical results are presented in the Fig. 4.27.

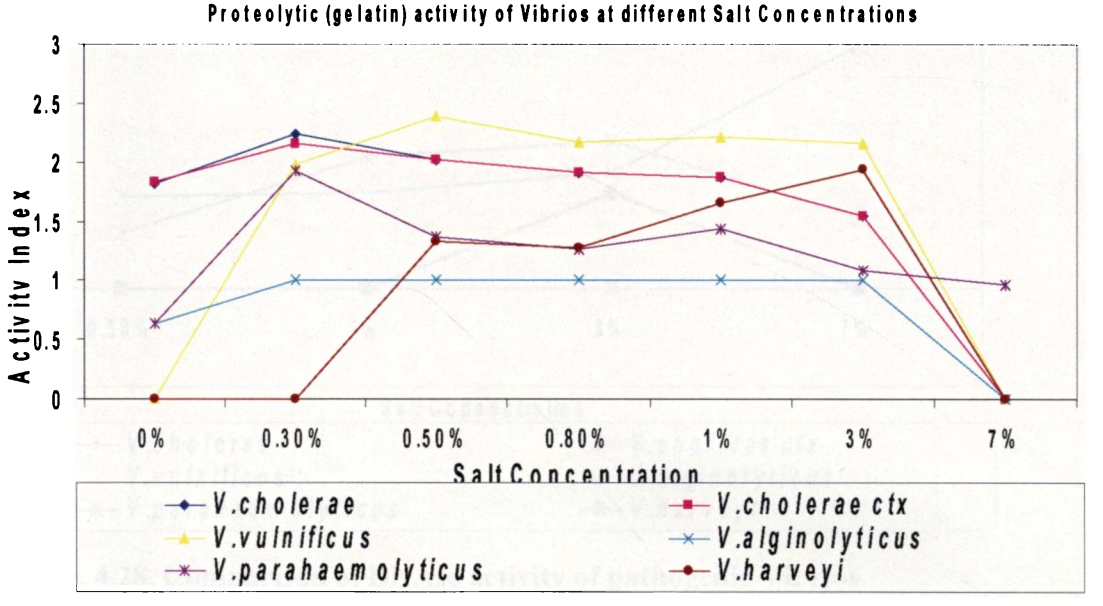


Fig. 4.27. Comparison of proteolytic activity of pathogenic Vibrios at different salt concentrations

Proteolytic activity using gelatin as protein source was observed in all the pathogenic Vibrio species. The pathogenic Vibrios showed proteolytic activity at a

relatively lower salt concentration (Fig. 4.27). 0.3% salt concentration was sufficient for *V.cholerae*, *V.cholerae ctx*, *V.alginolyticus*, *V.parahaemolyticus* to produce gelatinase. For *V.vulnificus* 0.5% salt concentration was found necessary. The maximum proteolytic activity index was observed with *V.vulnificus* at 0.5% (2.4) followed by *V.cholerae* at 0.3% and *V.cholerae ctx* at 0.3% (2.2). At higher salt concentration (7%) only *V.parahaemolyticus* was able to produce gelatinase.

4.2.4.7.3. Effect of salt on the DNAse activity pathogenic Vibrio cultures isolated from shrimp culture system

DNAse agar with different salt concentrations was prepared by adding the specific amount of NaCl (0.5%, 1%, 3% and 7% w/v). DNAse activity was determined as desribed in Materials and Methods. Typical results are presented in Fig. 4.28.

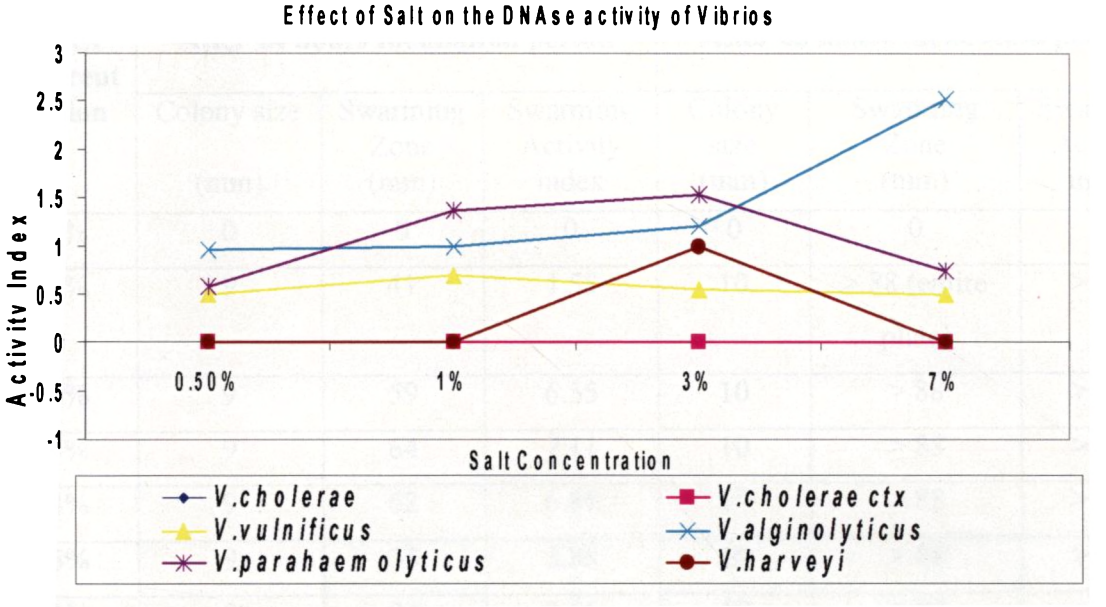


Fig. 4.28. Comparison of DNAse activity of pathogenic Vibrios at different salt concentrations

V.cholerae and choleratoxigenic *V.cholerae* isolates were negative for DNAse activity whereas *V.vulnificus*, *V.alginolyticus* and *V.harveyi* showed DNAse activity. DNAse activity was maximum in *V.alginolyticus*. The result of the study on the effect of salt on the DNAse activity is depicted in Fig. 4.28. At lower salt concentration the

swarming behaviour of *V.alginolyticus* made it difficult to estimate the activity index and as the colony size and activity zone appear same. *V.alginolyticus* showed an increasing trend of DNAse activity from 0.5%, to 7% salt concentration. The DNAse activity of *V.parahaemolyticus* increased from 0.5% to 3% and thereafter decreased.

4.2.4.8. Effect of salt on the swarming behaviour of *V.alginolyticus* isolated from shrimp culture system

10µl of overnight culture of *V.alginolyticus* grown in T_1N_3 was spotted on Tryptone Glucose agar with the corresponding concentration of salt (0% to 25% (w/v) NaCl). The size of the colony and the swarming zone for each NaCl concentration was measured and tabulated (Table 4.32).

Table 4.32. Effect of salt on the swarming behaviour of *V.alginolyticus*

NaCl Concentration	After 24 hours incubation period			After 48 hours incubation period		
	Colony size (mm)	Swarming Zone (mm)	Swarming Activity index	Colony size (mm)	Swarming Zone (mm)	Swarming Activity index
0%	0	0	0	0	0	0
1%	9	41	4.55	10	> 88 (entire plate)	> 8.8
2%	9	59	6.55	10	> 88	> 8.8
3%	9	64	7.11	10	> 88	> 8.8
4%	9	62	6.88	10	> 88	> 8.8
5%	9	35	3.88	10	> 88	> 8.8
6%	9	24	2.66	10	> 88	> 8.8
7%	8	8	1	8	34	4.25
8%	8	8	1	8	33	4.13
9%	8	8	1	8	14	1.75
10%	7 (faint growth)	7	1	8	8	1
12%	0	0	0	0	0	0
15%	0	0	0	0	0	0

18%	0	0	0	0	0	0
20%	0	0	0	0	0	0
25%	0	0	0	0	0	0

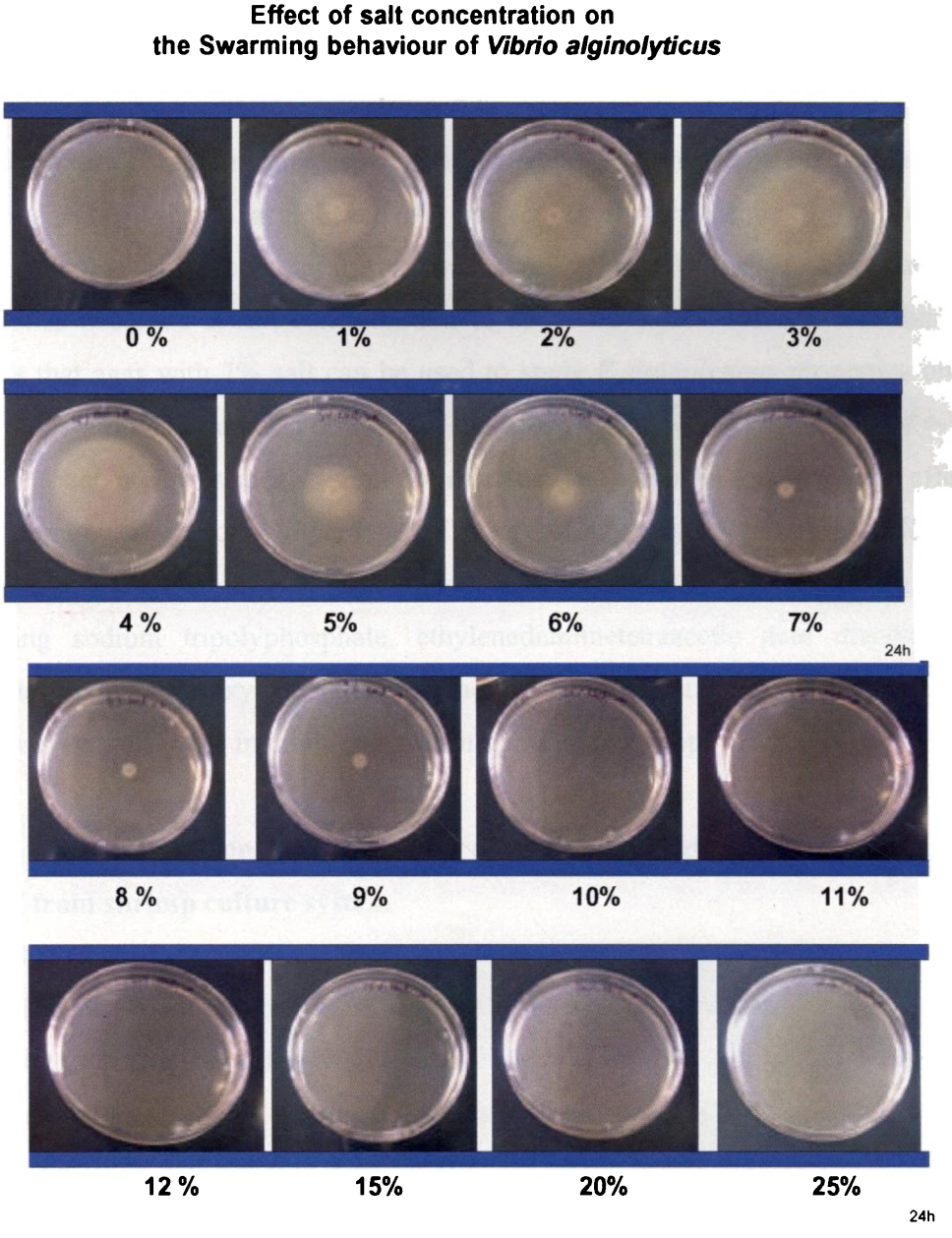


Fig. 4.29. Effect of salt on the swarming behaviour of *V.alginolyticus* (24 hours)

The effect of NaCl concentration on the swarming behaviour of *V.alginolyticus* is given in Table 4.32 and Fig. 4.29. The swarming zone of *V.alginolyticus* increased from 1% salt concentration to 3% salt concentration and thereafter showed a decreasing trend. No swarming was observed at 7% to 10% salt concentrations and no growth was observed at salt concentration of above 12%. The results at 48 hours showed that *V.alginolyticus* showed an increasing trend of swarming up to 6% salt concentration. Swarming was observed at 7% and 8% salt concentration. Minimal swarming was observed at 9% whereas no swarming was observed at 10% salt concentrations. No growth was observed at salt concentration of above 12%. The results from this study suggests that agar with 7% salt can be used to study *V.alginolyticus* properties on agar media; with minimal swarming problem of this species.

Surfactant agents such as cholic acid or its salts have been reported to be effective in inhibiting swarming of *V.alginolyticus* by way of inhibiting lateral flagella formation and movement (Boer *et al.*, 1975).Thampuran and Surendran (1993) developed a medium containing sodium tripolyphosphate, ethylenediaminetetraacetic acid disodium salt, dihydrate, sodium desoxycholate, supplemented with minerals and nutrient sources, which was very effective in inhibiting swarming of *Bacillus* spp.

4.2.4.9. Effect of salt on the utilization of sugars by pathogenic Vibrio cultures isolated from shrimp culture system

4.2.4.9.1. Effect of salt on the utilization of sucrose by pathogenic Vibrios

The effect of salt on the utilization of sucrose and mannitol by Vibrios was studied by preparing sugar fermentation broth containing the corresponding sugar at 1% level and to which NaCl was added at different concentrations. Initially the sugar utilization activity was tested at 0%, 3%, 6%, 9% and 12% salt concentrations. Based on the results at these concentrations (0%, 3%, 6%, 9% and 12%) further tests were carried out for each isolate in media containing intervening salt concentrations as outlined in the Materials and Methods section. Sucrose and mannitol were selected for the study. Sucrose is the sugar that is primarily employed to differentiate the pathogenic Vibrios on TCBS agar. Mannitol was chosen as all the pathogenic Vibrios utilized this sugar alcohol.

Table 4.33. Effect of salt on the utilization of Sucrose by pathogenic Vibrio cultures isolated from shrimp culture system

	0 %	0.1%	0.3%	0.5%	1%	3%	4%	5%	6%	7%	8%	9%	10%	11%	12%	15%	20%
VA 1*	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-
VA 2	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
VA 3	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
VA 4	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
VV1	-NG	-NG	-NG	-G	-G	- G	-G	- G	-G	-NG	-NG	-NG	-NG	-NG	-NG	-NG	-NG
VV 2	-NG	-NG	-NG	-G	-G	-G	-G	-G	-G	-NG	-NG	-NG	-NG	-NG	-NG	-NG	-NG
VV3	-NG	-NG	-NG	-G	-G	- G	-G	- G	-G	-NG	-NG	-NG	-NG	-NG	-NG	-NG	-NG
VV 4	-NG	-NG	-NG	-G	-G	-G	-G	-G	-G	-NG	-NG	-NG	-NG	-NG	-NG	-NG	-NG
VH 1	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
VH2	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
VC 1	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
VC 2	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
VC 3	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
VC 4	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
VCctx 1	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
VCctx 2	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
VCctx 3	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
VP1	-NG	-NG	-NG	-G	-G	- G	-G	-G	-G	- G	-G	-	-	-	-	-	-

VP2	-NG	-NG	-NG	-G	-G	-G	-G	-G	-G	-G	-G	-	-	-	-	-	-
VP3	-NG	-NG	-NG	-G	-G	- G	-G	-G	-G	- G	-G	-	-	-	-	-	-
VP3	-NG	-NG	-NG	-G	-G	-G	-G	-G	-G	-G	-G	-	-	-	-	-	-

+ : positive test result, - : negative test result (-)G : negative, but growth observed, (-) NG : No Growth

*VA- *V.alginolyticus*, VC- *V.cholerae*, VCctx -choleratoxigenic *V.cholerae*, VH – *V.harveyi*, VP- *V.parahaemolyticus*, VV- *V.vulnificus*

The utilization of sucrose by the pathogenic vibrios at different salt concentrations is given in Table 4.33. 100% isolates of *V.cholerae* and *V.cholerae ctx* isolates utilized sucrose between salt concentrations of 0% and 3%. 100% of *V.cholerae* and 66% of *V.cholerae ctx* isolates utilized sucrose at 4% salt concentration. 50% of the *V.cholerae* isolates utilized sucrose at 5% salt concentration whereas as none of the *V.cholerae ctx* isolates utilized sucrose at 4% salt concentration. *V.cholerae* and *V.cholerae ctx* isolates failed to utilized sucrose at salt concentrations of 6% and above. 100% isolates of *V.alginolyticus* isolates utilized sucrose between salt concentrations of 0.5% and 10%. 75% isolates of *V.alginolyticus* utilized sucrose at 0.3% salt concentration. None of the *V.alginolyticus* isolates could utilize sucrose at 0%, 0.1% and 11% above salt concentrations. *V.harveyi* isolates fermented sucrose at salt concentrations between 0.5% and 6%. *V.harveyi* isolates showed negative result at 0%, 0.1%, 0.3% and = 7% salt concentrations. *V.vulnificus* isolates showed growth but negative reaction for sucrose fermentation at salt concentrations between 0.5% and 6%. None of the *V.vulnificus* isolates showed growth at 0%, 0.1%, 0.3% and 7% and above salt concentrations. All the *V.parahaemolyticus* isolates showed growth but negative reaction for sucrose fermentation at salt concentrations between 0.5% and 8%. No growth was observed at salt concentration of 9% and above and at 0.3%, 0.1% and 0% salt concentrations.

4.2.4.9.2. Effect of salt on the utilization of Mannitol by pathogenic Vibrios

Table 4.34. Effect of salt on the utilization of Mannitol by of pathogenic Vibrio cultures isolated from shrimp culture system

	0 %	0.1%	0.3%	0.5%	1%	3%	4%	5%	6%	7%	8%	9%	10%	11%	12%	15%	20%
VA 1*	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-
VA 2	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-
VA3	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
VA 4	-	-	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-
VV 1	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
VV 2	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
VV 3	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
VV 4	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
VH 1	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
VH 2	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
VC 1	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
VC 2	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
VC 3	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
VC 4	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
VCctx 1	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-

VCctx 2	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
VCctx 3	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
VP1	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-
VP2	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-
VP3	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-
VP4	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-

*VA- *V.alginolyticus*, VC- *V.cholerae*, VCctx -choleratoxigenic *V.cholerae*, VH – *V.harveyi*, VP- *V.parahaemolyticus*, VV- *V.vulnificus*

+ : positive result, (-): negative result

The utilization of mannitol by the pathogenic vibrios at different salt concentrations is given in Table 4.34. 100% isolates of *V.cholerae* and *V.cholerae ctx* isolates utilized mannitol between salt concentrations of 0% and 3%. 100% of *V.cholerae* and 66% of *V.cholerae ctx* isolates utilized sucrose at 5% salt concentration. *V.cholerae* and *V.cholerae ctx* isolates failed to utilize mannitol at salt concentrations of = 6%. 100% isolates of *V.alginolyticus* isolates utilized mannitol between salt concentrations of 0.5% and 10%. 50% isolates of *V.alginolyticus* utilized mannitol at 0.3% salt concentration. None of the *V.alginolyticus* isolates could utilize sucrose at 0%, 0.1% and = 11% salt concentrations. *V.harveyi* isolates fermented mannitol at salt concentrations between 0.5% and 6%. *V.vulnificus* isolates utilized mannitol at salt concentrations between 0.5% and 6% while *V.parahaemolyticus* isolates utilized mannitol at salt concentrations between 0.5% and 8%.

4.2.4.9.3. Effect of salt on the utilization of Cellobiose by *V.vulnificus* isolated from shrimp hatchery

V.vulnificus isolates were tested for their ability to utilize cellobiose at different salt concentrations ranging between 0% and 20% NaCl (w/v) and the results given in Table 4.35.

Table 4.35. Effect of salt on the utilization of Cellobiose by *V.vulnificus*

	0 %	3%	4%	5%	6%	7%	8%	9%	12%	15%	20%
VV1*	-	+	+	+	+	-	-	-	-	-	-
VV2	-	+	+	+	+	-	-	-	-	-	-
VV3	-	+	+	+	+	-	-	-	-	-	-
VV4	-	+	+	+	+	-	-	-	-	-	-

*VV- *V.vulnificus* + : positive result, (-): negative result

The maximum salt concentration at which *V.vulnificus* isolates utilized cellobiose was 6%. No growth was observed at 0% salt concentration.

4.2.4.10. Effect of salt on the utilization of amino acids by pathogenic Vibrio cultures isolated from shrimp culture system

The effect of salt on the utilization of arginine, lysine and ornithine by Vibrios was studied by preparing Moller's decarboxylase broth containing the corresponding amino acid at 0.5% level and to which NaCl was added at different concentrations. Initially the amino acid utilization activity was tested at 0%, 3%, 6%, 9% and 12% salt concentrations. Based on the results at these concentrations (0%, 3%, 6%, 9% and 12%) further tests were carried out for each isolate in media containing intervening salt concentrations.

4.2.4.10.1. Effect of salt on the Arginine dihydrolase activity of Vibrios

Table 4.36. Effect of salt on the Arginine dihydrolase activity of pathogenic Vibrio cultures isolated from shrimp culture system

	0%	0.1%	0.2 %	0.5%	1%	3%	4%	5%	6%	7%	8%	9%	10%	11%	12%	15%	20%
VA 1*	NG	NG	-	-	-	-	-	-	-	-	-	-	-	-	±	NG	NG
VA 2	NG	NG	-	-	-	-	-	-	-	-	-	-	-	-	±	NG	NG
VA 3	NG	NG	-	-	-	-	-	-	-	-	-	-	-	-	±	NG	NG
VA 4	NG	NG	-	-	-	-	-	-	-	-	-	-	-	-	±	NG	NG
VV 1	NG	NG	NG	-	-	-	-	-	-	NG	NG	NG	NG	NG	NG	NG	NG
VV 2	NG	NG	NG	-	-	-	-	-	-	NG	NG	NG	NG	NG	NG	NG	NG
VV 3	NG	NG	NG	-	-	-	-	-	-	NG	NG	NG	NG	NG	NG	NG	NG
VV 4	NG	NG	NG	-	-	-	-	-	-	NG	NG	NG	NG	NG	NG	NG	NG
VH 1	NG	NG	NG	NG	-	-	-	-	-	NG	NG	NG	NG	NG	NG	NG	NG
VH 2	NG	NG	NG	NG	-	-	-	-	-	-	NG	NG	NG	NG	NG	NG	NG
VC 1	-	-	-	-	-	-	-	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
VC 2	-	-	-	-	-	-	-	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
VC 3	-	-	-	-	-	-	-	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG

VC 4	-	-	-	-	-	-	-	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
VCctx 1	-	-	-	-	-	-	-	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
VCctx 2	-	-	-	-	-	-	-	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
VCctx 3	-	-	-	-	-	-	-	NG	NG	NG	NG	NG	NG	NG	NG	NG	NG
VP 1	NG	NG	NG	-	-	-	-	-	-	-	-	NG	NG	NG	NG	NG	NG
VP 2	NG	NG	NG	-	-	-	-	-	-	-	-	NG	NG	NG	NG	NG	NG
VP 3	NG	NG	NG	-	-	-	-	-	-	-	-	NG	NG	NG	NG	NG	NG
VP 4	NG	NG	NG	-	-	-	-	-	-	-	-	NG	NG	NG	NG	NG	NG

+ : positive test result, - : negative test result, NG: No Growth

*VA- *V alginolyticus*, VC- *V.cholerae*, VCctx -choleratoxigenic *V.cholerae*, VH – *V.harveyi*, VP- *V.parahaemolyticus*, VV- *V.vulnificus*

The result of the study on the arginine dihydrolase activity of pathogenic vibrios is given in Table 4.36. *V. cholerae* and *V.cholerae ctx* isolates showed negative reaction for arginine dihydrolase activity between 0% and 4% salt concentrations. *V.alginolyticus* isolates showed negative reaction for arginine dihydrolase activity between 0.2% and 11% salt concentrations but at 12% salt concentration the reaction was not clear. *V.alginolyticus* isolates did not show arginine dihydrolase activity at 0%, 0.1%, 15% and 20% salt concentration. *V.vulnificus* showed negative reaction for arginine dihydrolase activity between 0.5% and 6% salt concentrations but did not show growth at 0%, 0.1%, 0.3% and =7% salt concentration. *V.parahaemolyticus* showed negative reaction for arginine dihydrolase activity between 0.5% and 8% salt concentrations but did not show growth at 0%, 0.1%, 0.3% and =9% salt concentration. The results are as expected, because Vibrio species are generally negative for arginine dihydrolase activity.

4.2.4.10.2. Effect of salt on the Lysine decarboxylase activity of Vibrios

Table 4.37. Effect of salt Lysine decarboxylase activity of pathogenic Vibrio cultures isolated from shrimp culture system

	0 %	0.1%	0.2%	0.5%	1%	3%	4%	5%	6%	7%	8%	9%	10%	11%	12%	15%	20%
VA 1*	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
VA 2	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
VA 3	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
VA 4	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
VV1	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
VV 2	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
VV3	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
VV4	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
VH 1	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-
VH 2	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-
VC 1	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
VC 2	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
VC 3	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
VC 4	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
VCctx 1	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
VCctx 2	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-
VCctx 3	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-

VP 1	-	-	-	+	+	+	+	+	+	+	+	+	-	-	-	-	-
VP 2	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-
VP 3	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-
VP 4	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-

+ : positive test result, - : negative test result

*VA- *V.alginolyticus*, VC- *V.cholerae*, VCctx -choleratoxigenic *V.cholerae*, VH – *V.harveyi*, VP- *V.parahaemolyticus*, VV- *V.vulnificus*

The result of the study on the lysine decarboxylase activity of pathogenic vibrios is given in Table 4.37. *V. cholerae* and *V.cholerae ctx* isolates showed lysine decarboxylase activity between 0% and 5% salt concentrations. *V.alginolyticus* isolates showed lysine decarboxylase activity between 0.2% and 11% salt concentrations. *V.alginolyticus* isolates did not show lysine decarboxylase activity at 0%, 0.1%, 12%, 15% and 20% salt concentration. *V.harveyi* isolates showed lysine decarboxylase activity between 1 % and 6% salt concentration. *V.vulnificus* showed lysine decarboxylase activity between 0.5% and 6% salt concentrations but did not show growth at 0%, 0.1%, 0.3% and =7% salt concentration. *V.parahaemolyticus* showed lysine decarboxylase activity between 0.5% and 8% but did not show growth at 0%, 0.1%, 0.3% and =9% salt concentration. One *V.parahaemolyticus* isolate showed lysine decarboxylase activity at 9% salt concentration.

4.2.4.10.3. Effect of salt on the ornithine decarboxylase activity of Vibrios

Table 4.38. Effect of salt on the ornithine decarboxylase activity of pathogenic Vibrio cultures isolated from shrimp culture system

	0 %	0.1%	0.2%	0.5%	1%	3%	4%	5%	6%	7%	8%	9%	10%	11%	12%	15%	20%
VA 1*	-	-	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
VA 2	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
VA 3	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
VA 4	-	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-
VV1	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
VV 2	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
VV3	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
VV4	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-
VH 1	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-
VH 2	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-
VC 1	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
VC 2	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
VC 3	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
VC 4	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
VCctx 1	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
VCctx 2	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-

VCctx 3	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
VP1	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-
VP2	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-
VP 3	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-
VP 4	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-

*VA- *V alginolyticus*, VC- *V.cholerae*, VCctx -choleratoxigenic *V.cholerae*, VH – *V.harveyi*, VP- *V.parahaemolyticus*, VV- *V.vulnificus* + : positive test result, - : negative test result

The result of the study on the ornithine decarboxylase activity of pathogenic vibrios is given in Table 4.38. *V. cholerae* and *V.cholerae ctx* isolates showed ornithine decarboxylase activity between 0% and 4% salt concentrations. 100% isolates of *V.alginolyticus* isolates showed ornithine decarboxylase activity between 0.2% and 9% salt concentrations whereas 75% isolates of *V.alginolyticus* isolates showed ornithine decarboxylase activity at 10% and 11% salt concentrations. *V.alginolyticus* isolates did not show lysine decarboxylase activity at 0%, 0.1%, 12%, 15% and 20% salt concentration. *V.harveyi* isolates showed ornithine decarboxylase activity between 1 % and 6% salt concentration. *V.vulnificus* showed ornithine decarboxylase activity between 0.5% and 6% salt concentrations but did not show growth at 0%, 0.1%, 0.3% and =7% salt concentration. *V.parahaemolyticus* showed ornithine decarboxylase activity between 0.5% and 8% and but did not show growth at 0%, 0.1%, 0.3% and =9% salt concentration..

V.cholerae, *V.cholerae* ctx, *V.vulnificus* and *V.harveyi* showed no significant variation in the effect of salt on the utilization of sugars (sucrose and mannitol) and amino acids (lysine and ornithine). All these Vibrios utilized sugars and amino acids within their normal salt tolerance for growth. Altermark et al (2007) noted that the optimal conditions for enzymatic activity coincide well with the corresponding optimal requirements for growth of bacteria. 100% of *V.alginolyticus* isolates utilized lysine even at 11% NaCl whereas these *V.alginolyticus* isolates could utilize sugars only upto 10% NaCl concentration. Similarly 25% of *V.parahaemolyticus* isolates utilized lysine at 9% NaCl but the maximum salt concentration at which *V.parahaemolytucus* utilized sugars was 8% NaCl.

4.2.4.11. Effect of temperature on the utilization of sugars by pathogenic Vibrio cultures isolated from shrimp culture system

Vibrio cholerae, V.alginolyticus, V.vulnificus, V.parahaemolyticus and V.harveyi cultures were inoculated in sugar fermentation broth supplemented with 3% NaCl and to which the corresponding sugar was added at 1% level. The tubes were then incubated at different temperatures viz., 4°C, 20°C, Room temperature RT (28-35°C), 42°C, 45°C and 56°C.

4.2.4.11.1. Effect of temperature on the utilization of sucrose by pathogenic Vibrios

Table 4.39. Effect of temperature on the utilization of Sucrose by pathogenic Vibrio cultures isolated from shrimp culture system

	4°C	20°C	RT (28-35°C)	30°C	42°C	45°C	56°C
VA 1*	+	+	+	+	+	+	-
VA 2	+	+	+	+	+	+	-
VA 3	+	+	+	+	+	+	-
VA 4	+	+	+	+	+	+	-
VV 1	-NG	-G	-G	-G	-G	-NG	-NG
VV 2	-NG	-G	-G	-G	-G	-NG	-NG
VV 3	-NG	-G	-G	-G	-G	-NG	-NG
VV 4	-NG	-G	-G	-G	-G	-NG	-NG
VH 1	-	+	+	+	-	-	-
VH 2	-	+	+	+	-	-	-
VC 1	-	+	+	+	+	-	-
VC 2	-	+	+	+	+	-	-
VC 3	-	+	+	+	+	-	-
VC 4	-	+	+	+	+	-	-
VCctx 1	-	+	+	+	+	-	-
VCctx 2	-	+	+	+	+	-	-
VCctx 3	-	+	+	+	+	-	-

VP 1	-NG	-G	-G	-G	-G	-NG	-NG
VP 2	-G	-G	-G	-G	-G	-NG	-NG
VP 3	-NG	-G	-G	-G	-G	-NG	-NG
VP 4	-NG	-G	-G	-G	-G	-NG	-NG

+ : positive test result, - : negative test result, NG: No Growth

*VA- *V.alginolyticus*, VC- *V.cholerae*, VCctx -choleratoxigenic *V.cholerae*, VH – *V.harveyi*, VP- *V.parahaemolyticus*, VV- *V.vulnificus*

The result of the effect of temperature on the utilization of sucrose by pathogenic Vibrios is given in Table 4.39. 100% isolates of *V.cholerae* and *V.cholerae ctx* isolates utilized sucrose between 20°C and 42°C. None of the *V.cholerae ctx* isolates could utilize sucrose at 4°C, 45°C or 56°C. 100% isolates of *V.alginolyticus* isolates utilized sucrose between 4°C to 45°C but no growth was observed at 56°C. *V.harveyi* isolates fermented sucrose between 20°C and 35°C. *V.vulnificus* and *V.parahaemolyticus* isolates showed growth but negative reaction for sucrose fermentation at temperatures between 20°C and 42°C. None of the *V.vulnificus* and *V.parahaemolyticus* isolates showed growth at 45°C or 56°C. One *V.parahaemolyticus* isolate showed growth at 4°C. The use of incubation temperature of 42°C has been found to give significantly higher recoveries of V.cholerae and to minimize problems with competing microorganisms (DePaola *et al.*, 1988).

4.2.4.11.2. Effect of temperature on the utilization of mannitol by pathogenic Vibrios

Table 4.40. Effect of temperature on the utilization of mannitol by pathogenic Vibrio cultures isolated from shrimp culture system

	4°C	20°C	RT (28-35°C)	30°C	42°C	44-45°C	56°C
VA 1*	+	+	+	+	+	-	-
VA 2	+	+	+	+	+	+	-
VA 3	+	+	+	+	+	+	-
VA 70	+	+	+	+	+	+	-
VV 1	-	+	+	+	+	-	-

VV 2	-	+	+	+	+	-	-
VV 3	-	+	+	+	+	-	-
VV 4	-	+	+	+	+	-	-
VH 1	-	+	+	+	-	-	-
VH 2	-	+	+	+	-	-	-
VC 1	-	+	+	+	+	-	-
VC 2	-	+	+	+	+	-	-
VC 3	-	+	+	+	+	-	-
VC 4	-	+	+	+	+	-	-
VCctx 1	-	+	+	+	+	-	-
VCctx 2	-	+	+	+	+	-	-
VCctx 3	-	+	+	+	+	-	-
VP 1	-NG	+	+	+	+	-	-
VP 2	+	+	+	+	+	-	-
VP 3	-NG	+	+	+	+	-	-
VP 4	-NG	+	+	+	+	-	-

+ : positive test result, - : negative test result, NG: No Growth

*VA- *V.alginolyticus*, VC- *V.cholerae*, VCctx -choleratoxigenic *V.cholerae*, VH – *V.harveyi*, VP- *V.parahaemolyticus*, VV- *V.vulnificus*

The result of the effect of temperature on the utilization of mannitol by pathogenic Vibrios is given in Table 4.40. 100% isolates of *V.cholerae* and *V.cholerae ctx* isolates utilized mannitol between 20°C and 42°C. None of the *V.cholerae ctx* isolates could utilize mannitol at 4°C, 45°C or 56°C. 100% isolates of *V.alginolyticus* isolates utilized mannitol between 4°C to 42°C but no growth was observed at 56°C. 75% *V.alginolyticus* isolates utilized mannitol at 45°C. *V.harveyi* isolates fermented mannitol between 20°C and 35°C. *V.vulnificus* and *V.parahaemolyticus* isolates gave positive reaction for mannitol fermentation at temperatures between 20°C and 42°C. 25% of *V.parahaemolyticus* isolates showed growth at 4°C. None of the *V.vulnificus* and *V.parahaemolyticus* isolates showed growth at 45°C or 56°C.

4.2.4.12. Effect of temperature on the utilization of amino acids by pathogenic Vibrio cultures isolated from shrimp culture system

Vibrio cholerae, *V.alginolyticus*, *V.vulnificus*, *V.parahaemolyticus* and *V.harveyi* cultures were inoculated in Moller's decarboxylase broth supplemented with 3% NaCl and to which the corresponding amino acid was added at 0.5% level. The tubes were then incubated at different temperatures viz., 4°C, 20°C, RT (28-35°C), 42°C, 45°C and 56°C.

4.2.4.12.1. Effect of temperature on the utilization of Arginine by pathogenic Vibrios

Table 4.41. Effect of temperature on the utilization of Arginine by pathogenic Vibrio cultures isolated from shrimp culture system

	4°C	20°C	RT (28-35°C)	30°C	42°C	45°C	56°C
VA 1	NG	-	-	-	-	NG	NG
VA 2	NG	-	-	-	-	NG	NG
VA 3	NG	-	-	-	-	NG	NG
VA 4	NG	-	-	-	-	NG	NG
VV 1	NG	-	-	-	-	NG	NG
VV 2	NG	-	-	-	-	NG	NG
VV 3	NG	-	-	-	-	NG	NG
VV 4	NG	-	-	-	-	NG	NG
VH 1	NG	-	-	-	NG	NG	NG
VH 2	NG	-	-	-	NG	NG	NG
VC 1	NG	-	-	-	-	NG	NG
VC 2	NG	-	-	-	-	NG	NG
VC 3	NG	-	-	-	-	NG	NG
VC 4	NG	-	-	-	-	NG	NG
VCctx 1	NG	-	-	-	-	NG	NG

VCctx 2	NG	-	-	-	-	NG	NG
VCctx 3	NG	-	-	-	-	NG	NG
VP 1	NG	-	-	-	-	NG	NG
VP 2	NG	-	-	-	-	NG	NG
VP 3	NG	-	-	-	-	NG	NG
VP 4	NG	-	-	-	-	NG	NG

+ : positive test result, - : negative test result, NG: No Growth

*VA- *V.alginolyticus*, VC- *V.cholerae*, VCctx -choleratoxigenic *V.cholerae*, VH – *V.harveyi*, VP- *V.parahaemolyticus*, VV- *V.vulnificus*

The result of the effect of temperature on the arginine dihydrolase activity of pathogenic Vibrios is given in Table 4.41. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus* and *V.parahaemolyticus* showed negative arginine dihydrolase activity (yellow colour) between 20°C and 42°C but at 4°C, 44-45°C and 56°C temperature none of the pathogenic bacteria showed growth. Results showed that the incubation temperature did not alter the utilization pattern, *ie* not able to utilize the arginine by the Vibrios.

4.2.4.12.2. Effect of temperature on Lysine decarboxylase activity by pathogenic Vibrios

Table 4.42. Effect of temperature on Lysine decarboxylase activity by pathogenic Vibrio cultures isolated from shrimp culture system

	4°C	20°C	RT (28-35°C)	30°C	42°C	44-45°C	56°C
VA 1*	-	+	+	+	+	-	-
VA 2	+	+	+	+	+	+	-
VA 3	-	+	+	+	+	-	-
VA 4	-	+	+	+	+	-	-

VV 1	-	+	+	+	+	-	-
VV 2	-	+	+	+	+	-	-
VV 3	-	+	+	+	+	-	-
VV 4	-	+	+	+	+	-	-
VH 1	-	+	+	+	+	-	-
VH 2	-	+	+	+	+	-	-
VC 1	-	+	+	+	+	-	-
VC 2	-	+	+	+	+	-	-
VC 3	-	+	+	+	+	-	-
VC 4	-	+	+	+	+	-	-
VCctx 1	-	+	+	+	+	-	-
VCctx 2	-	+	+	+	+	-	-
VCctx 3	-	+	+	+	+	-	-
VP 1	-	+	+	+	+	-	-
VP 2	+	+	+	+	+	-	-
VP 3	-	+	+	+	+	-	-
VP 4	-	+	+	+	+	-	-

+ : positive result, - : negative result

*VA- *V.alginolyticus*, VC- *V.cholerae*, VCctx -choleratoxigenic *V.cholerae*, VH – *V.harveyi*, VP- *V.parahaemolyticus*, VV- *V.vulnificus*

The result of the effect of temperature on the lysine decarboxylase activity of pathogenic Vibrios is given in Table 4.42. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus* and *V.parahaemolyticus* showed lysine decarboxylase between 20°C and 42°C. None of the pathogenic bacteria showed growth at 56°C temperature. 25% of *V.alginolyticus* isolates showed lysine decarboxylase at 4°C and 45°C whereas 25% of *V.parahaemolyticus* isolates showed lysine decarboxylase at 4°C.

4.2.4.12.3. Effect of temperature on ornithine decarboxylase activity by pathogenic Vibrios

Table 4.43. Effect of temperature on the utilization of Ornithine by pathogenic Vibrio cultures isolated from shrimp culture system

	4°C	20°C	RT (28-35°C)	30°C	42°C	44-45°C	56°C
VA 1*	-	-	+	+	+	-	-
VA 2	-	+	+	+	+	-	-
VA 3	-	+	+	+	+	-	-
VA 4	-	+	+	+	+	-	-
VV 1	-	+	+	+	+	-	-
VV 2	-	+	+	+	+	-	-
VV 3	-	+	+	+	+	-	-
VV 4	-	+	+	+	+	-	-
VH 1	-	+	+	+	-	-	-
VH 2	-	+	+	+	-	-	-
VC 1	-	+	+	+	+	-	-
VC 2	-	+	+	+	+	-	-
VC 3	-	+	+	+	+	-	-
VC 4	-	+	+	+	+	-	-
VCctx 1	-	+	+	+	+	-	-
VCctx 2	-	+	+	+	+	-	-
VCctx 3	-	+	+	+	+	-	-
VP 1	-	+	+	+	+	-	-
VP 2	-	+	+	+	+	-	-
VP 3	-	+	+	+	+	-	-
VP 4	-	+	+	+	+	-	-

+ : positive result, - : negative result

*VA- *V.alginolyticus*, VC- *V.cholerae*, VCctx -choleratoxigenic *V.cholerae*, VH – *V.harveyi*, VP- *V.parahaemolyticus*, VV- *V.vulnificus*

The result of the effect of temperature on the ornithine decarboxylase activity of pathogenic Vibrios is given in Table 4.43. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus* and *V.parahaemolyticus* showed ornithine decarboxylase activity between 20°C and 42°C but at 4°C, 44-45°C and 56°C temperature none of the pathogenic vibrios showed growth.

V.cholerae, *V.cholerae* ctx, *V.vulnificus* and *V.harveyi* showed no significant variation in the effect of temperature on the utilization of sugars (sucrose and mannitol) and amino acids (lysine and ornithine). All these Vibrios utilized sugars and amino acids within their normal temperature for growth. 100% of *V.alginolyticus* isolates utilized sucrose at 4°C and 45°C whereas only 25% of *V.alginolyticus* utilized lysine at 4°C and 75% of *V.alginolyticus* utilized lysine at 45°C. Sudha et al (1998) studied the effect of temperature on growth and biochemical properties of *V.parahaemolyticus, V.vulnificus, V.alginolyticus, V.mimicus* and *V.harveyi* and observed that 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.

V.alginolyticus isolates showed variation in the utilization of sugars and amino acids at different salt concentrations and at different temperatures. The effect of temperature was relatively more on the utilization of amino acids (lysine, ornithine) than on the utilization of sugars (sucrose and mannitol). On the other hand, the effect of salt concentration was more on the utilization of sugars (sucrose, mannitol) than on the amino acids (lysine, ornithine). 100% of *V.alginolyticus* utilized sucrose and mannitol at 4°C, 100% of *V.alginolyticus* utilized sucrose at 45°C and 75% of *V.alginolyticus* utilized mannitol at 45°C but only 25% of *V.alginolyticus* utilized lysine at 4°C and 45°C. None of the *V.alginolyticus* utilized ornithine at 4°C or 45°C. 100% of *V.alginolyticus* isolates utilized lysine even at 11% NaCl whereas these *V.alginolyticus* isolates could utilize sugars only upto 10% NaCl concentration. The ability of *V.alginolyticus* to utilize sugars and amino acids over a wide range of salinities and temperatures might be one of the

reason for the dominance of *V.alginolyticus*, both in shrimp hatcheries and in shrimp farms.

4.2.5. Effect of preservatives/ chemicals on the growth of pathogenic Vibrio cultures isolated from shrimp culture system

The effect of potassium chloride, potassium sorbate, sodium citrate and sodium tri polyphosphate on the growth and survivability of pathogenic Vibrios were studied. Muller-Hinton agar containing the chemical/preservative at different concentration (w/v) was used. Filtered sterilized (0.2µ pore size filter) solution of the chemical was added to molten and cooled (40 – 45°C) Mueller-Hinton agar as elaborated in Materials and Methods section. The volume of the chemical/preservative solution to be added was accounted in the final calculation of Muller-Hinton agar volume.

4.2.5.1. Effect of potassium chloride (KCl) on the growth of pathogenic Vibrio cultures isolated from shrimp culture system

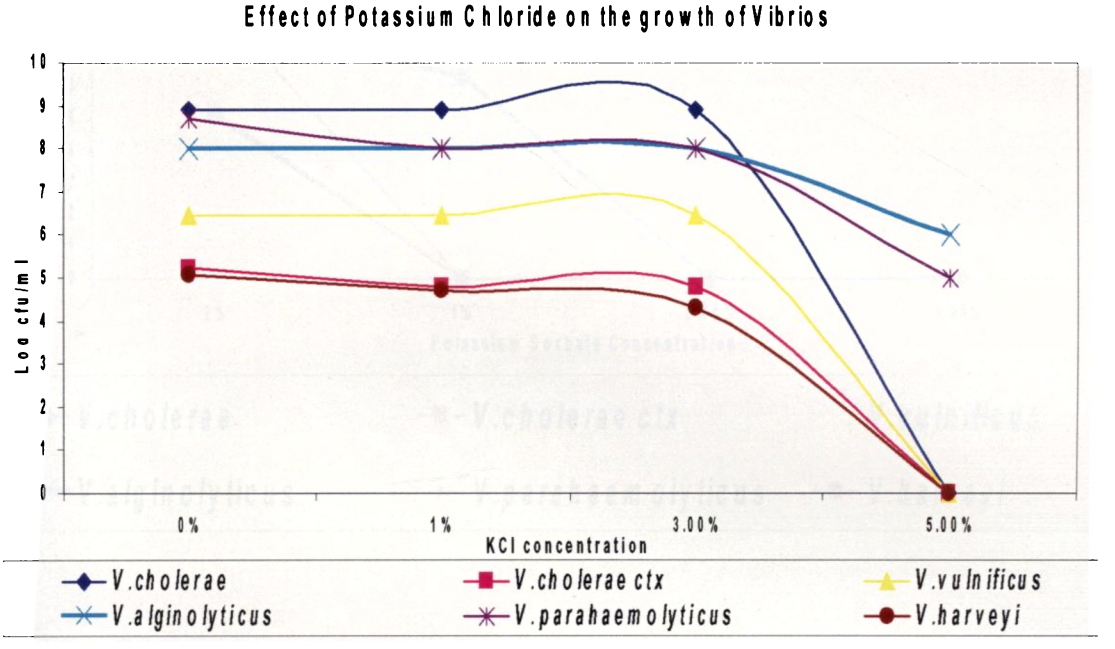


Fig. 4.30. Effect of potassium chloride (KCl) on the growth of pathogenic Vibrios

Potassium is vital in the human body and oral potassium chloride is the common means to replenish it. Potassium salt is being promoted as salt substitute for food as it can reduce hypertension. KCl is an approved food additive with an E-number of E508. KCl at 5% level inhibited the growth of *V.cholerae*, *V.cholerae ctx*, *V.vulnificus* and *V.harveyi* (Fig. 4.30). Nil to limited inhibition was observed between 0% and 3%. Inhibition by KCl was lower in the case of *V.parahaemolyticus* and *V.alginolyticus* isolates and they showed a 2 to 3 log decrease in counts even at 5% level. Saramma et al (1994) reported that sodium chloride could be replaced by potassium chloride without affecting growth and enzyme production of Vibrios. Orally, KCl is toxic in excess; the LD_{50} is around 2500 mg/kg.

4.2.5.2. Effect of potassium sorbate ($C_6H_7O_2K$) on the growth of pathogenic Vibrio cultures isolated from shrimp culture system

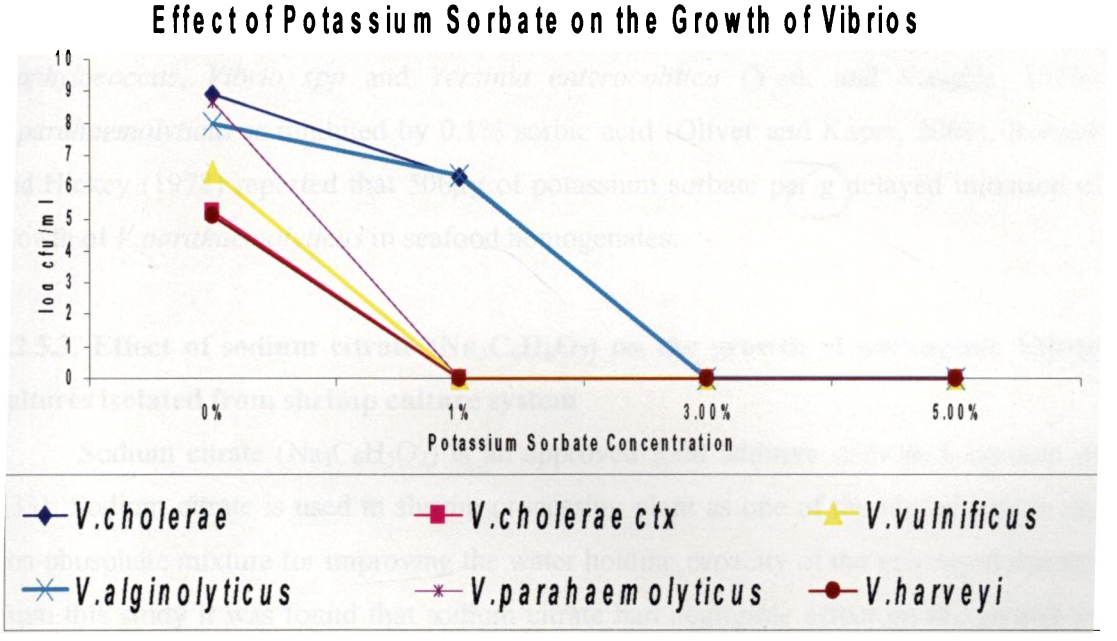


Fig. 4.31. Effect of potassium sorbate ($C_6H_7O_2K$) on the growth of pathogenic Vibrio cultures isolated from shrimp culture system

Potassium sorbate ($C_6H_7O_2K$) is an approved food additive with an E-number of E202. The potassium salt of sorbic acid is readily soluble in water and the undissociated form is 10 to 600 times more effective than the dissociated form (Eklund, 1983). Potassium sorbate at 1% level inhibited the growth of *V.cholerae ctx*, *V.vulnificus*, *V.parahaemolyticus* and *V.harveyi*. The counts decreased from 5-8 log to nil (Fig. 4.31). *V.cholerae* and *V.alginolyticus* showed a reduction of 2 log in counts at 1% level but were completely inhibited at 3% level. From this study it is known that all pathogenic Vibrio cultures isolated from shrimp culture system can be inhibited at 3% potassium sorbate level. This result finds application in the seafood processing plants wherein the use of potassium sorbate for the control of pathogenic vibrios can be explored.

One of the primary target of sorbic acids in bacterial cells appears to be the cytoplasmic membrane and it reduces the cytoplasmic membrane electrochemical gradient. Bacteria inhibited by sorbates include *Acinetobacter*, *Bacillus*, *Campylobacter*, *Clostridium*, *E.coli* O157:H7, *Listeria monocytogenes*, *Pseudomonas*, *Salmonella*, *Staphylococcus*, *Vibrio spp* and *Yersinia enterocolitica* (York and Vaughn, 1955). *V.parahaemolyticus* is inhibited by 0.1% sorbic acid (Oliver and Kaper, 2001). Robach and Hickey (1978) reported that 500µg of potassium sorbate per g delayed initiation of growth of *V.parahaemolyticus* in seafood homogenates.

4.2.5.3. Effect of sodium citrate ($Na_3C_6H_5O_7$) on the growth of pathogenic Vibrio cultures isolated from shrimp culture system

Sodium citrate ($Na_3C_6H_5O_7$) is an approved food additive with an E-number of E331. Sodium citrate is used in shrimp processing plant as one of the ingredients in the non-phosphate mixture for improving the water holding capacity of the processed shrimp. From this study it was found that sodium citrate had negligible effect on the growth of pathogenic Vibrios isolated from shrimp culture system. A maximum reduction of 1 log value was observed at 7% level (Fig. 4.32). Inhibition by citrate may be due to chelation. Buchanan and Golden (1994) found that undissociated citric acid is inhibitory against Gram positive *Listeria monocytogenes*. Ma et al (2005) reported that citric acid inhibits *Vibrio parahaemolyticus* and the MIC was 0.0008 g/ml.

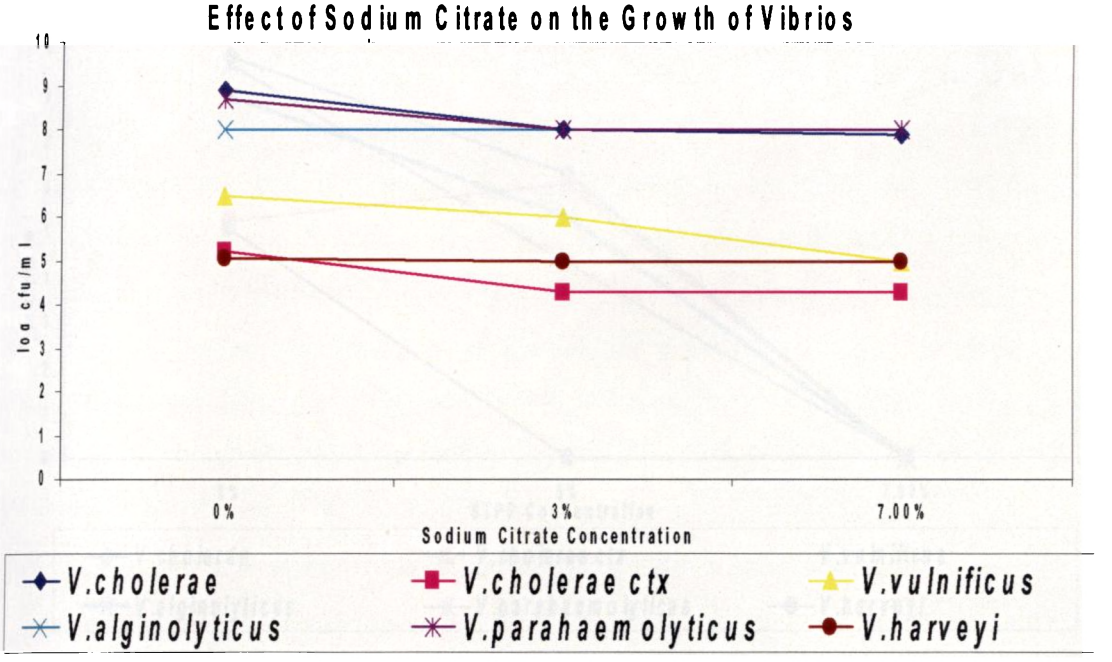


Fig. 4.32. Effect of sodium citrate ($Na_3C_6H_5O_7$) on the growth of pathogenic Vibrio cultures isolated from shrimp culture system

4.2.5.4. Effect of sodium tri polyphosphate ($Na_5P_3O_{10}$, STPP) on the growth of pathogenic Vibrio cultures isolated from shrimp culture system

Sodium tri polyphosphate ($Na_5P_3O_{10}$, STPP) is an approved food additive with an E-number of E451 (triphosphates). STPP in combination with salt is generally used in shrimp processing plants for increasing the water holding capacity of the processed shrimp. In this study, it was observed that 3% level of STPP inhibited *V.vulnificus* and *V.harveyi*. Others species showed 1- 3 log reduction in counts. At 7% level all the pathogenic vibrios were inhibited (Fig. 4.33).

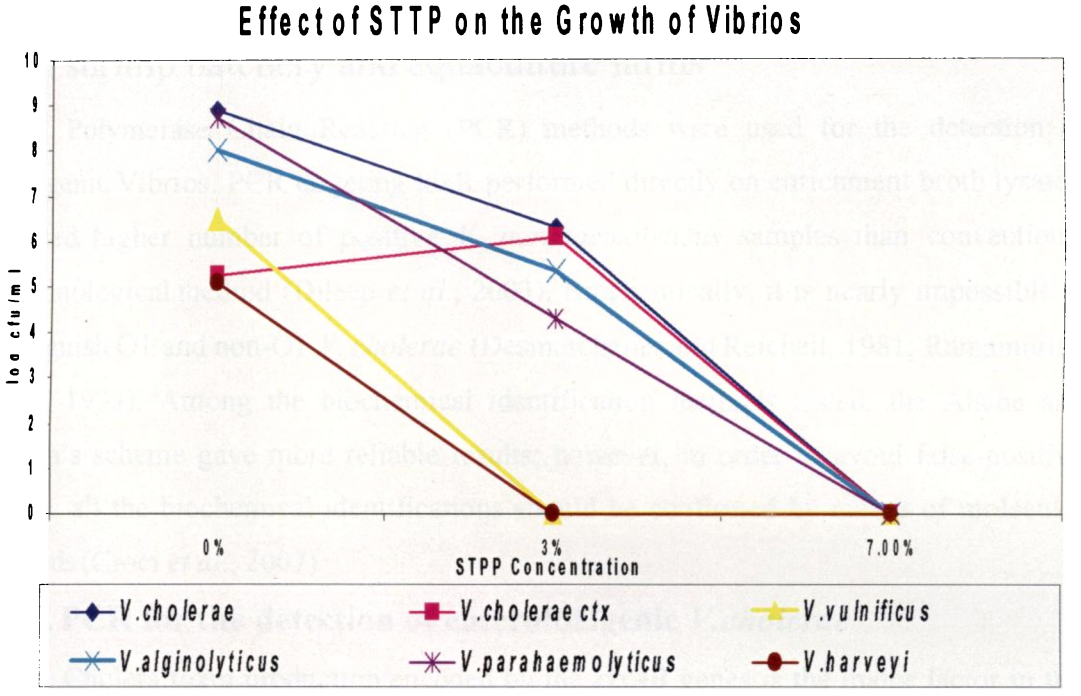


Fig. 4.33. Effect of sodium tri polyphosphate ($Na_5P_3O_{10}$, STPP) on the growth of pathogenic Vibrio cultures isolated from shrimp culture system

Gram positive bacteria are generally more susceptible to phosphates than are Gram negative bacteria. 0.5% STTP 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. Polyphosphates also 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*. 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 (Wong *et al.*, 1995).

4.3. Molecular characterization of pathogenic Vibrios isolated from shrimp hatchery and aquaculture farms

Polymerase Chain Reaction (PCR) methods were used for the detection of pathogenic Vibrios. PCR targeting toxR performed directly on enrichment broth lysates, detected higher number of positive *V. parahaemolyticus* samples than conventional microbiological method (Dileep *et al.*, 2003). Biochemically, it is nearly impossible to distinguish O1 and non-O1 *V. cholerae* (Desmarchelier and Reichelt, 1981; Ramamurthy *et al.*, 1993). Among the biochemical identification methods tested, the Alsina and Blanch's scheme gave more reliable results; however, in order to avoid false-positive results, all the biochemical identifications should be confirmed by means of molecular methods (Croci *et al.*, 2007).

4.3.1. PCR for the detection of enterotoxigenic *V.cholerae*

Cholera toxin production encoded by the *ctxAB* genes is the major factor in the pathogenesis of cholera. A PCR method that selectively amplifies a specific DNA fragment within the *ctxAB* operon of *V.cholerae* was used (USFDA-BAM, 2001) and sequence of cholera toxin PCR primers is given in Materials and Methods section. This PCR method detects only cholera toxin producing *V.cholerae*. The cholera toxin producing *V.cholerae* (*V.cholerae* ctx) strains yield an amplicon of 777bp in size.

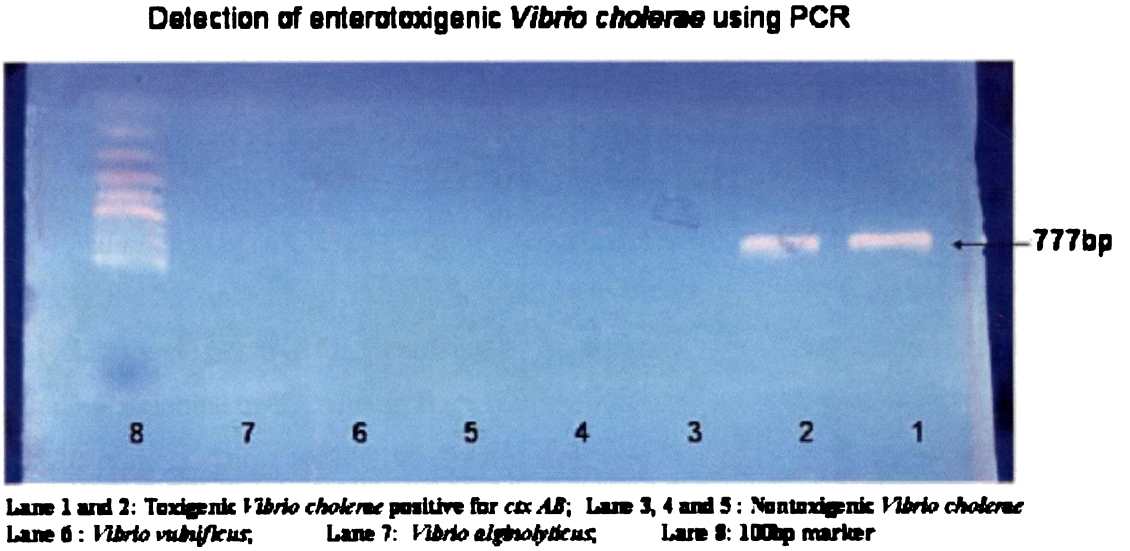


Fig. 4.34. PCR for the detection of enterotoxigenic *V.cholerae*

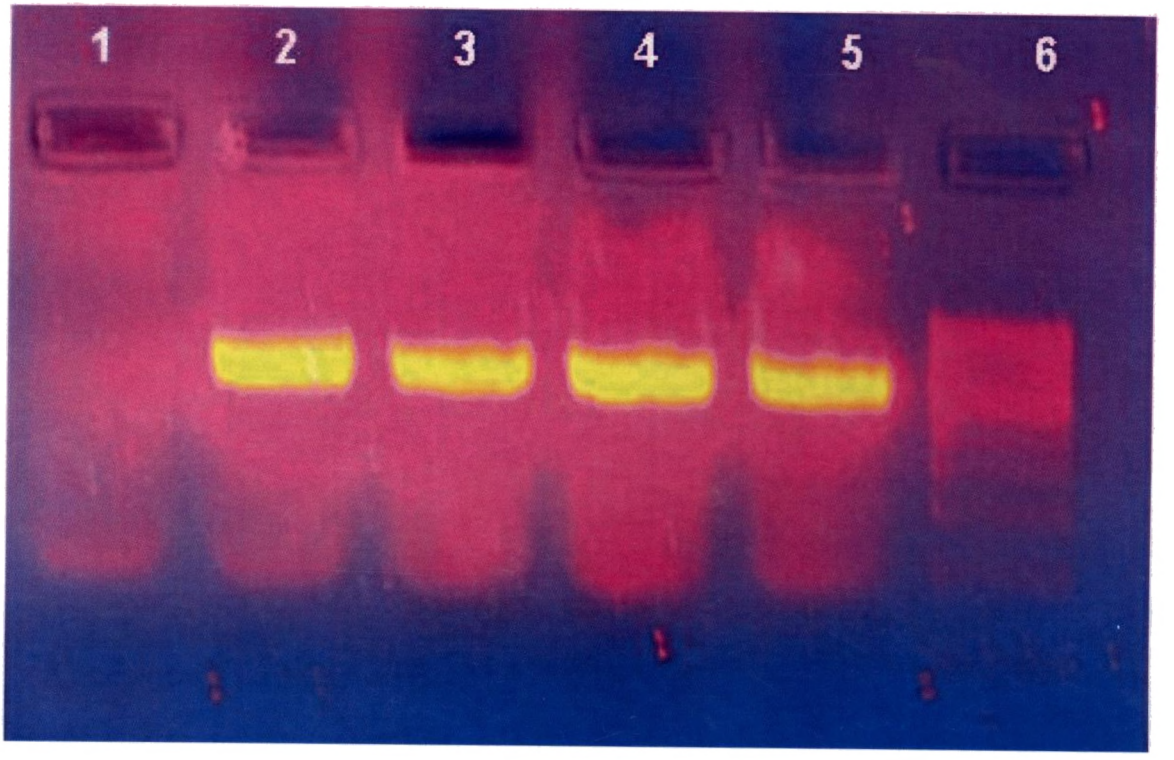


Fig. 4.35. PCR for the detection of enterotoxigenic *V.cholerae* from shrimp aquaculture farms

Lane 1: negative control; Lanes 2-4 : *V.cholerae* isolates from shrimp pond water, Lane 5: *V.cholerae* (MTCC 3906); Lane 6: 100bp DNA marker

The PCR was initially standardized using *V.cholerae* (*ctx* positive), *V.vulnificus*, *V.alginolyticus* type cultures. *V.cholerae* (MTCC 3906) yielded the 777bp amplicon whereas *V.vulnificus* (MTCC 1145), *V.alginolyticus* (ATCC 17749) were negative (Fig 4.34). Out of the 250 isolates of Vibrios isolated from the aquaculture farm environment only 42 isolates were positive for *V.cholerae* by biochemical tests (Noguerola and Blanch 2008; Bergey's manual of Systematic Bacteriology 2005). All the 42 isolates were tested using *ctxAB* primers. Three *V.cholerae* isolates were positive in this PCR and all of them yielded a single specific amplicon of 777bp size (Fig 4.35). 93% of the *V.cholerae* isolates were negative for the presence of cholera toxin genes (Fig. 4.36).

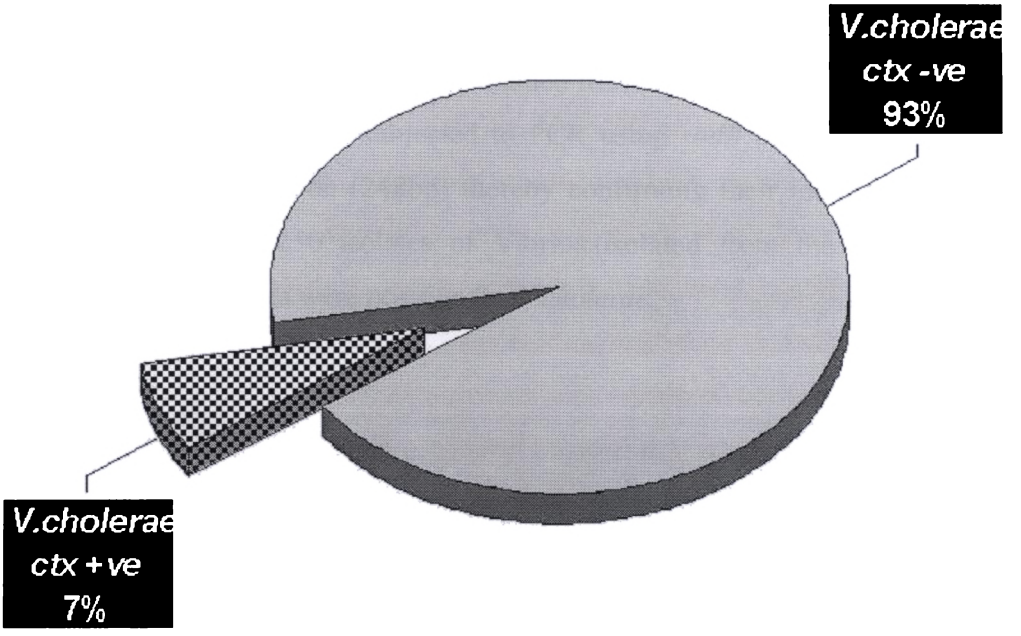


Fig. 4.36. Incidence of *ctx* positive *V.cholerae* in shrimp aquaculture farms (n=42)

The detection of ctx positive isolates among the Non O1 and Non O139 *V.cholerae* was reported in several studies. Jiang et al (2003) examined 137 non O1/O139 isolates from Newport, California and found that 17% of the strains were positive for *ctx* gene (*ctxA*). 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).

Karunasagar et al (2003) reported that all Vibrio cholerae isolates associated with aquaculture were found to be non-toxigenic as they were negative for *ctx, zot* and *tcp* genes. However, the detection of ctx positive isolates among Non O1 and Non O139 *V.cholerae* isolates from *P.monodon* shrimp aquaculture system is being reported in this study; probably this is the first report.

4.3.2. PCR for the detection of *V.cholerae* using species specific primers

This PCR uses species-specific primers that target the house keeping gene *sodB* of *V.cholerae* (Tarr *et al.*, 2007). As the house keeping genes are invariably present in all isolates this PCR method helps in the detection of all *V.cholerae* isolates irrespective of their toxigenic status. *V.cholerae* cultures yield an amplicon of 248bp. The PCR was

initially standardized using *V.cholerae* (MTCC 3906), *V.vulnificus* (MTCC 1145), *V.alginolyticus* (ATCC 17749) and *V.parahaemolyticus* (ATCC 17802) type cultures. *V.cholerae* yielded the 248bp amplicon whereas *V.vulnificus*, *V.alginolyticus* and *V.parahaemolyticus* were negative.

All the 42 isolates of *V.cholerae* obtained from aquaculture pond water, sediment, shrimp head and muscle were subjected to PCR using *sodB* primers. All the isolated yielded the specific amplicon (248bp) thereby confirming their identity as *V.cholerae* (Fig. 4.37) Out of the 250 isolates of Vibrios isolated from the aquaculture farm environment on 42 isolates were positive for *V.cholerae*.

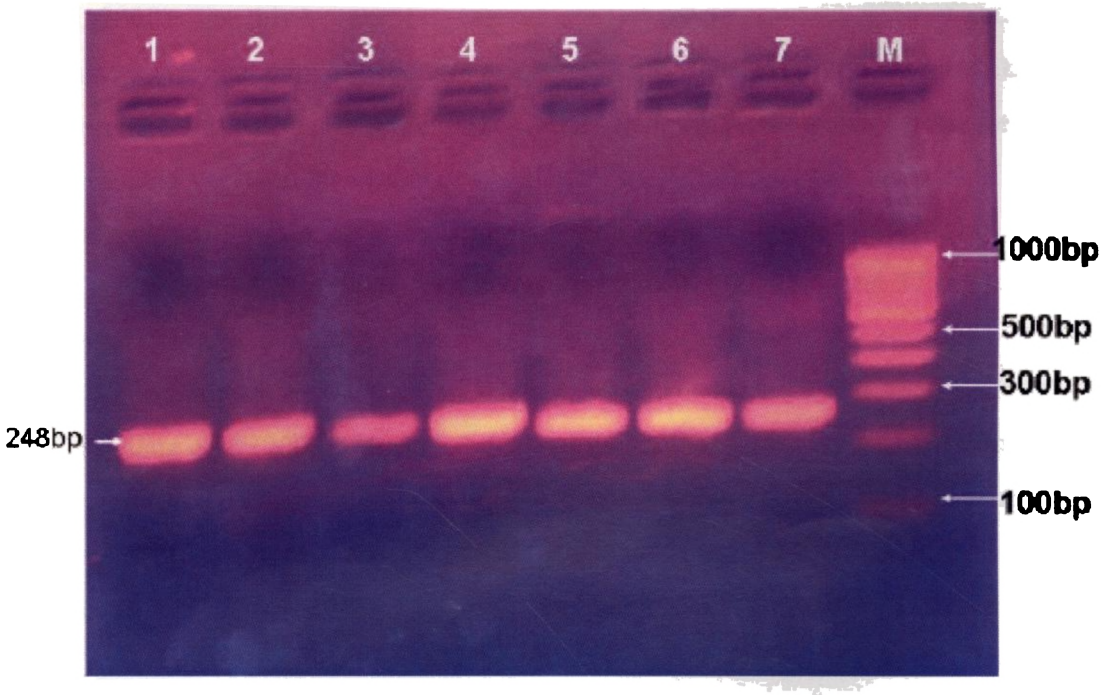


Fig. 4.37. PCR for the detection of *V.cholerae* using species specific primers.
Lane 1-3, *V.cholerae* isolates from shrimp pond water; Lane 4 – 5, *V.cholerae* isolates from shrimp pond sediment; Lane 6, *V.cholerae* isolates from shrimp muscle; Lane 7, *V.cholerae* (MTCC 3906); All *V.cholerae* yielded the species specific amplicon (248bp) with *sodB* primers; Lane8, 100bp DNA ladder (GeNei)

4.3.3. *V.cholerae*-duplex PCR for the simultaneous detection of *V.cholerae* and differentiation of cholera toxin producing *V.cholerae* isolates

PCR methods targeting a single gene/sequence, multiple genes within *V.cholerae* or target two or more pathogens in a single PCR assay have been used by several researchers (Brasher *et al.*, 1998, USFDA-BAM, 2001; Hervio-Heath *et al.*, 2003; Kong *et al.*, 2002; Singh *et al.*, 2002; Jing *et al.*, 2003; Karunasagar *et al.*, 2003; Lee *et al.*, 2003; Panicker *et al.*, 2004; DiPinto *et al.*, 2005; Fraga *et al.*, 2007; Tarr *et al.*, 2007; Khuntia *et al.*, 2008). The hlyA gene (hemolysin gene) was detected in 85% of the *V.cholerae* strains associated with aquaculture and ompU (outer membrane protein gene) in 77% of the strains (Karunasagar *et al.*, 2003). All the *Vibrio cholerae* isolates from the environment presented very similar virulence profiles by PCR, lacking *ctxA* and *tcpA* El Tor and containing *hlyA* (98·7%), *rtxA* (99·0%), *toxR* (98·7%) and *stn-sto* (1·9%) (Fraga *et al.*, 2007). The *toxR, hlyA*, and *ompU* genes were present in 100, 98.6, and 87.0% of the environmental isolates *V. cholerae* isolates, respectively (Rivera *et al.*, 2001). Hypothesis of the existence of a TCP-independent mechanism for infection by CTX? was proposed by Jiang et al (2003). 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). PCR targeting virulence genes might not detect all *V.cholerae* isolates present in food. Rapid Alert System for Food and Feed (RASFF) alert notifications were issued for the presence of *V.cholerae*. The presence of the pathogen is sufficient. In such a scenario the target sequence should always be present. The combination of the species-specific 205-bp vvh fragment along with viuB in a multiplexed PCR enabled to confirm the presence of potentially pathogenic strains of *V. vulnificus* (Panicker *et al.*, 2004).

An effective PCR method for detecting enterotoxigenic *Vibrio cholerae* in food samples was described in Bacteriological Analytical Manual (USFDA-BAM, 2001). This PCR selectively amplifies a specific DNA fragment within the *ctxAB* operon of *V.cholerae*. This PCR method detects only cholera toxin producing *V.cholerae* but does not provide information on non-cholera-toxigenic *V.cholerae*. Tarr et al (2007) developed

a multiplex PCR for *V. vulnificus*, *V.parahaemolyticus*, *V. mimicus and V. cholerae* wherein the intra-specific variation in the conserved housekeeping gene viz., *sodB* was used as a source of marker for *V.cholerae* and this PCR method helps in the detection of all *V.cholerae* isolates irrespective of their toxigenic status.

The draw back of the PCR targeting *ctxAB* genes is that it detects only enterotoxigenic strains of *V.cholerae*. It does not detect non-toxigenic (*ctxAB* negative) strains. The lacunae of using the PCR targeting species-specific *sodB* primers is that it identifies *V.cholerae* but does not state its toxigenic status with respect to *ctxAB* genes. In this study a *V.cholerae*-duplex PCR method was developed by utilizing *V.cholerae* species specific (Tarr *et al.*, 2007) and *ctxAB* genes specific primers (USFDA-BAM, 2001). A *V.cholerae*-duplex PCR that detects all *V.cholerae* isolates and provides information on the choleratoxigenic potential is essential for risk analysis of food. A *V.cholerae*-duplex PCR method was designed to detect *V.cholerae* isolates and differentiate *ctx* toxin producing strains.

PCR cycle condition for the *ctxAB* primers was as follows : 34 cycles: 1 min at 94°C, 1 min at 55°C, 1 min at 72°C. The thermal cycling profile for the *sodB* was as follows: 15 min at 93°C, 35 cycles : 40sec at 92°C, 1 min at 57°C, 1.5 min at 72°C; and 7 min at 72°C. As the annealing temperature for the *ctxAB* primers (55°C) and *sodB* primers (57°C) were nearer the *ctxAB* and *sodB* primers were selected for developing *V.cholerae*-duplex PCR and the primer annealing condition of 1 min at 57°C was used in *V.cholerae*-duplex PCR. A simple template preparation procedure was used for the *V.cholerae*-duplex PCR and the crude lysate of *V.cholerae* culture was found sufficient for obtaining specific result in the *V.cholerae*-duplex PCR method.

A simple template preparation procedure was standardized for the *V.cholerae*-multiplex PCR. 1ml of *V.cholerae* grown in T_1N_1 broth (37°C/24h) is centrifuged; supernatant was carefully discarded and the cell pellets were resuspended in 100µl of Tris-EDTA (TE) buffer. The microcentrifuge tubes were placed in a dry bath at 95°C for 5 min. This crude lysate was used as template for PCR reaction immediately. This template preparation was found to be sufficient for this *V.cholerae*-duplex PCR. PCR

reaction preparation and PCR cycle conditions were described in Materials and Methods section.

The *V.cholerae*-duplex PCR was initially standardized using *V.cholerae* (MTCC 3906), *V.vulnificus* (MTCC 1145), *V.alginolyticus* (ATCC 17749) and *V.parahaemolyticus* (ATCC 17802) cultures and *ctx* negative *V.cholerae* (CIFT culture collection) cultures. *V.cholerae* (*ctx* negative) cultures yielded a single amplicon (248bp); *ctxAB* positive *V.cholerae* cultures yielded two amplicons (248bp and 777bp). Other Vibrio spp. did not yield the specific amplicons. The results indicate that the *V.cholerae*-duplex PCR was specific to *V.cholerae* and the PCR cycle conditions were adequate for obtaining the desired result.

All the 42 *V.cholerae* isolates obtained from shrimp aquaculture system were subjected to *V.cholerae*-duplex PCR. Thirty nine *V.cholerae* cultures yielded a single amplicon of 248bp indicating that they are *V.cholerae* but non-choleratoxigenic. Three *V.cholerae* cultures yielded two amplicons viz., species specific 248bp and cholera toxin specific 777bp, thereby indicating that they were cholera toxin producing strains of *V.cholerae* (Fig. 4.38).

The specificity of the *V.cholerae*-duplex PCR was confirmed by screening the remaining 208 Vibrios cultures isolated from aquaculture ponds. None of the samples yielded the specific amplicons in *V.cholerae*-duplex PCR indicating the specificity of *V.cholerae*-duplex PCR. The *V.cholerae*-duplex PCR and the dichotomous scheme (Noguerola and Blanch, 2008) yielded the same result; in both the cases the same cultures were identified as *V.cholerae*.

V.cholerae strains that carry the *ctx* genes in the *ctx* genetic element can produce CT and these strains are termed as toxigenic strains. The *ctx* element is responsible for the cholera gravis form of *V.cholerae*. Cholera toxin production encoded by the *ctxAB* genes is the major factor in the pathogenesis of cholera and the presence of *ctxAB* is a prerequisite for full blown cholera disease (*Cholera gravis*) to occur. *Cholera gravis* is caused by strains of *V.cholerae* which produce cholera toxin (Varnam and Evans, 1996).

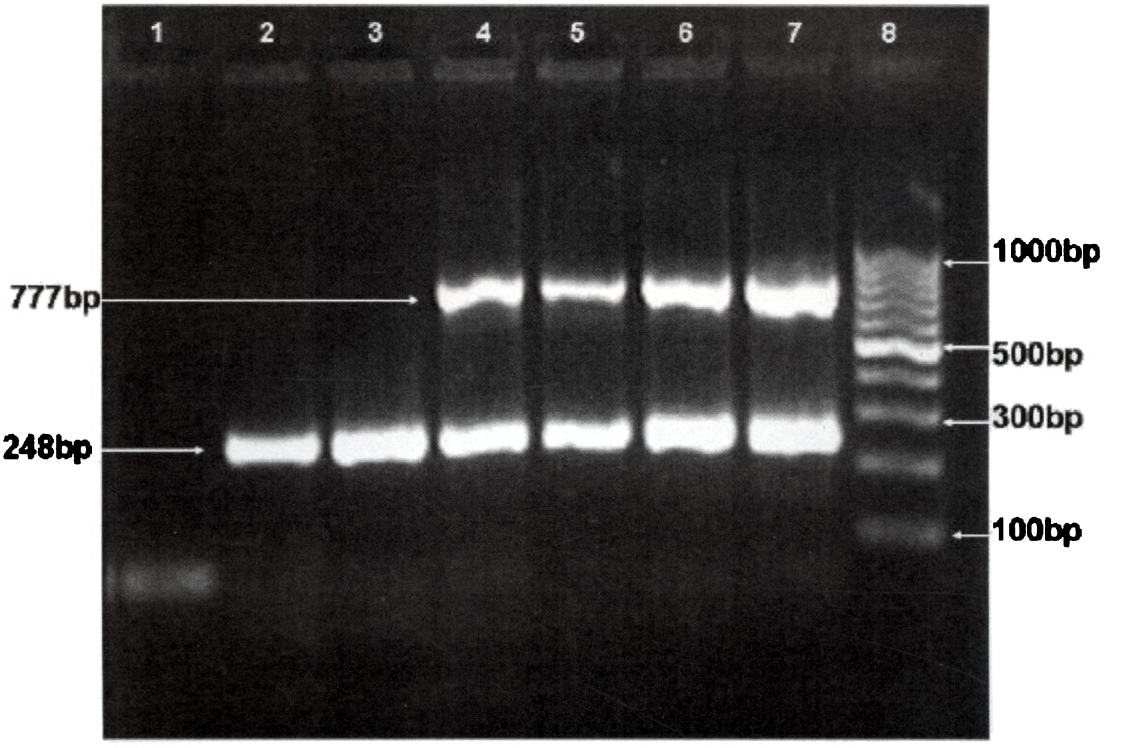


Fig. 4.38. Duplex PCR for the simultaneous detection of *V.cholerae* and differentiation of cholera toxin producing *V.cholerae* isolates.

Lane 1, Negative control; Lane 2–3, Cholera toxin negative *V.cholerae* isolates from shrimp pond water; Lane 4-6, Cholera toxin positive *V.cholerae* isolates from shrimp pond water; Lane 7, *V.cholerae* (MTCC 3906); Lane 8, 100bp DNA ladder (Gene Ruler^{TM}, Fermentas)

Significance of Choleratoxigenic *V.cholerae* in shrimp aquaculture system:

Even though *V.cholerae* was detected in shrimp pond sediment, shrimp head and shrimp muscle samples these isolates were found to be non-choleratoxigenic as the isolates did not yield the *ctxAB* specific amplicon in *V.cholerae*-duplex PCR. 93% of the *V.cholerae* isolates were negative for the presence of cholera toxin genes and only 7% of the *V.cholerae* isolates were potentially cholera-toxigenic (Fig. 4.36). The result

indicates that the majority of the *V.cholerae* isolates obtained from aquaculture farm water, soil and shrimp were non-toxigenic with respect to cholera toxin. The three *V.cholerae* isolates which were positive for the presence of cholera toxin genes were obtained from pond water. These three *V.cholerae* isolates agglutinated neither with polyclonal somatic O antiserum nor with O139 antiserum thereby grouping them as *V.cholerae* Non 1 and Non O139 serogroup. These three isolates failed to yield O1 specific or O139 specific amplicon when tested in PCR (Fig. 4.39) reconfirming that the isolates were indeed VC Non O1 and Non O139.

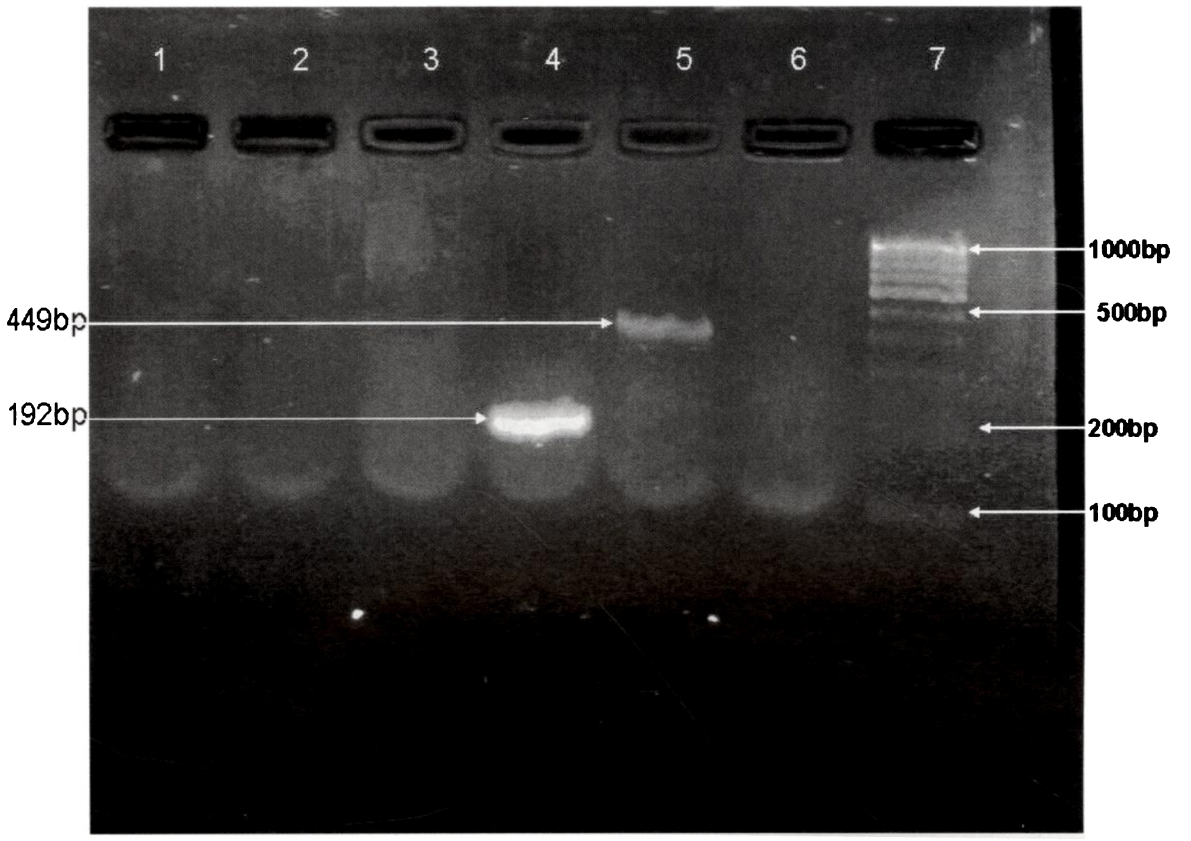


Fig. 4.39. PCR for testing the O1 and O139 status of *ctx* bearing *V.cholerae* isolated from farm water

Lanes 1-3, *ctx* positive *V.cholerae* isolates from shrimp aquaculture system failed to yield O1 specific or O139 specific amplicon indicating that the isolates were VC Non O1 and Non O139; lane 4, Known *V.cholerae* O1 isolate from laboratory culture collection; lane 5, *V.cholerae* (MTCC 3906) – *V.cholerae* O139; lane 6, Known *V.cholerae* Non O1 and Non O139 from laboratory culture collection; lane 7, 100bp DNA ladder (GeNei)

The autochthonous existence of *V.cholerae* in environment has been previously reported by several researchers (Colwell *et al.*, 1981; Kaneko and Colwell, 1978; Kaper *et al.*, 1979; Lee *et al.*, 1982). However the presence of *ctx* positive *V.cholerae* indicates human influence as it is known that the environmental strains do not carry the cholera toxin genes (DePaola *et al.*, 1983; Faruque *et al.*, 1998; Kaysner *et al.*, 1987; Twedt *et al.*, 1981). In this study it was observed that the *E.coli* were detected in aquaculture farms but not in shrimp hatchery samples (Table 4.2) and the mean *E.coli* levels were high in pond water (123±87 cfu/g) than in shrimp head (16±16.7 cfu/g) and shrimp muscle (12±11 cfu/g). This may suggest that the *ctx* positive *V.cholerae* in pond water might have entered through faecal pollution either through the source water or through feed. Farm made feeds were reported positive for *V.cholerae* (Raghavan, 2003). However, pond sediment samples had higher mean *E.coli* levels (204±133 cfu/g) than pond water (123±87 cfu/g) but *ctx* positive *V.cholerae* could not be isolated from these sources which suggests that the relationship between faecal bacteria and presence of ctx positive *V.cholerae* is non-existent. A negative correlation was observed between total vibrio counts and *E.coli* (r = -0.54) in the shrimp culture system. The incidence of *V.cholerae* observed in aquaculture ponds might also have been due to natural inhabitation. The presence of *V. cholerae* could not be correlated with faecal indicators (Kaper *et al.*, 1979; Hood and Ness, 1982; Nayyarahamed and Karunasagar, 1994; Filetici *et al.*, 1997).

The structural genes for the ctx element reside on a filamentous phage ctx? (Waldor and Mekalanos, 1996). CTX? is found in all epidemic *V.cholerae* isolates but is rarely recovered from the non O1 non O139 VC environmental isolates (Albert, 1996). A DNA probe study showed that a small percentage of environmental strains of VC Non O1 have the *ctx* gene (Nair *et al.*, 1988). 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.*, 2000) and CT genes are highly mobile among environmental isolates. The spread of CT genes in the environment can be facilitated by the exposure of CTX? positive strains to sunlight (Faruque *et al.*, 2000). Genetic and phenotypic evidence strongly suggests that the O139 strain arose from a VC O1 strain by

horizontal gene transfer (Bik *et al.*, 1995; Bik *et al.*, 1996; Comstock *et al.*, 1996; Johnson *et al.*, 1994; Waldor and Mekalanos, 1994; Faruque *et al.*, 2000a). *V.cholerae* Non O1 and Non O139 strains can also acquire toxigenic genes for toxin production by transduction and therefore might be the source of new epidemics. 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.

Even though the existence of *ctx* carrying Non O1 and Non O139 *V.cholerae* isolates was very low in shrimp culture system, the ecological significance of *ctx* genes among these *V.cholerae* Non O1 and Non O139 isolates in the shrimp aquaculture environment needs to be further investigated. The detection of ctx positive isolates among Non O1 and Non O139 *V.cholerae* isolates from *P.monodon* shrimp aquaculture system is being reported for the first time in this study.

The autochthonous existence of *V.cholerae* especially Non O1 and Non O139 in aquatic environment has been reported from several areas world over. In such a scenario, rejection of fish/shrimp by the importing nations based on the presence of what appears to be autochthonous bacterial flora in the shrimp culture system appears to be stringent. It is proposed that the mere presence of *V.cholerae* Non O1 and Non O139 need not be the criterion for rejection but as a safety measure *ctx* carrying *V.cholerae* Non O1 and Non O139 may be considered as potential public health risks. However, further studies are needed to establish *V.cholerae* Non O1 and O139 as native flora of black tiger shrimp culture system.

4.3.3.1. End point dilution of *V.cholerae*-duplex PCR

The sensitivity of *V.cholerae*-duplex PCR was determined by making serial 10 fold dilutions of *V.cholerae* culture (grown in T_1N_1, incubated at 37°C for 24h) in normal saline ranging from undiluted(10^6cells/ml), 10^{-1} (10^5 cells/ml), 10^{-2} (10^4 cells/ml), 10^{-3} (10^3 cells/ml), 10^{-4} (10^2 cells/ml) to 10^{-5} (10 cells/ml) and 10^{-6} (1 cell/ml). The *ctxAB* specfic primers yielded amplicon only when the concentration of *V.cholerae* was above 1000 cells/ml. Whereas, the species specific sodB primers yielded amplicon at

concentration of 100 cells/ml (Fig. 4.40). The intensity and thickness of band decreased with decrease in cell numbers.

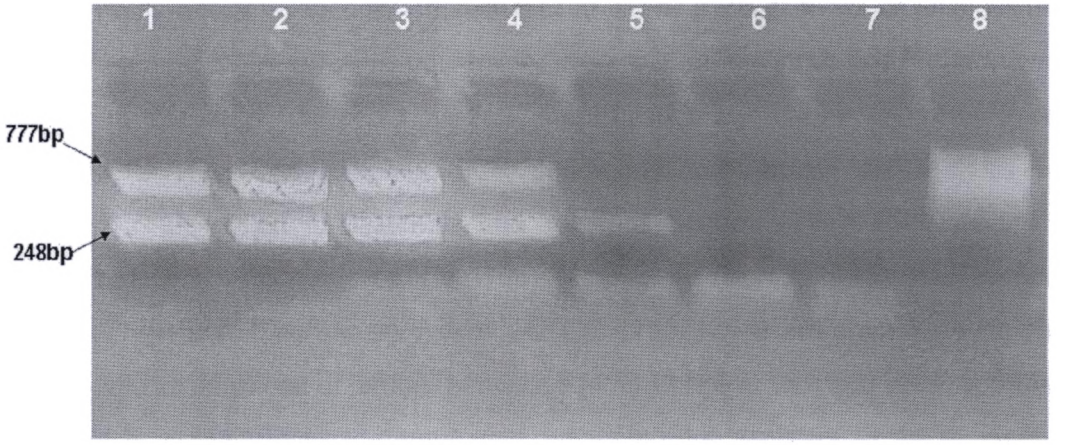


Fig. 4.40. End point dilution of *V.cholerae*-duplex PCR.
Lane 1, 10^6 *V.cholerae* cells; lane 2, 10^5 *V.cholerae* cells; lane 3, 10^4 *V.cholerae* cells; lane 4, 10^3 *V.cholerae* cells; lane 5, 100 *V.cholerae* cells; lane 6, 10 *V.cholerae* cells; lane 7, 1 *V.cholerae* cell; lane 8 , 100bp DNA ladder(GeNei)

The sensitivity attained was similar to previously reported sensitivities for multiplex PCR. The sensitivity of the multiplex PCR approach for the detection of *V.cholerae* (targeting *ctxA, ace, zot, tcpA* and *toxR*) reached to 10^2 cfu/ml (Jing *et al.*, 2003). A duplex PCR targeting the genes gyrB and tl for specific identification of *V.parahaemolyticus* could detect as few as 250 cfu/ml in pure cultures (Vongxay *et al.*, 2006). In the multiplex PCR (vvh fragment and viuB) for *V.vulnificus*, the sensitivity of detection for both targeted genes was 10 pg of purified DNA, which correlated with 10^3 cfu/ml of pure culture (Panicker *et al.*, 2004)

4.3.4. PCR for the detection of *V.alginolyticus* (Zhou et al., 2007)

Ribotyping and PCR fingerprinting methods were used to characterize different *V. alginolyticus* strains (Zanetti *et al.*, 1999; George *et al.*, 2005). Amplification of the R72H sequence (Robert-Pillot *et al.*, 2002) and gyrB gene (Venkateswaran *et al.*, 1998)

were used to differentiate *Vibrioparahaemolyticus* from *V. alginolyticus*. However, both methods can only be used for the detection of *V. parahaemolyticus*. Simultaneous detection of *V. alginolyticus*, *V.cholerae* and *V. parahaemolyticus* was demonstrated by a multiplex PCR assay (Di Pinto *et al.*, 2005).

A SYBR Green I Real time PCR assay targeting the house keeping gene *gyrB* of *V.alginolyticus* was developed by Zhou et al (2007). The gyrB gene which encodes the B subunit protein of DNA gyrase is universally present in all the strains of *V.alginolyticus*. However in this study, the real time PCR amplification conditions were employed in regular PCR. The DNA template, PCR conditions and agarose gel analysis were performed as described in Materials and Methods Section. The PCR was initially standardized using *V.cholerae* (MTCC 3906), *V.vulnificus* (MTCC 1145), *V.alginolyticus* (ATCC 17749) and *V.parahaemolyticus* (ATCC 17802) type cultures. *V.alginolyticus* cultures yield an amplicon of 340bp (between 814 and 1154 of the open reading frame of *gyrB*) which is specific to *V.alginolyticus* whereas other bacteria failed to yield the specific amplicon.

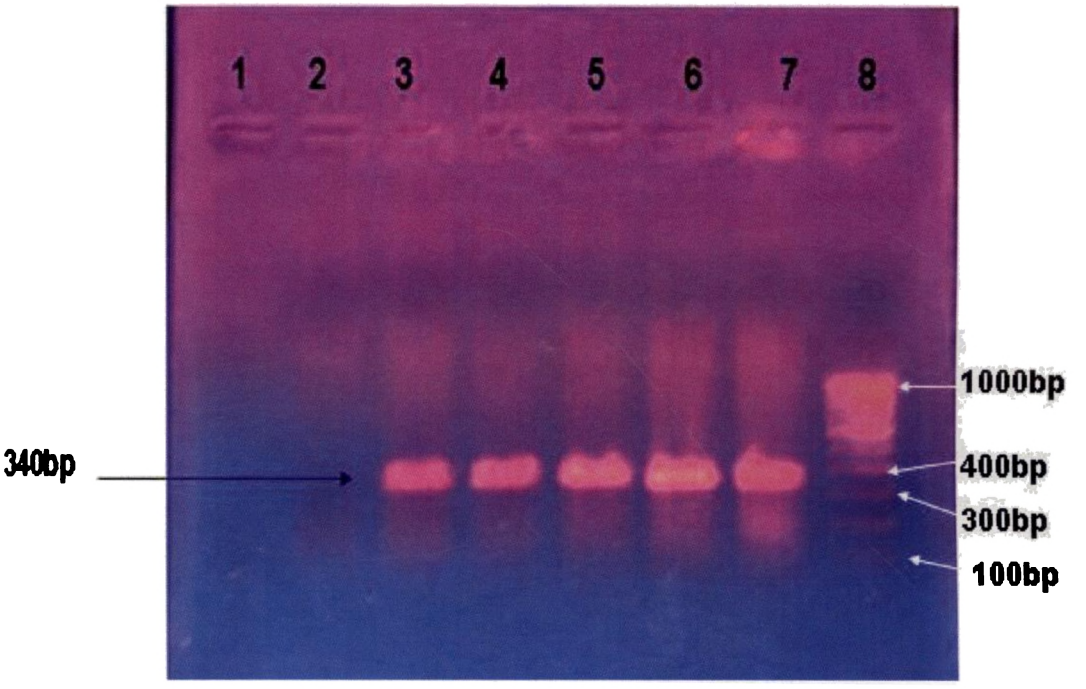


Fig. 4.41. PCR for the detection of *V.alginolyticus*.

Lane 2- Negative control, Lane 3-7 V.alginolyticus isolates (340bp), Lane 8-100bp DNA ladder

24.3% (51 isolates out of 210 Vibrio cultures) vibrio isolated from hatcheries and 38.4% (96 isolates out of 250 Vibrio cultures) vibrios isolates from aquaculture farms were found to be *V.alginolyticus*. All the *V.alginolyticus* isolates yielded the 340bp *V.alginolyticus* species specific amplicon (Fig. 4.41).

4.3.4.1. End point dilution of *V.alginolyticus* PCR

The sensitivity of *V.alginolyticus* specific PCR was determined by making serial 10 fold dilutions of *V.alginolyticus* culture (grown in T_1N_1, incubated at 37°C for 24h) in normal saline ranging from undiluted (9×10^7 cells/ml), 10^{-1} (9×10^6 cells/ml), 10^{-2} (9×10^5 cells/ml), 10^{-3} (9×10^4 cells/ml), 10^{-4} (9×10^3 cells/ml) to 10^{-5} (9×10^2 cells/ml) and 10^{-6} (90 cells/ml). From each tube 1 ml was withdrawn for template preparation. PCR for the detection of *V.alginolyticus* was carried out as elaborated in Material and Methods section.

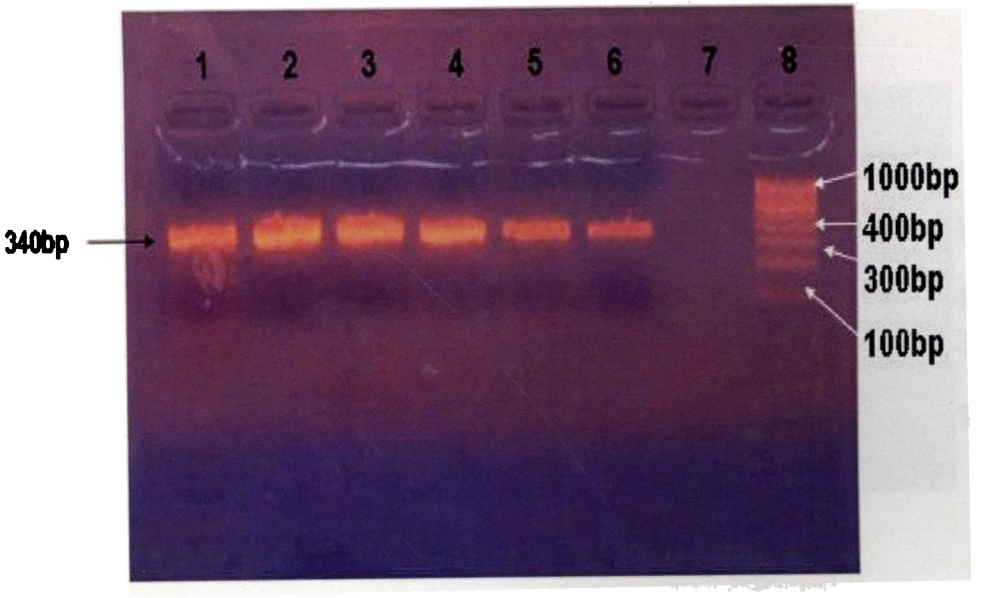


Fig. 4.42. End point dilution of *V.alginolyticus* PCR.

Lane 1-10^{-1} (9×10^6 cells/ml), Lane 2-10^{-2} (9×10^5 cells/ml), Lane 3-10^{-3} (9×10^4 cells/ml), Lane 4-10^{-4} (9×10^3 cells/ml) Lane 5-10^{-5} (9×10^2 cells/ml) and Lane6-0^{-6} (90 cells/ml).

V.alginolyticus specific PCR could detect 90 cells/ml (Fig. 4.42). The intensity and thickness of band decreased with decrease in cell numbers.

4.3.5. *V.alginolyticus*-duplex PCR for the using species specific and genus specific primers

A *V.alginolyticus*-duplex PCR method was developed by utilizing *V.alginolyticus* species specific primers (Zhou *et al.*, 2007) and Vibrio genus specific primers (Tarr *et al.*, 2007) This method was designed to detect *Vibrio species and V.alginolyticus*. One set of primer targets the *16S rRNA* genes (Tarr *et al.*, 2007) which are specific to the genus Vibrio. The second set of primers target sequences within the house keeping gene *gyrB* which is specific to *V.alginolyticus* (Zhou *et al.*, 2007). *V.alginolyticus* isolates were grown in T_1N_1 and incubated at 37C for 24h and used for *V.alginolyticus*-duplex PCR. Template DNA preparation (3.2.7.3.), PCR reaction preparation, PCR cycle conditions and agarose gel analysis of PCR products were performed as described in the Materials and Methods section.

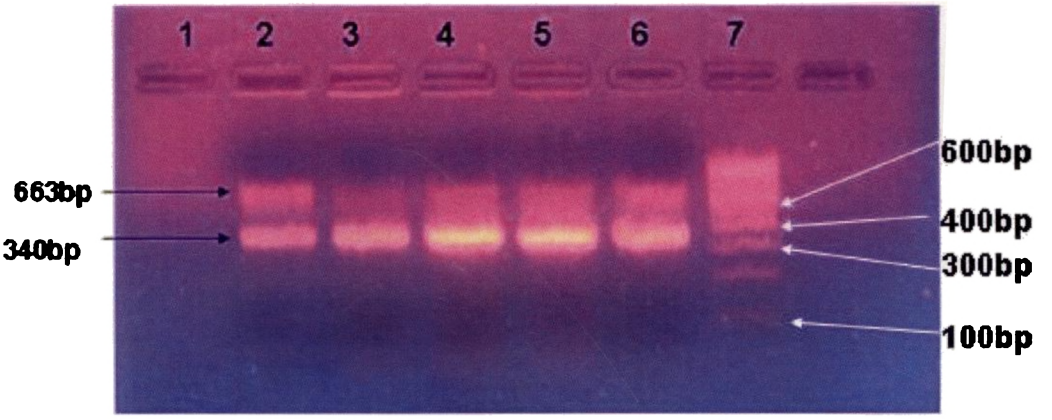


Fig. 4.43. *V.alginolyticus*-duplex PCR.

Lanes 2-6 *V.alginolyticus* specific amplicon (340bp) and Vibrio genus specific amplicon (663bp). Lane 7, 100bp DNA ladder.

All *V.alginolyticus* cultures yield 2 amplicons of sizes viz., 340bp and 663 bp (Fig. 4.43). Other Vibrio cultures yield only a single amplicon of 663bp size.

4.3.6. PCR for detection of *V.vulnificus*

Rapid Alert System for Food and Feed (RASFF) alert notifications were issued for the presence of *V.vulnificus* (Table 1.2). The presence of the pathogen is sufficient. In such a scenario the target sequence should always be present.

V.vulnificus-PCR uses species-specific primers that target the house keeping gene *hsp60* of *V.vulnificus* (Tarr *et al.*, 2007). As the house keeping genes are invariably present in all isolates this PCR helps in the detection of all *V.vulnificus* isolates. Template preparation, PCR reaction preparation, PCR cycle conditions and agarose gel analysis of PCR products were performed as described in Materials and Methods section. The PCR was initially standardized using *V.cholerae* (MTCC 3906), *V.vulnificus* (MTCC 1145), *V.alginolyticus* (ATCC 17749) and *V.parahaemolyticus* (ATCC 17802) type cultures. Only the *V.vulnificus* cultures yielded the specific amplicon of 410bp (Fig. 4.44).

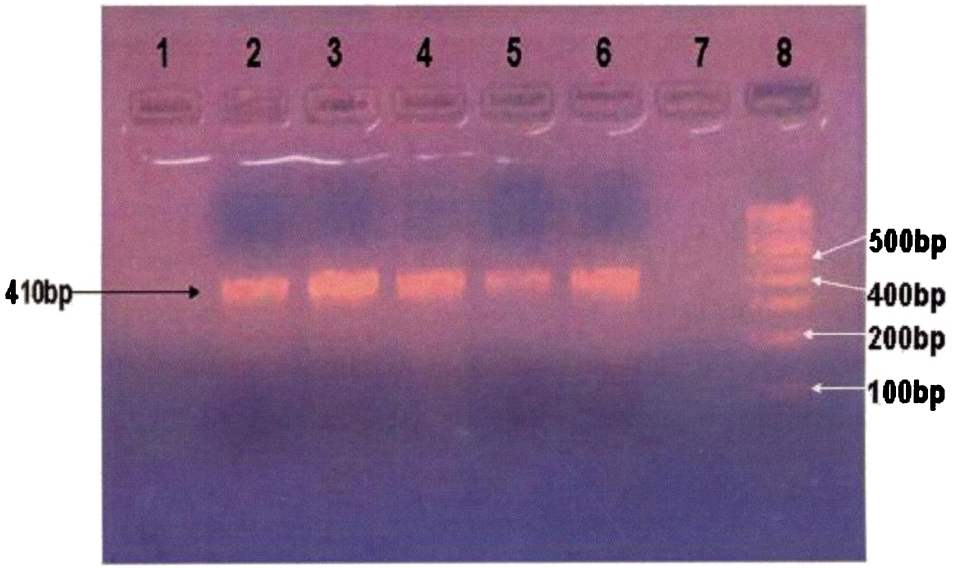


Fig. 4.44. PCR for detection of *V.vulnificus*.

Lane 2-6 *V.vulnificus* isolates (410bp), Lane 8-100bp DNA ladder

14 *V.vulnificus* isolates were obtained from hatchery waters and 4 *V.vulnificus* isolates were obtained from post-larvae. *V.vulnificus* was not detected in aquaculture farm samples.

4.3.7. PCR for detection of *V.parahaemolyticus*

PCR-based methods that amplify regulatory *toxR* sequences (Kim *et al.*, 1999), conserved sequences such as *gyrB* (Venkateswaran *et al.*, 1998), conserved chromosomal sequences (Lee *et al.*, 1995; Karunasagar *et al.*, 1996; Karunasagar *et al.*, 1997), and hemolysin sequences such as *tdh* (Kelly and Stroh, 1988; Tada *et al.*, 1992; Nishibuchi and Kaper, 1995; Dileep *et al.*, 2003), *trh* (Tada *et al.*, 1992), or *tlh* (thermolabile hemolysin) (Bej *et al.*, 1999) have been used by various workers.

Rapid Alert System for Food and Feed (RASFF) alert notifications were issued for the presence of *V.parahaemolyticus* (Table 1.2). The mere presence of the pathogen is the criterion for rejection. In such a scenario the target sequence should always be present. The PCR assay targeting the *toxR* gene achieved the highest performance (100% inclusivity and exclusivity); PCR protocols based on *tlh* gene detection, although showing the same inclusivity (100%), differed in the exclusivity (50 and 91%, respectively) and the results provided by the PCR assays targeting the *gyrB* gene and *pR72H* fragment were less reliable (Croci *et al.*, 2007).

V.parahaemolyticus-PCR uses species-specific primers that target the *flaE* sequence in the flagellin gene of *V.parahaemolyticus* (Tarr *et al.*, 2007). Template preparation, PCR reaction preparation, PCR cycle conditions and agarose gel analysis of PCR products were performed as described in Materials and Methods section. The PCR was initially standardized using *V.cholerae* (MTCC 3906), *V.vulnificus* (MTCC 1145), *V.alginolyticus* (ATCC 17749) and *V.parahaemolyticus* (ATCC 17802) type cultures. Only the *V.parahaemolyticus* cultures yielded the specific amplicon of 897bp.

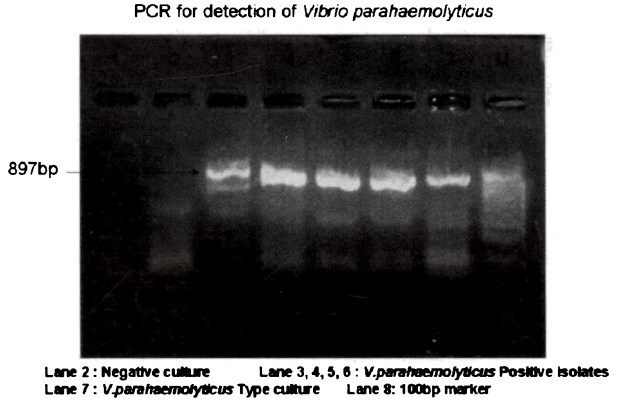
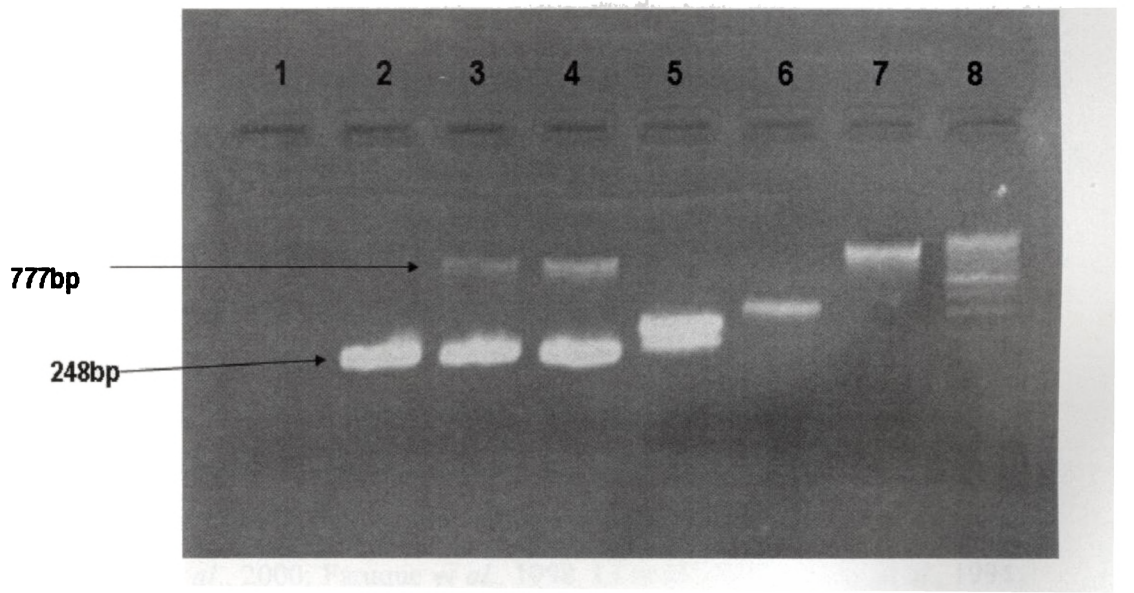


Fig. 4.45.PCR for detection of *V.parahaemolyticus*.

was planned to include all these primers in a single reaction with an annealing temperature of 57°C.

The anticipated result was obtained during the standardization of the pathogenic Vibrio-multiplex PCR employing *V.cholerae* (MTCC 3906), *V.vulnificus* (MTCC 1145), *V.alginolyticus* (ATCC 17749) and *V.parahaemolyticus* (ATCC 17802) type cultures and *V.cholerae* (ctx negative) cultures.



Lane 1: Negative Control, Lane 2 : *V.cholerae* (248bp),
Lane 3 & 4: *V.cholerae ctx positive* (248bp & 777bp), Lane 5 : *V.alginolyticus* (340bp),
Lane 6 : *V.vulnificus* (410bp), Lane 7 : *V.parahaemolyticus* (897bp),
Lane 8: 100bp DNA ladder

Fig. 4.46.Pathogenic Vibrio-multiplex PCR

When specific DNA was added as template to the PCR mix, only the corresponding primers specifically reacted and yielded that particular amplicon (single amplicon in the presence of multiple primers). Non cholera toxin producing *V.cholerae* cultures yielded an amplicon of 248bp; *ctxAB* positive *V.cholerae* cultures yielded two amplicons viz., 248bp and 777bp; *V.alginolyticus* cultures yielded an amplicon of 340bp; *V.vulnificus* cultures yielded an amplicon of 410bp and *V.parahaemolyticus* cultures yielded an amplicon of 897bp (Fig. 4.46).

4.3.8.1. Multiplex PCR for pathogenic Vibrios using unknown culture.

Unidentified colonies (yellow) from TCBS agar plate were transferred to T_1N_1 broth tubes and incubated at 37°C for 24h. These cultures were used for PCR template preparation. The bacterial colony was suspended in normal T_1N_1 broth, centrifuged and the pellet suspended in TE buffer and pathogenic Vibrio-Multiplex PCR was performed as described in Materials and Methods section. All the cultures yielded a single amplicon of 340bp size indicating that the unknown cultures were *V.alginolyticus* cultures. The results indicate the usefulness of the pathogenic Vibrio-Multiplex PCR in rapid identification of any of these common pathogenic Vibrios.

4.3.9. PCR fingerprinting of *V.cholerae* isolates using RS-PCR, REP-PCR and ERIC-PCR

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). 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 (Brazil *et al.*, 2002; Chakraborty *et al.*, 2000; Faruque *et al.*, 1998, Li *et al.*, 2002, Mintz *et al.*, 1994; Sechi *et al.*, 2000; Singh *et al.*, 2001). Molecular typing methods were employed to study the genetic heterogeneity of pathogenic Vibrios. 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 and comparison of ribotype patterns showed a high degree of genetic divergence within *V. cholerae* non-O1. Keymer et al (2007) studied the genomic diversity of coastal *V.cholerae* strains and observed that autochthonous environmental isolates of this species routinely display more extensive genetic diversity than the primarily pathogenic strains.

4.3.9.1. RS-PCR targeting Ribosomal Gene Spacer Sequence

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). Wong and Lin (2001) suggested that 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.

The *V.cholerae* isolates (n = 35) obtained from shrimp aquaculture system yielded fewer amplification bands ranging between 2 and 4 in the RS-PCR (Fig. 4.47). The bands were tightly grouped in a narrow spectrum between 530bp and 870bp. The *V.cholerae* isolates that yielded only 2 amplification bands were isolated from shrimp muscle and pond sediment. The first two amplification bands (lower size bands) were present in all the isolates.

The electrophoretic patterns of the *V.cholerae* isolates obtained using RS-PCR, were analysed using Gel Compar^{R} II software, 2005 (Applied Maths, Belgium) and dendograms were constructed based on unweighted pair-group method with arithmetic means (UPGMA). The dendograms of *V.cholerae* isolates from farms using RS-PCR shows that relatively strong similarity exists amongst the *V.cholerae* isolates obtained from shrimp aquaculture (Fig. 4.48a, Fig. 4.48b). The similarity between the *V.cholerae* isolates ranged between 75 and 100%. The dendogram (Fig. 4.48a) shows the result for 22 *V.cholerae* isolates. The 22 isolated showed 10 fingerprint patterns. 100% similarity was observed between certain groups of the *V.cholerae*. A maximum of 7 isolates showed the same fingerprint pattern (Fig. 4.52b). The choleratoxigenic (ctx +ve) *V.cholerae* (1 and 2) isolated from aquaculture environment were grouped under separate clones. The *ctx* +ve *V.cholerae* showed closer genetic similarity with *ctx* negative *V.cholerae* than with amongst *ctx* +ve *V.cholerae* isolates. The similarity is expected as the *ctx* positive *V.cholerae* belonged to Non O1 and Non O139 serogroup and all the *ctx* negative *V.cholerae* isolates also obtained belonged to the Non O1 and Non O139 group. The 100% similarity of *ctx* positive *V.cholerae* with ctx –ve *V.cholerae* isolates indicates

that the *ctx* positive strains might have originated from autochthonous *V.cholerae* in the aquatic niche.

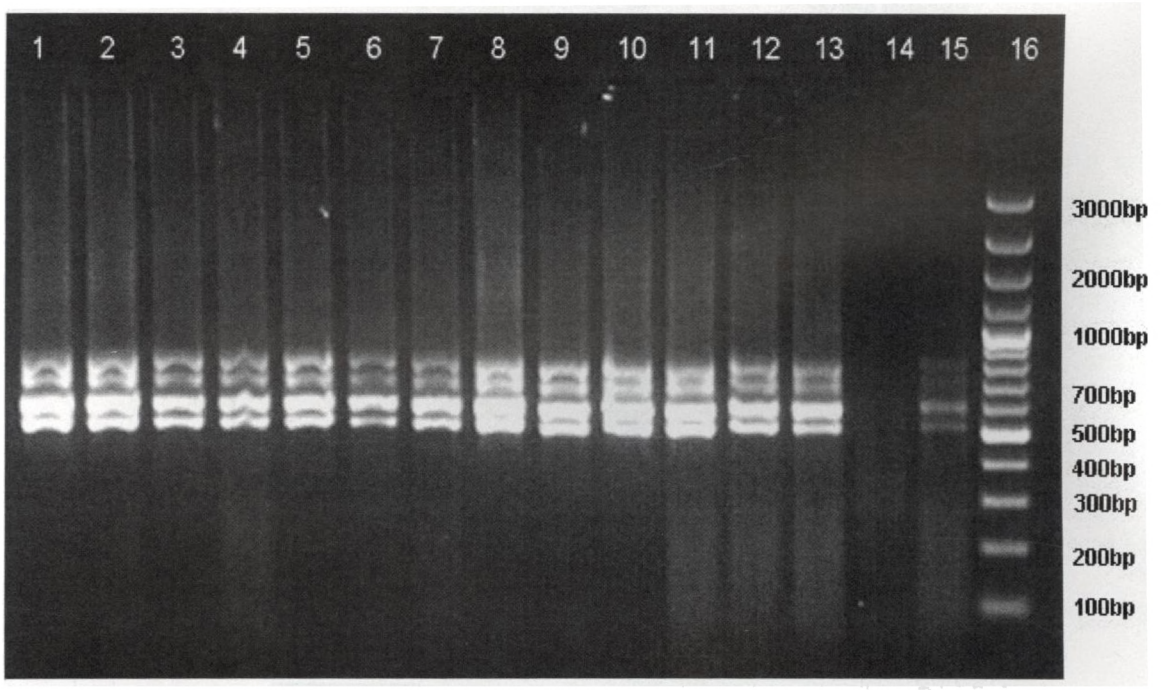


Fig. 4.47. DNA finger print pattern of *V.cholerae* isolates from farms using RS-PCR

Lane 1-14 : *V.cholerae* isolates from shrimp aquaculture system (Pond water in lanes 9, 10, 11 & 13; Pond sediment in lanes 1, 2, 7, 8 & 12; shrimp head in lanes 4 & 6; shrimp muscle in lanes 3 & 5); **Lane 11**: ctx +ve *V.cholerae* isolate; **Lane 15**: *V.cholerae* MTCC 3906, **Lane 16**: DNA molecular weight marker 100bp to 3000bp (Gene Ruler^{TM}, Fermentas)

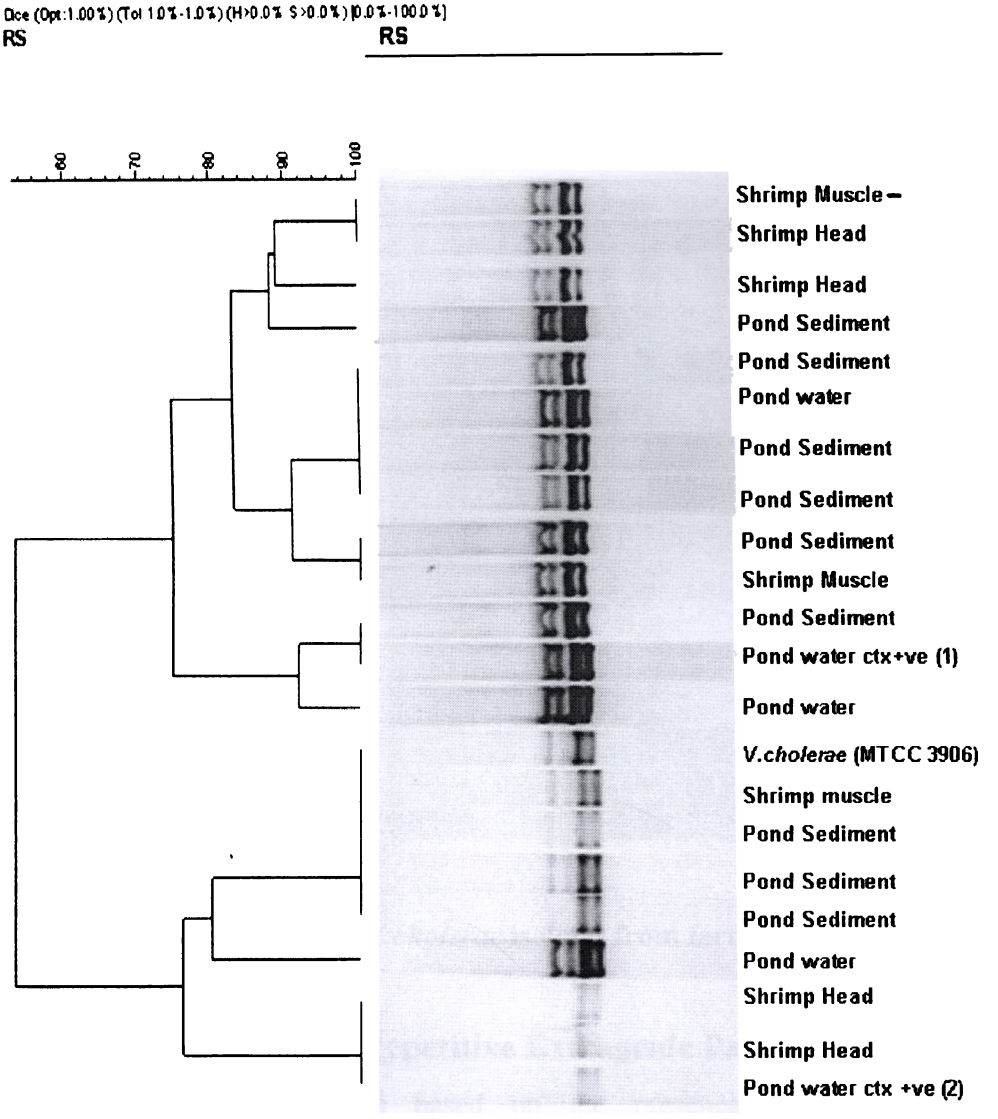


Fig. 4.48a. Dendogram of *V.cholerae* isolates from farms using RS-PCR

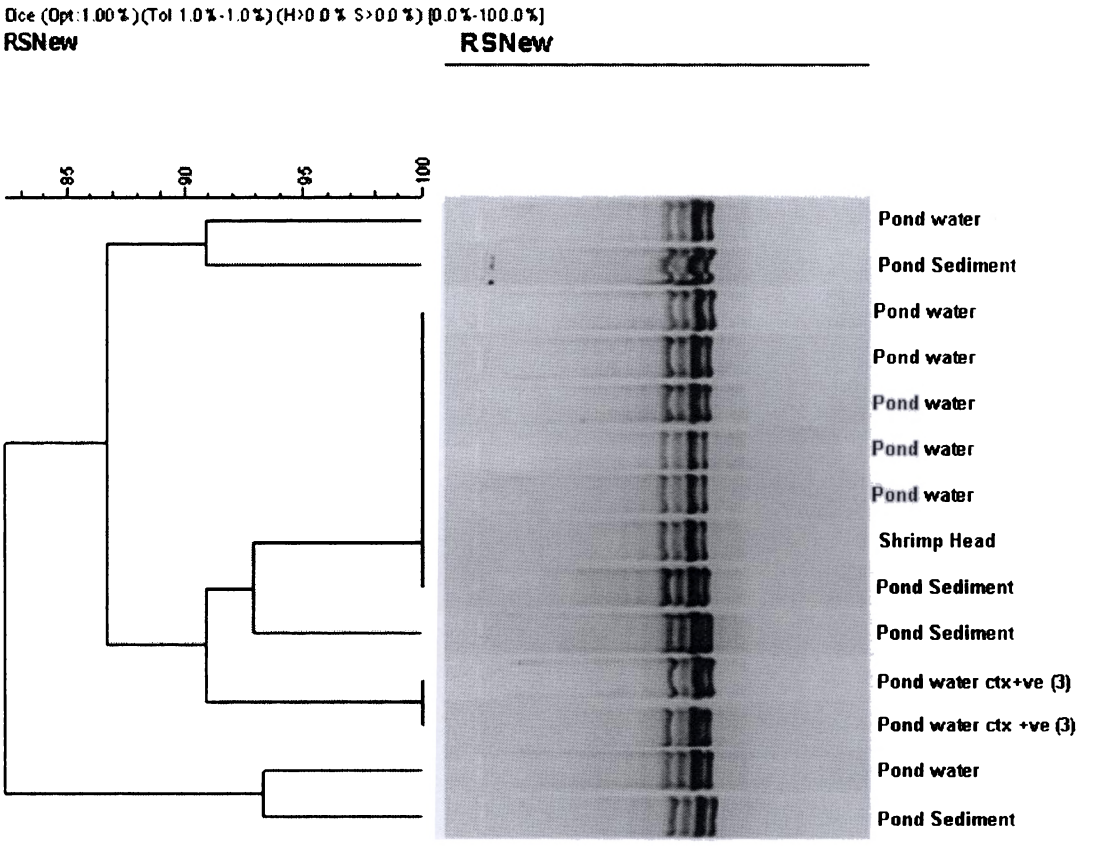


Fig. 4.48b. Dendograms of *V.cholerae* isolates from farms using RS-PCR

4.3.9.2. REP-PCR targeting Repetitive Extragenic Palindromes

The REP-PCR method is based on the presence of 38-bp REPs in *Enterobacteriaceae* and other bacteria and has been applied to many species (Marshall *et al.*, 1999; Rodriguez *et al.*, 1995; Stern *et al.*, 1984; Stubbs *et al.*, 1999). Shangkuan et al (1997) studied the diversity of DNA sequences among *Vibrio cholerae* O1 and non-O1 isolates detected by whole-cell repetitive element sequence based polymerase chain reaction (rep-PCR) and the results indicated that rep-PCR can be used to identify and differentiate different toxigenic O1, non-toxigenic O1 and non-O1 *V.cholerae* isolates.

In this study, REP-PCR showed more extensive genetic diversity among the *V.cholerae* isolates (n = 33) obtained from shrimp aquaculture. The number of amplification bands varied from one band to 10 amplification bands. The size of the

bands ranged between 1700bp to 100bp (Fig. 4.49). The amplification bands viz., 1200bp, 960bp, 350bp and 220bp were present in most of the *V.cholerae* isolates.

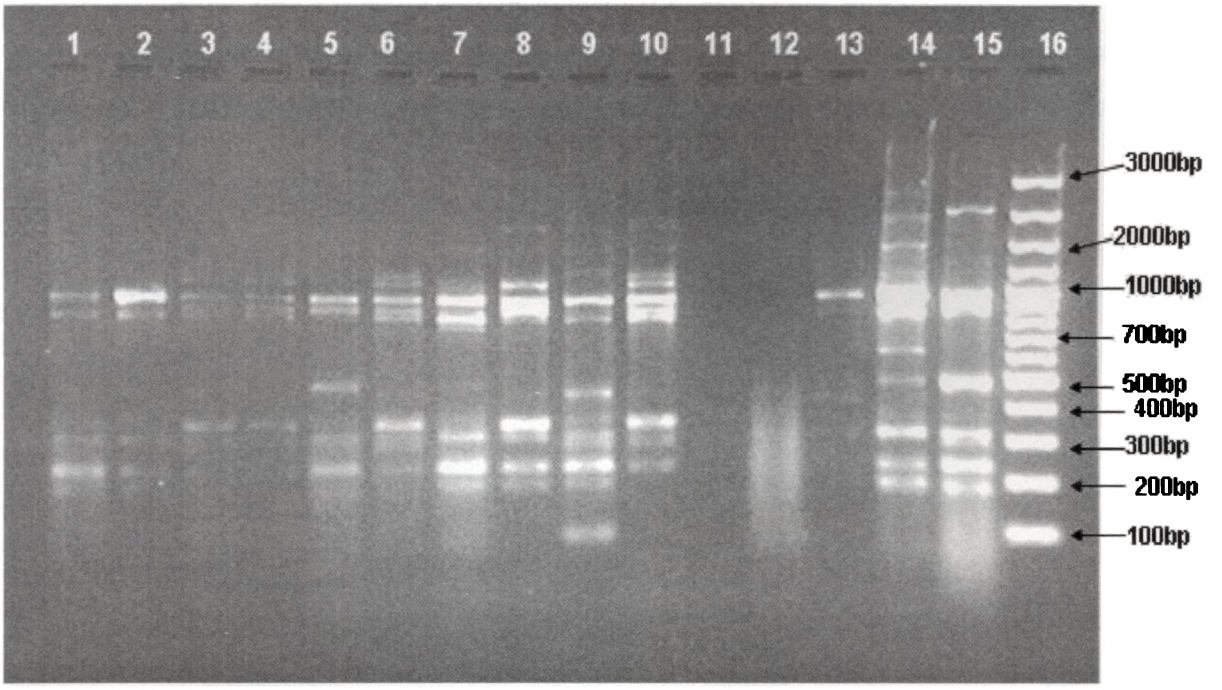


Fig. 4.49. DNA finger print pattern of *V.cholerae* isolates from farms using REP-PCR

Lane 1-14 : *V.cholerae* isolates from shrimp aquaculture system (Pond water in lanes 1, 2, 3, 4, 5, 6, 8, 9 & 11; Pond sediment in lanes 7, 12, 13 & 14; shrimp muscle in lane 10); **Lane 2 & 8**: ctx +ve *V.cholerae* isolates; **Lane 15**: *V.cholerae* MTCC 3906, **Lane 16**: DNA molecular weight marker 100bp to 3000bp (Gene Ruler^{TM}, Fermentas)

The dendogram of *V.cholerae* isolates from shrimp aquaculture farms using REP-PCR showed relatively extensive genetic diversity among the *V.cholerae* isolates (Fig 4.50a, Fig. 4.50b). The similarity between the *V.cholerae* isolates ranged between 60 and 95%.The dendogram (Fig. 4.50a) constructed with 25 isolates showed 25 different fingerprint patterns. Only 4 isolates showed similarity of above 90% and none of the isolates showed 100% similarity with other isolates. The ctx +ve *V.cholerae* isolates (1 and 3) showed greater similarity (75%) in REP-PCR and were grouped under different

arms of the same clone. Shangkuan et al (1997) obtained sixteen different rep-PCR types from 24 non-O1 *V.cholerae* strains isolated from human and seafood from Taiwan. All the *V.cholerae* isolates obtained in this study belonged to Non O1 and Non O139 serogroup. Diverse set of fingerprint types obtained might be due to the diversity of the Non O1 and Non O139 *V.cholerae*. The Non O1 and Non O139 comprise of nearly 180 serogroups representing O2 through O138 and other groups (Shimada *et al.*, 1994; Yamai *et al.*, 1997)

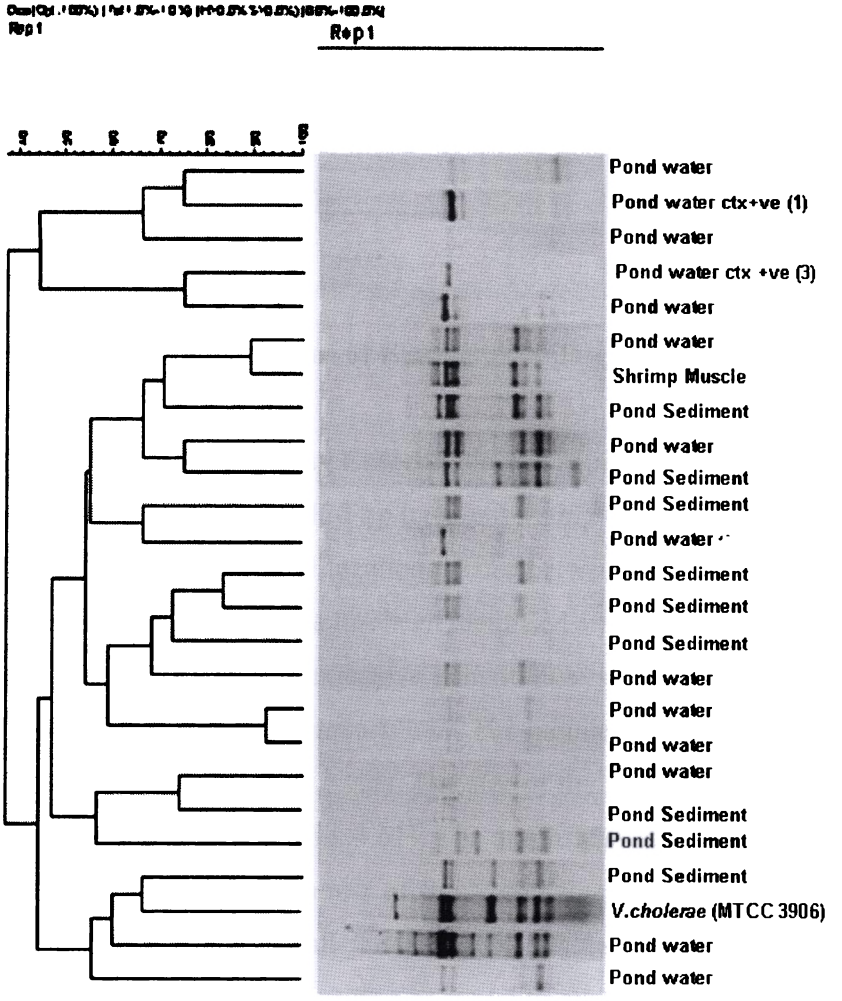


Fig. 4.50a. Dendograms of *V.cholerae* isolates from farms using REP-PCR

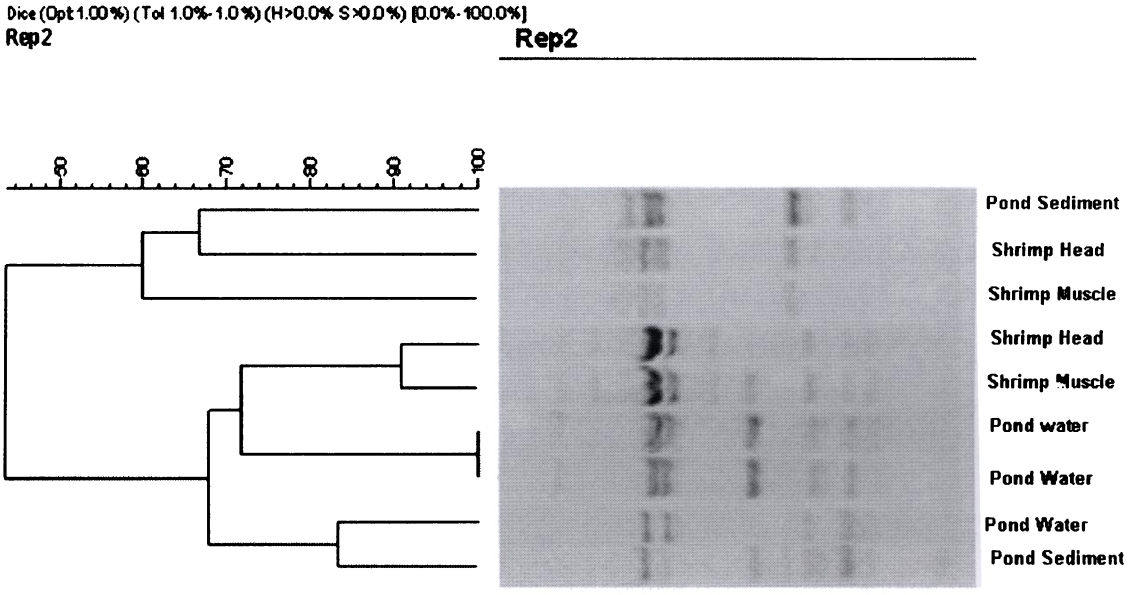


Fig. 4.50b. Dendograms of *V.cholerae* isolates from farms using REP-PCR

4.3.9.3. ERIC-PCR targeting Enterobacterial repetitive intergenic consensus

ERIC sequences are 126 bp long 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,

ERIC-PCR showed extensive genetic diversity among the *V.cholerae* isolates (n = 36) obtained from shrimp aquaculture. The number of amplification bands varied from one band to 12 amplification bands. The size of the bands was spread over a wider range between 3000bp to 150bp (Fig. 4.51). The amplification bands viz., 3000bp, 2250bp and 240bp size were present in most of the V.cholerae isolates. The amplification band of 470bp size was present in all the *V.cholerae* isolates except one. Rivera et al (1995) reported that a 0.5-kb fragment was common to all strains and serogroups of *V. cholerae* analyzed and the number of amplification bands for Non O1 *V.cholerae* ranged between 2 and 8 with band sizes ranging between 180 to 2700bp using ERIC-PCR.

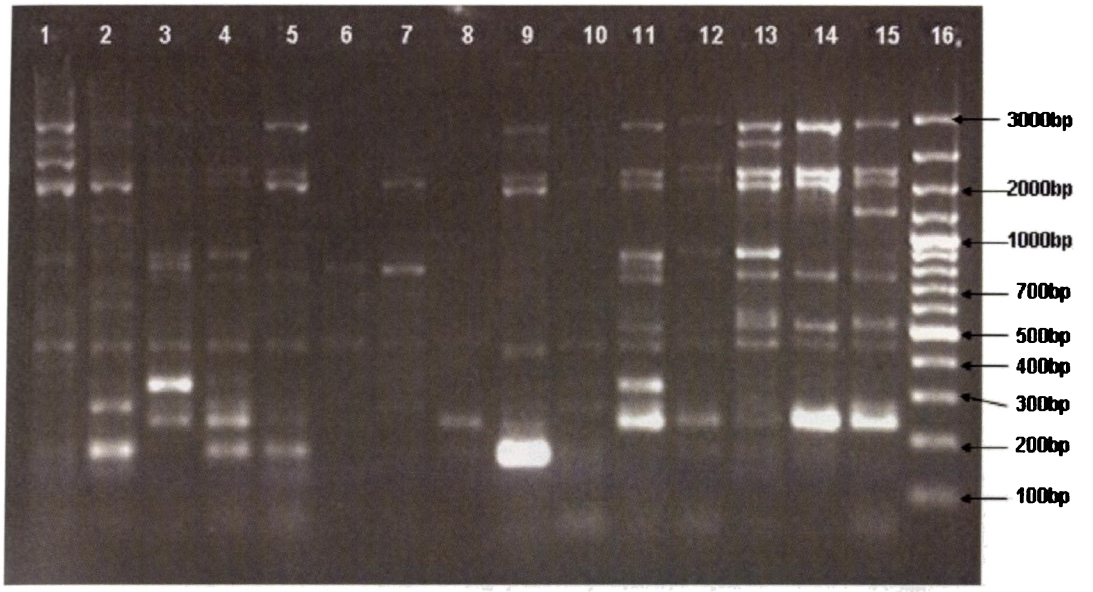


Fig. 4.51. DNA finger print pattern of *V.cholerae* isolates from farms using ERIC-PCR

Lane 1-14 : *V.cholerae* isolates from shrimp aquaculture system (Pond water in lanes 1, 2, 3, 4, 5, 6, 7, 10 & 11; Pond sediment in lanes 9, 12, 13, 14 & 15; shrimp head in lane 8); **Lane 2 & 10**: ctx +ve *V.cholerae* isolates; **Lane 16**: DNA molecular weight marker 100bp to 3000bp (Gene Ruler^{TM}, Fermentas)

The dendogram of *V.cholerae* isolates from shrimp aquaculture farms using ERIC-PCR showed greater genetic diversity among the *V.cholerae* isolates (Fig. 4.52a, Fig 4.52b). The similarity between the *V.cholerae* isolates ranged between 40 and 95%.The dendogram (Fig. 4.52a) constructed with 24 isolates showed 24 different fingerprint patterns. None of the *V.cholerae* isolates showed 100% similarity with other *V.cholerae* isolates obtained from shrimp aquaculture system. The ctx positive V.cholerae isolates were grouped under different clones. The *ctx* positive isolates showed greater similarity with *ctx* negative *V.cholerae* isolates than with other *ctx* positive isolates. Rivera et al (1995) obtained a total of 15 different patterns among the *V. cholerae* non-O1 strains.

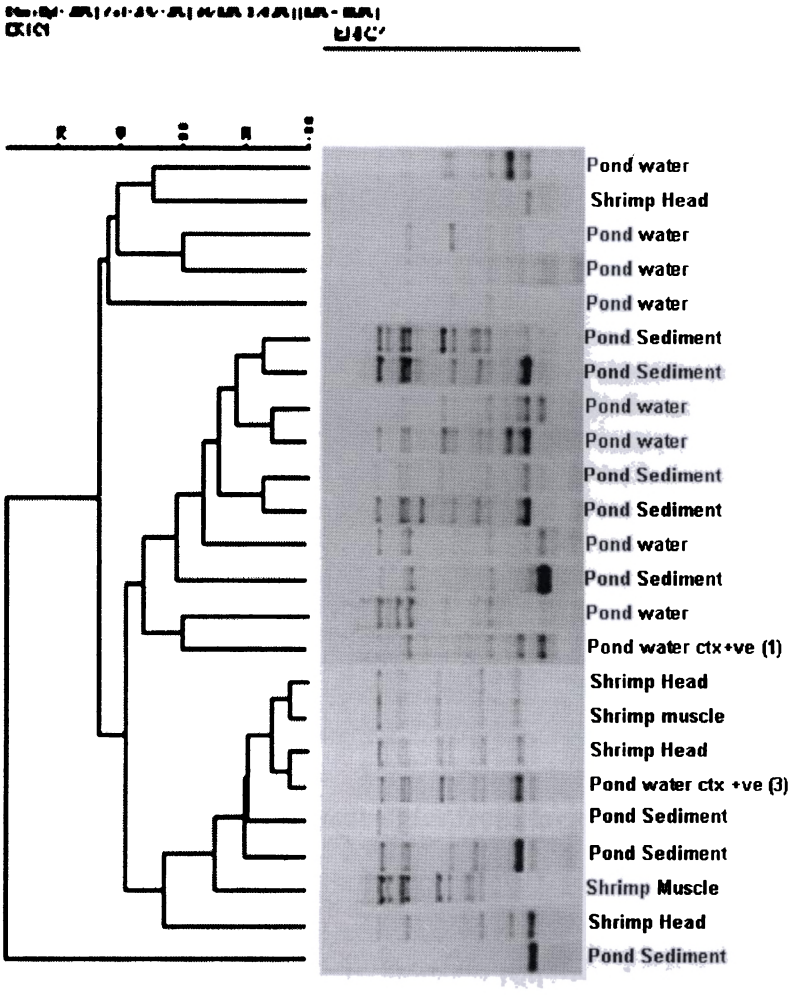


Fig. 4.52a. Dendograms of *V.cholerae* isolates from farms using ERIC-PCR

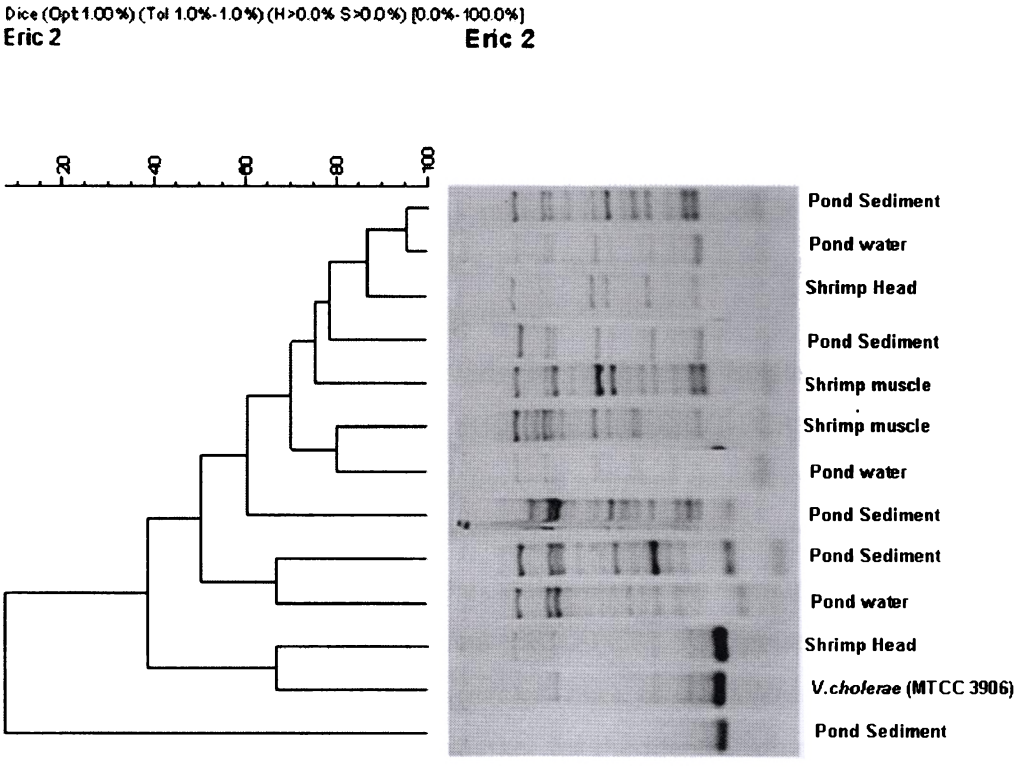


Fig. 4.52b. Dendograms of *V.cholerae* isolates from farms using ERIC-PCR

4.3.9.4. Similarity among patterns of the *V. cholerae* isolates obtained from shrimp aquaculture farms obtained by three different PCR fingerprinting methods.

RS-PCR yielded fewer bands (maximum 4) when compared to REP-PCR (maximum 10 bands) and ERIC-PCR (maximum 12 bands). The dendogram of RS-PCR (Fig. 4.48a) for 22 *V.cholerae* isolates showed 10 fingerprint patterns whereas the dendogram of REP-PCR (Fig. 4.50a) for 25 isolates and dendogram of ERIC-PCR (Fig. 4.52a) for 24 isolates showed 25 fingerprint patterns and 24 fingerprints, respectively. 100% similarity between *V.cholerae* isolates obtained from shrimp aquaculture was noticed only in RS-PCR. None of the isolates showed 100% similarity either in REP-PCR or ERIC-PCR.

Autochthonous environmental isolates of *V. cholerae* routinely display more extensive genetic diversity than the primarily pathogenic strains (Keymer et al., 2007). Maluping et al (2005) demonstrated genetic variability within the *V.parahaemolyticus* strains isolated from shrimps (*Penaeus monodon*) and concluded that REP-PCR is inferior to ERIC-PCR owing to the fact that it is less reproducible and it yielded a relatively small number of products. Wong and Lin (2001) suggested, while analysing *V.parahaemolyticus*, that REP-PCR was preferable to ERIC-PCR because of greater reproducibility of its fingerprints while RS-PCR might be a practical method because it generates fewer amplification bands and patterns.

From this PCR fingerprinting study on V.cholerae isolated from *P.monodon* shrimp farms, it can be concluded that REP-PCR and ERIC-PCR are best suitable to study the genetic variation amongst *V.cholerae* at a higher sensitivity level. On the other hand, the RS-PCR will be helpful in comparing the genetic similarity among *V.cholerae*. The isolates that were less genetically apart will be shown as similar in RS-PCR but will be shown as different in REP-PCR and ERIC-PCR. The presence of 470bp size amplification band in all the isolates (except one) of *V.cholerae* using ERIC-PCR analysis suggests it's potential use as a marker for the identification of *V.cholerae*. The greater similarity of *ctx* positive *V.cholerae* with ctx –ve *V.cholerae* isolates indicates that the *ctx* positive strains (Non O1 and Non O139) might have originated from autochthonous *V.cholerae* in the aquatic niche.

4.3.10. Real time PCR for *V.cholerae*

Real time PCR is a quantitative PCR and Real time PCR chemistries allow for the detection of PCR amplification during the early phases of the reaction. An increase in the reporter fluorescent signal is directly proportional to the number of amplicons generated. 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 more template present at the beginning of the amplification reaction, the fewer number of cycles kit takes to reach C_T (cycle

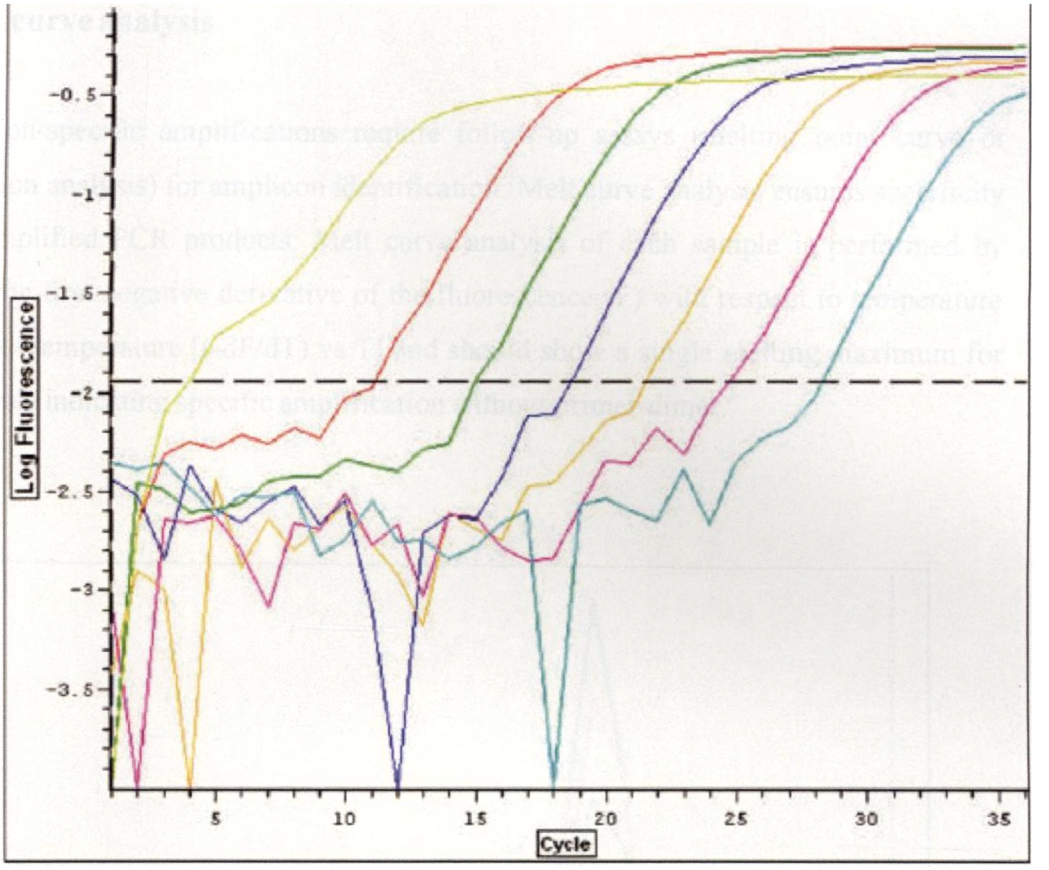


Fig. 4.53. Real time PCR data Sheet for *V.cholerae* using species specific primers

Table 4.44. Quantity Calculations of Real time PCR of *V.cholerae*

Well	Dye	Content	Efficiency	C(T)	ng
A1	SYB	Standard	85.14%	11.15	105
B1	SYB	Standard	113.56%	14.97	10
C1	SYB	Standard	76.17%	18.67	1
D1	SYB	Standard	80.82%	21.59	0.1
E1	SYB	Standard	47.61%	24.73	0.01
F1	SYB	Standard	84.24%	28.29	0.001
G1	SYB	Sample	44.99%	3.99	9035

Melting curve analysis

Non-specific amplifications require follow-up assays (melting point curve or dissociation analysis) for amplicon identification. 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.

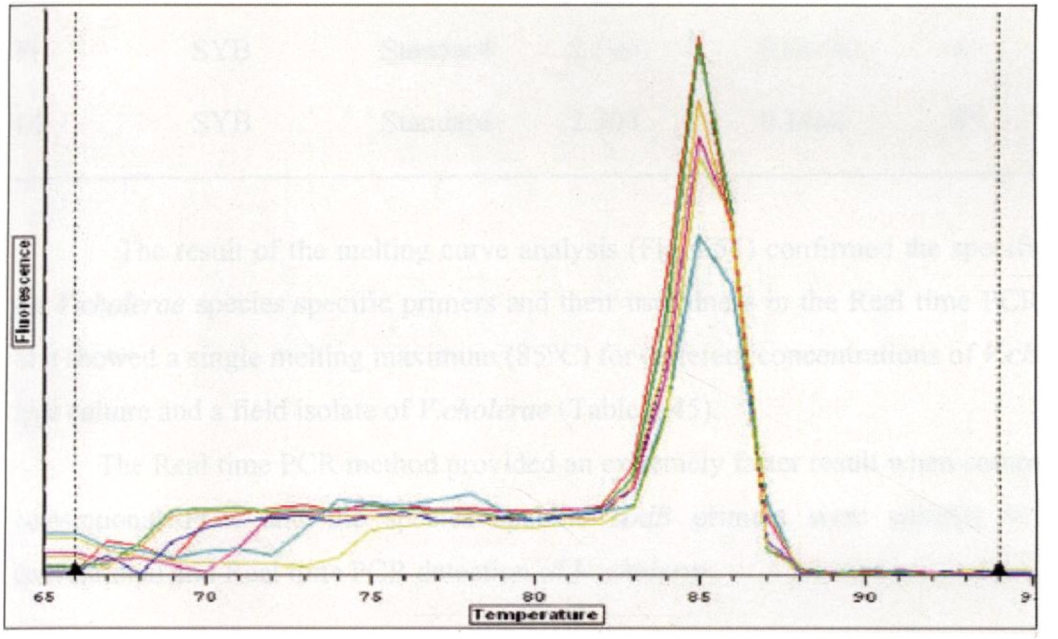


Fig.4.54. Melt curve analysis of the Real time PCR products obtained using *V.cholerae* species specific primers

Table 4.45. Melting temperature calculation of the Real Time PCR products

Well	Dye	Content	FWHM	-dI/dT max)	Tm
A1	SYB	Standard	2.343	0.1871	85
B1	SYB	Standard	2.242	0.1768	85
C1	SYB	Standard	2.255	0.1629	85
D1	SYB	Standard	2.252	0.156	85
E1	SYB	Standard	2.362	0.1376	85
F1	SYB	Standard	2.506	0.08842	85
G1	SYB	Standard	2.303	0.1468	85

The result of the melting curve analysis (Fig 4.54) confirmed the specificity of the *V.cholerae* species specific primers and their usefulness in the Real time PCR assay as it showed a single melting maximum (85°C) for different concentrations of *V.cholerae* type culture and a field isolate of *V.cholerae* (Table 4.45).

The Real time PCR method provided an extremely faster result when compared to conventional PCR and the species specific *sodB* primers were suitable for both conventional and Real time PCR detection of *V. cholerae*.

CHAPTER · 5

SUMMARY

5. SUMMARY

Vibrios are important during hatchery rearing, aquaculture phase and post-harvest quality of black tiger shrimp, *Penaeus monodon*. *Vibrio spp* are of concern to shrimp farmers and hatchery operators because certain species cause Vibriosis. Vibrio species are of concern to humans because certain species cause serious diseases. Vibrios related to post harvest shrimp quality are mainly *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus*. Rapid Alert System for Food and Feed (RASFF) of the European Commission has issued alert notifications with respect to *P. monodon* and other shrimp exported to Europe from India because of the presence of pathogenic vibrios. Recent rejections vis-à-vis Vibrios in black tiger shrimps were mainly due to the presence of *V. cholerae* and V. *parahaemolyticus*. The export rejections cause serious economic loss to the shrimp industry and might harm the brand image of the shrimp products from the country.

There is a need for an independent study on the incidence of different pathogenic vibrios in shrimp aquaculture and investigate their biochemical characteristics to have a better understanding about the growth and survival of these organisms in the shrimp aquaculture niche. PCR based methods (conventional PCR, duplex PCR, multiplex-PCR and Real Time PCR) for the detection of the pathogenic Vibrios is important for rapid post-harvest quality assessment. Studies on the genetic heterogeneity among the specific pathogenic vibrio species isolated from shrimp aquaculture system provide valuable information on the extent of genetic diversity of the pathogenic vibrios the shrimp aquaculture system. The present study was undertaken with this goal.

Samples of water (n=7) and post-larvae (n=7) were obtained from seven *Penaeus monodon* hatcheries and samples of water (n=5), sediment (n=5) and shrimp (n=5) were obtained from five *Penaeus monodon* aquaculture farms located on the East Coast of India. The microbiological examination of water, sediment, post-larvae and shrimp samples was carried out employing standard methods and by using standard media.

In aquaculture pond samples the mean TPC of pond sediment ($2.9 \times 10^5 \pm 1.4 \times 10^4$ cfu/g) was 2 logs higher than pond water ($3.5 \times 10^3 \pm 790$ cfu/ml). The TPC of pond sediments ranged from 2.8×10^5 to 3.04×10^5 cfu/g while the TPC of pond waters ranged from 2.6×10^3 to 4.4×10^3 cfu/ml in shrimp aquaculture farms. The higher bacterial loads

in pond sediments obtained in this study can be attributed to the accumulation of organic matter at the pond bottom which stimulated bacterial growth. Shrimp head ($4.78 \times 10^5 \pm 3.0 \times 10^4$ cfu/g) had relatively higher bacterial load when compared to shrimp muscle $2.7 \times 10^5 \pm 1.95 \times 10^4$ cfu/g). In shrimp hatchery samples, the post-larvae ($2.2 \times 10^6 \pm 1.9 \times 10^6$ cfu/g) had higher bacterial load than water ($5.6 \times 10^3 \pm 3890$ cfu/ml).

The mean *E.coli* counts were higher in aquaculture pond sediment (204±133 cfu/g) and pond water (124±88 cfu/ml). Relatively lower *E.coli* counts were obtained from shrimp samples (12±11 to 16±16.7 cfu/g). The presence of *E.coli* in aquaculture environment might have been from the source water. *E.coli* was not detected in hatchery waters and post-larvae. Higher mean *E.coli* counts were obtained from pond sediment (204± 132.9) and pond water 123.6 ± 87.8) samples. The *E.coli* counts ranged from 140 to 440 cfu/g in pond sediment and from 48 to 260 cfu/ml in pond water samples. *E.coli* was detected in in three shrimp head and three shrimp muscle samples but the counts were lower. *E.coli* in shrimp head samples ranged between 0 and 40 cfu/g and in shrimp muscle it ranged between 0 and 20 cfu/g. In the present study a negative correlation was observed between total vibrio counts and *E.coli* ($r = -0.54$) in the shrimp culture system which is in accordance with previous reports that state that the counts of vibrios were either negatively correlated or showed no correlation with counts of indicator bacteria (*Escherichia coli*, *Enterococci*, fecal coliforms, and total coliforms). A poor correlation between the level of faecal indicator organisms and the incidence of Vibrios indicate that the Vibrios are a part of the natural microflora of the shrimp culture environment.

Vibrio loads were higher in *P. monodon* hatchery samples than in aquaculture pond samples. Post-larvae had maximum loads of Vibrios ($2.1 \times 10^5 \pm 1.1 \times 10^5$ cfu/g). Shrimp head portion had relatively higher counts of Vibrios ($3.5 \times 10^4 \pm 2.2 \times 10^4$ cfu/g) than shrimp muscle portion ($1.4 \times 10^4 \pm 1.42 \times 10^4$cfu/g). Hatchery waters had higher Vibrio loads (2400± 2200 cfu/ml) than pond waters (150 ± 42 cfu/ml). In the present study the mean TVC of pond sediment (1.5×10^3 cfu/g) was 10 times higher than the mean TVC of pond water (1.5×10^2 cfu/ml). Sucrose non-fermenting vibrios were higher in shrimp head portion (59%) and hatchery waters (49%) whereas more than 90% of the vibrios in post-larvae, pond water, pond sediment and shrimp muscle portions were sucrose fermenters.

A total of 210 Vibrio cultures isolated and purified from the water (105 Vibrio cultures) and post-larvae samples (105 Vibrio cultures) from hatchery samples were screened for the presence of pathogenic *Vibrio spp* based on their biochemical reactions. *V.alginolyticus* (24.3%) was the most common pathogenic *Vibrio spp* detected in hatchery samples followed by *V. vulnificus* (9.1%), *V. parahaemolyticus* (8.6%) and *V. harveyi* (3.8%). The pathogenic Vibrios were most commonly encountered in the water samples (34.76%) than the post-larvae samples (10.95%). *V.alginolyticus* was the most dominant pathogenic *Vibrio spp* in hatchery water (17%) and post-larvae samples (7.1%). The incidence of *V.parahaemolyticus* (7.1%) was slightly higher than *V.vulnificus* (6.7%) in hatchery water samples whereas the incidence of *V.vulnificus* (2.4%) was slightly higher than *V.parahaemolyticus* (1.4%) in post-larvae. *V.harveyi* was detected in hatchery waters (3.85%) but in post-larvae. The lesser incidence of *V.harveyi* in hatchery samples in the present study can be attributed to hatchery water quality management and the size of the post-larvae samples used for analysis.

A total of 250 Vibrio cultures isolated and purified from farm water (75 Vibrio cultures), farm sediment (75 Vibrio cultures) and farmed shrimp (100 Vibrio cultures) samples were screened for the presence of pathogenic *Vibrio spp* based on their biochemical reactions. *V. alginolyticus* (38.4%) was the most common pathogenic *Vibrio spp* detected in aquaculture samples followed by *V. cholerae* (16.8%). Other pathogenic *Vibrio spp* that were detected in hatchery samples viz., *V.parahaemolyticus, V.vulnificus* and *V.harveyi* were not detected in aquaculture samples. *V.alginolyticus* was the most predominant pathogenic *Vibrio spp* in pond sediment (16.4%), pond water (12.4%) and shrimp samples (9.6%). *V.cholerae* incidence was higher in pond water (7.6%) than in pond sediment (5.2%) and shrimp (4%). Shrimp head portion had relatively higher incidence of *V.alginolyticus* (6%) and *V.cholerae* (3.6%) when compared to shrimp muscle where the incidence levels were 2.4% and 1.6%, respectively.

A total of 96 isolates of *V.alginolyticus* were obtained from aquaculture samples of which 42.7% were isolated from culture pond sediment, 32.3% from pond water, 15.6% from shrimp head portion and 9.4% from shrimp muscle. It indicated that farm sediment had a higher presence of *V.alginolyticus* compared with their presence in farm water. But the incidence of *V.alginolyticus* had been relatively very low in the farmed

shrimp. In the farmed shrimp itself, the head region had a higher percentage of *V.alginolyticus* (15.6%) than the shrimp muscle (9.4%).

A total of 42 isolates of *V.cholerae* were obtained from aquaculture samples. Of these, 45.2% were isolated from pond water, 31% from pond sediment, 14.3% from shrimp head portion and 9.5% from shrimp muscle. It indicated that shrimp farm water had a higher presence of *V.cholerae* compared with their presence in farm sediment. But the incidence of *V.cholerae* had been relatively very low in the farmed shrimp. In the farmed shrimp, the head region had a higher percentage of *V.cholerae* (14.3%) than the shrimp muscle (9.5%).

The 42 *V.cholerae* isolates obtained from shrimp aquaculture system (19 isolates from pond water; 13 isolates from pond sediment, 6 isolates from shrimp head and 4 isolates from shrimp muscle) gave negative agglutination reaction with polyvalent somatic O antiserum and with *V.cholerae* O139 antiserum thereby grouping them as Non O1 and Non O139 *V.cholerae*.

Utilization of sugars and sugar derivatives by Vibrio cultures isolated from hatcheries / farms was studied. *V.cholerae*, *V.cholerae ctx* (cholera toxigenic *V.cholerae)*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* failed to utilize ribose and xylose. Only *V.parahaemolyticus* isolates utilized arabinose. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* utilized glucose, fructose, mannose and mannitol. Only *V.alginolyticus* isolates were negative for galactose. *V.cholerae*, *V.cholerae ctx*, *V.alginolyticus* and *V.harveyi* utilized sucrose whereas *V.vulnificus* and *V.parahaemolyticus* failed to ferment sucrose. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* utilized maltose and all these Vibrios failed to utilize lactose. *V.vulnificus* and *V.harveyi* utilized cellobiose. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* failed to utilize aesculin and salicin. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* utilized glycogen.

Utilization of amino acids by Vibrio cultures isolated from hatcheries / farms was studied using 4 critical amino acids, namely, arginine, lysine, ornithine and histidine. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and

V.harveyi showed decarboxylase activity with lysine and ornithine but not with histidine. All these pathogenic *Vibrio spp* were negative for arginine dihydrolase activity.

All the *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* were studied for their potential to produce enzymes like amylase, gelatinase and other proteases, DNAses, lipases, phospholipases and phosphatase. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, and *V.parahaemolyticus* showed amylolytic activity. *V.harveyi* isolates were found to be negative for amylase activity. The mean amylolytic activity index was higher in *V.parahaemolyticus* and *V.alginolyticus*. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* were positive for gelatinase activity; they liquefied gelatin and showed gelatinase activity on gelatin agar. The mean gelatinase activity index was higher in *V.vulnificus*, *V.cholerae* and *V.cholerae ctx*. 25% of *V.cholerae*, 25% of *V.cholerae ctx*, 50% of *V.parahaemolyticus*, 100% of *V.alginolyticus* and 100% of *V.vulnificus* isolates showed proteolytic activity on fish powder agar. *V.harveyi* failed to show proteolytic activity on fish powder agar. *V.alginolyticus* and *V.vulnificus* showed higher proteolytic activity index on fish protein. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* isolated from shrimp culture environment were positive for proteolytic activity on shrimp protein agar. *V.vulnificus*, *V. harveyi* and *V.cholerae ctx* showed higher proteolytic activity index on shrimp protein. In this study it was noticed that all the pathogenic Vibrio species isolated from shrimp hatchery and farms were able to utilize shrimp protein thereby suggesting their capability to invade the shrimp tissue. The proteolytic activity differed with the protein substrate. 75% of *V.cholerae*, 75% of *V.cholerae ctx*, and 100% of *V.harveyi* isolates failed to show proteolytic activity on fish powder agar whereas all these vibrios showed proteolytic activity on gelatin and shrimp protein. All *V.cholerae* and *V.cholerae ctx* isolates were negative for DNAse activity whereas *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* showed DNAse activity. Neutral lipid (tributyrin) was not utilized by *V.alginolyticus* and *V.harveyi*. Phospholipid (lecithin in egg yolk) was utilized by all the pathogenic vibrio species. Maximum lipolytic activity index on phospholipid was given by *V.alginolyticus* followed by *V.vulnificus* and *V.parahaemolyticus*. The lipolytic activity index of *V.cholerae* and *V.cholerae ctx* was

slightly higher on neutral lipid than on phospholipid. *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* were positive for phosphatase activity.

The result of the study on the growth of pathogenic vibrios at different temperature showed that all the pathogenic vibrios showed good growth between 22°C and 42°C. At 45°C, only *V.alginolyticus* showed growth. *V.parahaemolyticus* and *V.alginolyticus* showed growth at lower temperature (4°C to 8°C). *V.cholerae*, *V.cholerae ctx*, *V.vulnificus*, *V.alginolyticus*, *V.parahaemolyticus* and *V.harveyi* were negative for growth at 0°C and 55°C temperatures. Study on the growth of pathogenic vibrios at different pH showed that these organisms grew at pH between 7 and 9. None of the pathogenic vibrios isolated from shrimp culture system showed growth either at pH 4 or pH 12.

Effect of salt on the growth of pathogenic Vibrio cultures isolated from shrimp culture system was studied. Maximum growth of *V.cholerae* was observed between 0% and 2% salt concentration (log 8 cfu/ml to 8.3cfu/ml) followed by 3% (log 7cfu/ml) and 6% (log 5.6 cfu/ml) salt concentrations. No growth was observed above 6% salt concentration. In the present study the *V.cholerae* isolate showed growth at 6% salt concentration. This *V.cholerae* isolate belonged to the *V.cholerae* Non O1 and Non O139 group. The Non O1 and O139 *V.cholerae* are a diverse group with high genetic variation between them. The growth at higher salt concentration (6%) might be attributed to the genetic potential of the isolate. In the case of choleratoxigenic *V.cholerae* (*V.cholerae* ctx) no growth was observed at 6% and above salt concentrations. Maximum growth of *V.vulnificus* was observed at 3% followed by 2% and 1% salt concentrations. No growth was observed at 0% and at above 8% salt concentration. Maximum growth of *V.parahaemolyticus* was observed at 3% (log 9.77 cfu/ml) followed by 2% (log 9.47 cfu/ml), 1% (log 9.3 cfu/ml) and 6% (log 9.3 cfu/ml) salt concentrations. No growth was observed at 0%, 12% and 15% salt concentration. The ability of this *V. parahaemolyticus* isolate obtained from shrimp hatchery to grow at a very low salinity of 0.3% is reported in this study. The growth of *V. parahaemolyticus* at lower salinities was reported in few cases. 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. The result of the study on the influence of salt on the growth of *V.alginolyticus* showed that maximum growth was observed at 2% salt concentration (log 9.6 cfu/ml) followed by 3% (log 9.47 cfu/ml) and 0.5% salt concentrations (log 8.78 cfu/ml). No growth was observed at 0% and 15% salt concentrations.

Effect of salt on the enzymatic activity pathogenic Vibrio cultures isolated from shrimp culture system was studied. All isolates showed amylolytic activity at 0.8% salt concentration. At 0% salt concentration, only *V.cholerae* and *V.cholerae ctx* showed amylolytic activity. Amylolytic activity was maximum at 0.8% salt concentration for *V.cholerae*, *V.cholerae ctx*, *V.parahaemolyticus*; at 0.5% concentration for *V.alginolyticus* and at 1% concentration for *V.vulnificus* and *V. harveyi*. At higher salt concentration (7%) only *V.parahaemolyticus* and *V.alginolyticus* produced amylase. 0.3% salt concentration was sufficient for *V.cholerae*, *V.cholerae ctx*, *V.alginolyticus*, *V.parahaemolyticus* to produce gelatinase. For *V.vulnificus* 0.5% salt concentration was found necessary. The maximum proteolytic activity index was observed with *V.vulnificus* at 0.5% followed by *V.cholerae* at 0.3% and *V.cholerae ctx* at 0.3%. At higher salt concentration (7%) only *V.parahaemolyticus* was able to produce gelatinase. *V.alginolyticus* showed an increasing trend of DNAse activity from 0.5%, to 7% salt concentration. The DNAse activity of *V.parahaemolyticus* increased from 0.5% to 3% and thereafter decreased.

The effect of NaCl concentration on the swarming behaviour of *V.alginolyticus* was studied. The swarming zone of *V.alginolyticus* increased from 1% salt concentration to 3% salt concentration and thereafter showed a decreasing trend. No swarming was observed at 7% to 10% salt concentrations and no growth was observed at salt concentration of above 12%. The results from this study suggests that agar with 7% salt can be used to study *V.alginolyticus* properties on agar media; with minimal swarming problem of this species.

Effect of salt and temperature on the utilization of sugars (sucrose and mannitol) and amino acids (arginine, lysine and ornithine) by pathogenic Vibrio cultures isolated from shrimp culture system was studied.

Effect of preservatives/ chemicals on the growth of pathogenic Vibrio cultures isolated from shrimp culture system was studied. Potassium chloride (KCl) at 5% level inhibited the growth of *V.cholerae*, *V.cholerae ctx*, *V.vulnificus* and *V.harveyi*. Inhibition by KCl was lower in the case of *V.parahaemolyticus* and *V.alginolyticus* isolates and they showed a 2 to 3 log decrease in counts even at a level as high as 5%. From this study it was found that sodium citrate had negligible effect on the growth of pathogenic Vibrios isolated from shrimp culture system. A maximum reduction of 1 log value was observed at 7% level. It was observed that 3% level of sodium tri polyphosphate (STPP) inhibited *V.vulnificus* and *V.harveyi*. Others species showed 1- 3 log reduction in counts. At 7% level all the pathogenic vibrios were inhibited. Potassium sorbate at 1% level inhibited the growth of *V.cholerae ctx*, *V.vulnificus*, *V.parahaemolyticus* and *V.harveyi*. The counts decreased from 5-8 log to nil. *V.cholerae* and *V.alginolyticus* showed a reduction of 2 log in counts at 1% level but were completely inhibited at 3% level. From this study it is known that all pathogenic Vibrio cultures isolated from shrimp culture system can be inhibited at 3% potassium sorbate level. This result finds application in the seafood processing plants wherein the use of potassium sorbate for the control of pathogenic vibrios can be explored.

Cholera toxin production encoded by the *ctxAB* genes is the major factor in the pathogenesis of cholera. A PCR method that selectively amplifies a specific DNA fragment within the *ctxAB* operon of *V.cholerae* was used. All the 42 isolates were tested using *ctxAB* primers. Three *V.cholerae* isolates were positive in this PCR and all of them yielded a single specific amplicon of 777bp size. 93% of the *V.cholerae* isolates were negative for the presence of cholera toxin genes. The detection of *ctx* positive isolates (7%) among Non O1 and Non O139 *V.cholerae* isolates from *P.monodon* shrimp aquaculture system is being reported for the first time in this study.

PCR method employing species-specific primers that target the house keeping gene *sodB* of *V.cholerae* was used. All the 42 isolates of *V.cholerae* obtained from aquaculture pond water, sediment, shrimp head and muscle yielded the specific amplicon (248bp) thereby confirming their identity as *V.cholerae*.

The draw back of the PCR targeting *ctxAB* genes is that it detects only enterotoxigenic strains of *V.cholerae*. It does not detect non-toxigenic (*ctxAB* negative)

strains. The lacunae of using the PCR targeting species-specific *sodB* primers is that it identifies *V.cholerae* but does not state its toxigenic status with respect to *ctxAB* genes. An experiment was planned as *V.cholerae*-duplex PCR for detection and confirmation of toxigenic *V. cholerae*. For this, the method of Tarr et al (2007) and USFDA-BAM method (2001) were integrated. The amplification cycle conditions for the *V.cholerae*-duplex PCR were standardized and a simple template preparation procedure was used for the *V.cholerae*-duplex PCR. The *V.cholerae*-duplex PCR was initially standardized using *V.cholerae* (MTCC 3906), *V.vulnificus* (MTCC 1145), *V.alginolyticus* (ATCC 17749) and *V.parahaemolyticus* (ATCC 17802) cultures and *ctx* negative *V.cholerae* (laboratory culture collection) cultures. *V.cholerae* (*ctx* negative) cultures yielded a single amplicon (248bp); *ctxAB* positive *V.cholerae* cultures yielded two amplicons (248bp and 777bp). Other *Vibrio spp.* did not yield these specific amplicons. The results indicate that the *V.cholerae*-duplex PCR was specific to *V.cholerae* and the PCR cycle conditions were adequate for obtaining the desired result. All the 42 *V.cholerae* isolates obtained from shrimp aquaculture system were subjected to *V.cholerae*-duplex PCR. Thirty nine *V.cholerae* cultures yielded a single amplicon of 248bp indicating that they are *V.cholerae* but non-choleratoxigenic. Three *V.cholerae* cultures yielded two amplicons viz., species specific 248bp and cholera toxin specific 777bp, thereby indicating that they were cholera toxin producing strains of *V.cholerae*. The result indicates that the majority of the *V.cholerae* isolates obtained from aquaculture farm water, soil and shrimp were non-toxigenic with respect to cholera toxin. The three *V.cholerae* isolates which were positive for the presence of cholera toxin genes were obtained from pond water. These three *V.cholerae* isolates agglutinated neither with polyclonal somatic O antiserum nor with O139 antiserum thereby grouping them as *V.cholerae* Non 1 and Non O139 serogroup. These three isolates failed to yield O1 specific or O139 specific amplicon when tested in PCR reconfirming that the isolates were indeed *V. cholerae* Non O1 and Non O139.

Cholera toxin production encoded by the *ctxAB* genes is the major factor in the pathogenesis of cholera and the presence of *ctxAB* is a prerequisite for full blown cholera disease (Cholera gravis) to occur. In this study a negative correlation was observed between total vibrio counts and *E.coli* ($r = -0.54$) in the shrimp culture system. The

incidence of *V.cholerae* observed in aquaculture ponds might also have been due to natural inhabitation. The structural genes for the ctx element reside on a filamentous phage ctx? (Waldor and Mekalanos, 1996). CTX? is found in all epidemic *V.cholerae* isolates but is rarely recovered from the non O1 non O139 VC environmental isolates. The spread of CT genes in the environment can be facilitated by the exposure of CTX? positive strains to sunlight (Faruque *et al.*, 2000). Genetic and phenotypic evidence strongly suggests that the O139 strain arose from a VC O1 strain by horizontal gene transfer. *V.cholerae* Non O1 and Non O139 strains can also acquire toxigenic genes for toxin production by transduction and therefore might be the source of new epidemics. Even though the existence of *ctx* carrying Non O1 and Non O139 *V.cholerae* isolates was very low in shrimp culture system, the ecological significance of *ctx* genes among these *V.cholerae* Non O1 and Non O139 isolates in the shrimp aquaculture environment needs to be further investigated. The detection of *ctx* positive isolates among Non O1 and Non O139 *V.cholerae* isolates from *P.monodon* shrimp aquaculture system is being reported for the first time in this study.

The autochthonous existence of *V.cholerae* especially Non O1 and Non O139 in aquatic environment has been reported from several areas world over. In such a scenario, rejection of fish/shrimp by the importing nations based on the presence of what appears to be autochthonous bacterial flora in the shrimp culture system appears to be stringent. It is proposed that the mere presence of *V.cholerae* Non O1 and Non O139 need not be the biohazard criterion for rejection of cultured *P.monodon* shrimp but as a safety measure *ctx* carrying *V.cholerae* Non O1 and Non O139 may be considered as potential public health risks. However, further studies are needed to establish *V.cholerae* Non O1 and O139 as native flora of black tiger shrimp culture system.

The sensitivity of *V.cholerae*-duplex PCR was determined. The *ctxAB* specfic primers yielded amplicon only when the concentration of *V.cholerae* was above 1000 cells/ml whereas the species specific sodB primers yielded amplicon at concentration of 100 cells/ml.

A SYBR Green I Real time PCR assay targeting the house keeping gene *gyrB* of *V.alginolyticus* was developed by Zhou et al (2007). However in this study, the real time PCR amplification conditions were employed in regular PCR. 24.3% (51 isolates out of

210 Vibrio cultures) vibrio isolated from hatcheries and 38.4% (96 isolates out of 250 Vibrio cultures) vibrios isolates from aquaculture farms were found to be *V.alginolyticus*. as they yielded the *V.alginolyticus* species specific 340bp amplicon. *V.alginolyticus* specific PCR could detect 90 cells/ml. A *V.alginolyticus*-duplex PCR method was developed by utilizing *V.alginolyticus* species specific primers (Zhou *et al.*, 2007) and Vibrio genus specific primers (Tarr *et al.*, 2007).

V.vulnificus-PCR uses species-specific primers that target the house keeping gene *hsp60* of *V.vulnificus* (Tarr *et al.*, 2007). Only the *V.vulnificus* cultures yielded the specific amplicon of 410bp. 14 *V.vulnificus* isolates were obtained from hatchery waters and 4 *V.vulnificus* isolates were obtained from post-larvae. *V.vulnificus* was not detected in aquaculture farm samples.

V.parahaemolyticus-PCR uses species-specific primers that target the *flaE* sequence in the flagellin gene of *V.parahaemolyticus* (Tarr *et al.*, 2007). Only the *V.parahaemolyticus* cultures yielded the specific amplicon of 897bp. 18 vibrio isolates (15 from water, 3 from PL) were found to be *V.parahaemolyticus*.

A pathogenic Vibrio-multiplex PCR method was developed to detect common pathogenic Vibrios. The pathogenic Vibrio-multiplex PCR utilized *V.cholerae* species specific primers (Tarr *et al.*, 2007) and *V.cholerae ctxAB* genes specific primers (Bacteriological Analytical Manual, 2001), *V.alginolyticus* specific *gyrB* primers (Zhou *et al.*, 2007), *V.vulnificus* specific *hsp60* primers (Tarr *et al.*, 2007) and *V.parahaemolyticus* specific *flaE* primers (Tarr *et al.*, 2007). Multiplex PCR was performed using a single PCR reaction mix which contained 5 sets of primers. The multiplex PCR can help in identifying the above mentioned human pathogenic Vibrios from an unknown colony on TCBS agar. When specific DNA was added as template to the PCR mix, only the corresponding primers specifically reacted and yielded that particular amplicon (single amplicon in the presence of multiple primers). Non cholera toxin producing *V.cholerae* cultures yielded an amplicon of 248bp; *ctxAB* positive *V.cholerae* cultures yielded two amplicons viz., 248bp and 777bp; *V.alginolyticus* cultures yielded an amplicon of 340bp; *V.vulnificus* cultures yielded an amplicon of 410bp and *V.parahaemolyticus* cultures yielded an amplicon of 897bp.

Real time PCR was performed for the detection and quantification of *V.cholerae*. The *V.cholerae* species specific (*sodB*) primers used in regular PCR were tried in the Real Time PCR method. Different concentrations of DNA (105ng to 0.001ng) extracted from *V.cholerae* was used in Real Time PCR. The C_T (Threshold cycle) values decreased proportionally with the increase in the specific DNA concentration. The Real time PCR method provided an extremely faster result when compared to conventional PCR. The result of the melting curve analysis confirmed the specificity of the *V.cholerae* species specific primers and their usefulness in the Real time PCR assay as it showed a single melting maximum (85°C) for different concentrations of *V.cholerae* type culture and a field isolate of *V.cholerae*. The Real time PCR method provided an extremely faster result when compared to conventional PCR and the species specific *sodB* primers were suitable for both conventional and Real time PCR detection of *V. cholerae*.

PCR fingerprinting of *V.cholerae* isolates was performed using RS-PCR, REP-PCR and ERIC-PCR methods. RS-PCR yielded fewer bands (maximum 4) when compared to REP-PCR (maximum 10 bands) and ERIC-PCR (maximum 12 bands). The dendogram of RS-PCR for 22 *V.cholerae* isolates showed 10 fingerprint patterns whereas the dendogram of REP-PCR for 25 isolates and dendogram of ERIC-PCR for 24 isolates showed, 25 fingerprint patterns and 24 fingerprints, respectively. 100% similarity between *V.cholerae* isolates obtained from shrimp aquaculture was noticed only in RS-PCR. None of the isolates showed 100% similarity either in REP-PCR or ERIC-PCR. From this PCR fingerprinting study on *V.cholerae* isolated from *P.monodon* shrimp farms, it can be concluded that REP-PCR and ERIC-PCR are best suitable to study the genetic variation amongst *V.cholerae* at a higher sensitivity level. On the other hand, the RS-PCR will be helpful in comparing the genetic similarity among *V.cholerae*. The isolates that were less genetically apart will be shown as similar in RS-PCR but will be shown as different in REP-PCR and ERIC-PCR. The presence of 470bp size amplification band in almost all the isolates of *V.cholerae* using ERIC-PCR analysis suggests it's potential use as a marker for the identification of *V.cholerae*. The greater similarity of *ctx* positive *V.cholerae* with ctx –ve *V.cholerae* isolates indicates that the *ctx* positive strains (Non O1 and Non O139) might have originated from autochthonous *V.cholerae* in the aquatic niche.

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ANNEXURE

A. Composition of bacteriological media

1. Alkaline Peptone Water (APW)

Peptone	10g
Sodium chloride	5g
Distilled water (DW)	1 Litre
	pH: 9.1± 0.1

2. Brilliant Green Bile Broth (BGLB)

Peptone	1 g
Lactose	1 g
Bile salt	2 g
Brilliant green	0.00133g
DW	100 ml
	pH: 7.4 ± 0.1

3. Decarboxylase Broth

Basal medium

Yeast extract	0.3g
Glucose	0.1g
Bromocresol purple (BCP)	0.0016g
DW	100ml
	pH : 6.5 ± 0.1

One amino acid to 100ml of the basal medium.

1 .L-Lysine Hydrochloride	0.5g
2. L-Arginine Hydrochloride	0.5g
3. L-Ornithine Hydrochloride	0.5g

4. DNAse Agar with Toluidine blue

Tryptose	20g
NaCl	5g
DNA	2g
Toluidine blue	0.1g
Agar	15g
Distilled water	1litre
	pH: 7.3 ± 0.2

5. EC broth

Tryptone	2 g
Lactose	0.5 g
Bile salt (No.3)	0.15 g
K_2HPO_4	0.4 g
KH_2PO_4	0.15 g
NaCl	0.5 g
DW	100 ml
	pH: 6.9 ± 0.1

6. Egg Yolk Agar

Nutrient Agar	100ml
50% Egg Yolk solution	5ml
	pH 7± 0.2

7. Eosin Methylene Blue (EMB Agar)

Peptone	10g
Lactose	10g
K_2HPO_4	2g
Eosin Y	0.4g
Methylene blue	0.065g
Agar	15g
DW	1 litre
	pH : 7.1 ± 0.1

8. Fish powder agar

Peptone	10g
Beef extract	3g
Fish powder	30g
Agar	15 g
DW	1 litre
	pH: 7.0 ± 0.1

Fish powder agar with different salt concentrations was prepared by adding the required quantity of NaCl to the media along with all other ingredients before autoclaving.

9. Gelatin Agar

Nutrient Agar	1000ml
KH_2PO_4	0.5g
K_2HPO_4	1.5g
Gelatin	4g
Glucose	0.05g
	pH 7.0± 0.2

10. Gelatin Medium

Peptone	10g
Beef extract	3g
NaCl	5 g
Gelatin	120 g
DW	1 litre
	pH: 7.0 ± 0.1

11. Hugh & Leifson Glucose O/F Medium (H&L)

Peptone	1g
NaCl	0.5g
K_2HPO_4	0.4g
Dextrose	1g
Agar	0.3g
DW	100ml
	pH: 7.1 ± 0.1

Add 1ml of 0.1% solution of phenol red indicator

12. Kligler Iron Agar (KIA)

Peptone	20g
Yeast extract	3g
Beef extract	3g
NaCl	5g
Lactose	10g
Glucose	1g
Ferric citrate	0.3g
Sodium thiosulphate	0.3g
Phenol red	0.05g
Agar	15g
DW	1 litre
	pH 7.4 ±0.2

13. Methyl Red Vogues Prausker Medium (MRVP)

Peptone	0.5g
D-glucose	0.5g
K_2HPO_4	0.5g
DW	100ml
	pH: 6.9 ± 0.1

14. Nitrate Broth

Peptone	1g
Potassium nitrate	0.1g
NaCl	0.5g
DW	100ml
	pH: 7.1 ± 0.1

15. Mueller Hinton Agar

Casein acid hydolysate	17.5g
Beef infusion	300g
Starch	1.5g
Agar	17g
DW	1000ml
	pH 7.3± 0.2

16. Normal Saline (NS) (Physiological Saline)

NaCl	8.5 g
DW	1 litre

17. Nutrient Agar

Peptone	10g
Beef extract	3g
NaCl	5 g
Agar	15 g
DW	1 litre
	pH: 7.0 ± 0.1

18. Nutrient Broth

Peptone	10g
Beef extract	3g
NaCl	5 g
DW	1 litre
	pH: 7.0 ± 0.1

19. ONPG (O-nitrophenyl galactopyranoside)

ONPG solution :

ONPG	6g
Na_2HPO_4 buffer	1000ml

Dissolve and filter

Peptone water:

Peptone	10g
NaCl	5g
DW	1000ml

Test: Take 1 part of ONPG solution and 3 parts of peptone water in small tubes. Inoculate heavily with the test culture. Incubate at 37°C. Read after 20 min to 24h. Yellow colour indicates positive result

20. $P_1 N_0$ Medium

Peptone	1g
Distilled water	100ml
	pH: 7.0 ± 0.1

21. Phosphate Buffer Diluent

KH_2PO_4	34g
Distilled water	500ml

Adjust pH to 7.2 ± 0.1 with 1 N NaOH and bring volume to 1 litre with distilled water. Take 1.25 ml of the above stock solution and bring volume to 1 litre with distilled water. Dispense in flasks or tubes and sterilize at 121°C for 15min.

22. Purple Broth Base (For Sugar Fermentation)

Peptone	10g
NaCl	5g
Bromocresol purple	0.02 g
	pH to 7.0 ± 0.2

Add the required sugar to 1% level.

23. Shrimp powder agar

Peptone	10g
Beef extract	3g
Shrimp powder	30g
Agar	15 g
DW	1 litre
	pH: 7.0 ± 0.1

Shrimp powder agar with different salt concentrations was prepared by adding the required quantity of NaCl to the media along with all other ingredients before autoclaving.

24. Simmon's Citrate Agar

Sodium citrate	0.2 g
NaCl	0.5g
K_2HPO_4	0.1g
$NH_4H_2PO_4$	0.1g
$MgSO_4$	0.02g
Bromothymol blue	0.008g
Agar	1.5g
DW	100 ml
	pH: 7.0 ± 0.2

25. Skim milk agar

Casein enzymatic hydrolysate	5g
Yeast Extract	2.5g
Dextrose	1g
Skim milk powder	28g
Agar	15g
	pH 7.0± 0.2

26. Starch Agar

Peptone	10g
Beef extract	3g
NaCl	5 g
Soluble Starch	10g
Agar	15 g
DW	1 litre
	pH: 7.0 ± 0.1

27. Sugar Fermentation Media

Peptone	10 g
Sodium chloride	5 g
DW	1 litre
	pH: 7.2 ± 0.1

Add 10ml of phenol red indicator, adjust pH.
Add the required sugar to 1% level.

28. Tergitol-7 Agar (T-7)

Peptone	10g
Yeast extract	6 g
Beef extract	5 g
Lactose	20g
Tergitol-7	0.1g
Bromothymol blue	0.05g
Agar	15g
DW	1 litre
	pH: 7.2 ± 0.2

Before pouring the plates, after melting and cooling, add 0.25ml of 1% solution of sterile Triphenyl Tetrazolium Chloride (TTC) per 100 ml media.

29. a) Thiosulfate Citrate Bile Salt Sucrose Agar (TCBS)

Yeast extract	5g
Peptone	10g
Sucrose	20g
Sodium thiosulphate	10g
Sodium Citrate Dihydrate	10g
Sodium cholate	3g
Ox-gall	5g
Sodium chloride	10g
Ferric citrate	1g
Bromothymol blue (BTB)	0.04g
Thymol blue (TB)	0.04g
Agar	15g
DW	976ml
	pH: 8.6 ± 0.1

b) TCBS Agar (Difco)

Formula Per Liter

Bacto Yeast Extract	5 g
Bacto Proteose Peptone No. 3	10 g
Sodium Citrate	10 g
Sodium Thiosulfate	10 g
Bacto Oxgall	8 g
Bacto Saccharose	20 g
Sodium Chloride	10 g
Ferric Citrate	1 g
Bacto Bromo Thymol Blue	0.04 g
Thymol Blue	0.04 g
Bacto Agar	15 g
Final pH 8.6 ± 0.2 at 25°C	

30. T_1N_0 and T_1N_3 Medium

Trypticase	2.0 g
DW	200 ml
pH: 7.2 0 ± 0.2	

Dissolve; adjust the pH; divide into two lots. To one lot add 3 g NaCl, dissolve and dispense in 5 ml quantities. This is T_1N_3 medium. The second lot is dispensed in 5 ml quantities in test tubes. This is T_1N_0 medium.

31. Tributyrin agar

Peptic digest of animal tissue	5g
Yeast Extract	3g
Agar	15g
DW	1000ml
pH 7.5 ±0.2	

Add 10ml of Tributyrin (glycerol tributyrate) to 1litre of the medium.

32. Triple Sugar Iron Agar (TSI)

Peptone	20g
Yeast extract	3g
Beef extract	3g
Lactose	5g
Sucrose	10g
Glucose	1g
Ferric citrate	0.3g
Sodium thiosulfate	0.3g
Phenol red (0.2% soln:)	12 ml
Agar	12g
DW	988 ml
pH: 7.4 ± 0.2	

33. Trypticase Salt Broth

Trypticase	10 g
DW	1L.
	pH: 7.2 ± 0.2

Add 10, 30, 60, 80 and 100g NaCl, as the case may be, to make media with salt concentration of 3, 6, 8 and 10%. Distribute 5 ml quantities in test tubes.

34. Tryptone Broth (Indole Medium)

Tryptone	1 g
NaCl	0.5 g
DW	100 ml
	pH 7.1± 0.1

35. Tryptone Glucose Agar (TGA)

Tryptone	0.5 g
Beef Extract (Lab lemco)	0.3 g
NaCl	0.5 g
D-Glucose	0.1 g
Agar agar	1.5 g
Distilled water (DW)	100 ml
	pH: 7.1± 0.1

36. Tryptone Soy Agar (Difco™)

Pancreatic digest of casein	15g
Soybean peptone	5g
NaCl	5g
Agar	15g
DW	1000ml

37. Urea Agar

Peptone	1g
Dextrose	1g
Sodium Chloride	5g
Potassium di hydrogen phosphate	2g
Phenol red	0.012g
Agar Agar	15g
Distilled water	1000ml
	pH: 6.8 ± 0.1

After sterilizing, cool to 50°C and aseptically add 0.5 ml of a 40 % solution of Urea per tube, mix well and allowed to cool and form slants.

B. Test Reagents

1. Kovac's cytochrome oxidase reagent

N:N:N:N.Tetramethyl-p-phenylene diamine -hydrochloride	100mg
DW	10 ml

2. Kovac's Indole reagent

p-dimethyl amino benzaldehyde	5g
N-butyl alcohol (or amyl alcohol)	75ml

3. VP test reagents and test

Solution A

α-naphthol	0.25g
Alcohol	5ml

Solution B

KOH	2g
DW	5 ml

4. Reagents for nitrate reduction test

Solution A

α- Naphthylamine	0.5 g
Dilute Sulphuric Acid (1:20)	100 ml

Dissolve, filter and store in a brown bottle.

Solution B

Sulphanilic Acid	0.8 g
Dilute Sulphuric Acid (1:125)	100 ml

5. Mercuric chloride solution

$HgCl_2$	15g
HCl	20ml
DW	100ml

LIST OF PUBLICATIONS BY THE AUTHOR

a) Publications in peer reviewed journals:

1. **B. Madhusudana Rao** and M. V. Subba Rao. (1999). An indirect IgM-ELISA for diagnosis of sheep pox virus infection. ***Indian Veterinary Journal***, vol 76 (12), pp 1050-1054
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12. **B. Madhusudana Rao** and D.I. Khasim (2009). Hydrogen sulphide producing bacteria as indicators of spoilage of freshwater fish, rohu (*Labeo rohita*). ***Journal of Food Science and Technology***, 46(4), 377-379
13. **B. Madhusudana Rao**, US Jayaprakash Babu and D Imam Khasim. Microbiological quality of fermented fish silage. ***Journal of Food Science and Technology***, 46(5), 488-490.

b) Papers presented in Seminars :

1. **B. Madhusudana Rao** and P.K.Surendran. (2000). Bacteriophages as indicators of pollution in Fish and Aquaculture farms, presented at The National Seminar in Official Language on 'Pollution in Aquatic environment and its impact on Fishery

Resources' on 6th & 7th September 2000, Central Institute of Fisheries Technology, Cochin.

2. **B. Madhusudana Rao,** Prasad, M. M. and Sibsankar Gupta. (2002). Bacteriological Quality of Fish and Fishery products of Visakhapatnam Region presented at the Symposium on Seafood Safety: Status and Strategies, 28th to 30th May 2002, Cochin.
3. **B. Madhusudana Rao** and P. K. Surendran. (2003). Coliphage test: a quick and easy method to detect faecal pollution in Water and Fish. In : ***Seafood Safety*** (Surendran P.K., Mathew, P.T., Thampuran, N., Nambiar, V.N., Joseph, J., Boopendranath, M.R., Lakshmanan, P.T. & Nair, P.G.V., Eds.) pp: 554-561, Society of Fisheries Technologists (India), Cochin.
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c) <u>Popular articles:</u>

1. **B. Madhusudana Rao**, J. Charles Jeeva and Imam Khasim. Palm Impression Technique: A tool to popularize hygiene literacy among fish handlers – **Fishing Chimes**, vol 24, No 7, October 2004, pp 32-33; **Seafood News**, July & August

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2. A. K.Chattopadhyay, **B. Madhusudana Rao** and Sibsankar Gupta (2006) Edible Fish Powder from Small Sized Freshwater Carps. ***Fish Technology Newsletter***, vol XVII, No 4, December 2006 p-3.
3. D. Imam Khasim, **B. Madhusudana Rao** and A. K.Chattopadhyay (2009). CIFT attempts to address the problem of hypertension among people through potassium supplemented fish products. **Fish Technology Newsletter**, Vol XX, No 1, Jan-Mar 2009, pp 2-4. **ICAR News,** Jul – Sep 2009, Vol 15 (3), pp 20
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d) <u>Papers approved for publication by The Director, CIFT, Cochin</u>

- Activity index: a tool to compare enzymatic activity potential of bacteria by P.K.Surendran, O.K.Sindhu and B. Madhusudana Rao

<u>Radio talks:</u>

1. Gave a radio talk on '*chepala nanyathaku patinchavalasina vidhanulu* in Telugu (Steps to improve the quality of fish)' broadcasted by All India Radio, Visakhapatnam on 1st Aug 2006.
2. Gave a radio talk on '*chepala nanyatha : sookshmajeevula pathra* in Telugu (Fish quality : Role of Microorganisms)' broadcasted by All India Radio, Visakhapatnam on 8th Nov 2009.

<u>Awards for publications:</u>

- ✓ Awarded Dr S Vancheeswara Iyer Memorial Gold Medal by the Indian Veterinary Association in 2001 for the paper published in Indian Veterinary Journal, Vol 76, 1999
- ✓ Best paper award given by the Society of Fisheries Technologists (India), Cochin in 2004 for the paper published in Fishery Technology, Vol 42, No 2, 2003.

Papers from the Thesis

- Duplex PCR for the simultaneous detection of *V. cholerae* and differentiation of cholera toxin producing *V. cholerae* and incidence of potential cholera-toxigenic *V. cholerae* in shrimp aquaculture system' by B. Madhusudana Rao and P K. Surendran (approved by the Director, CIFT for publication)

- Genetic heterogeneity of Non O1 and Non O139 *Vibrio cholerae* isolates from shrimp aquaculture system : a comparison of RS, REP and ERIC PCR fingerprinting approaches' by B. Madhusudana Rao and P. K. Surendran (approved by the Director, CIFT for publication)