# 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).

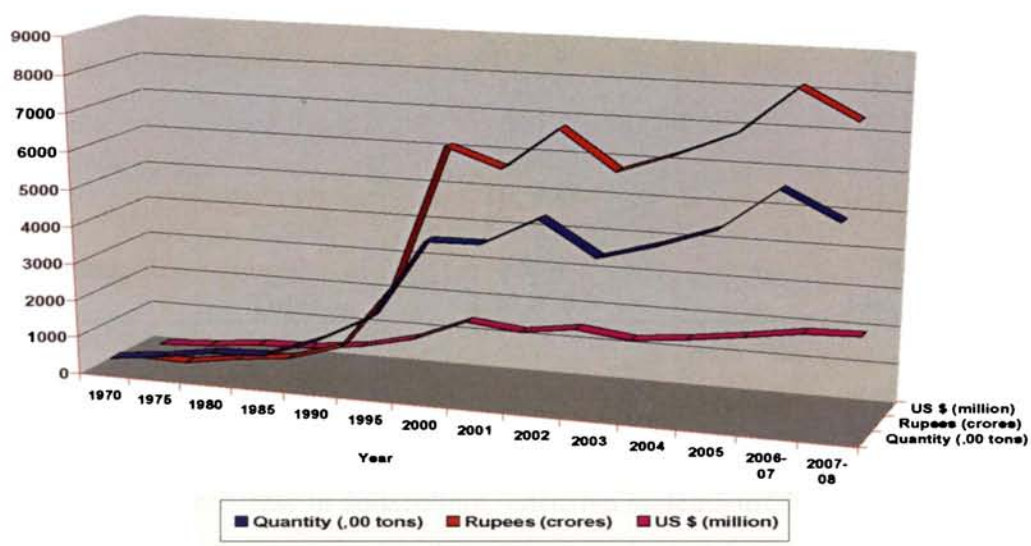


**Fig 1.1. Growth of Indian Marine Exports**

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).

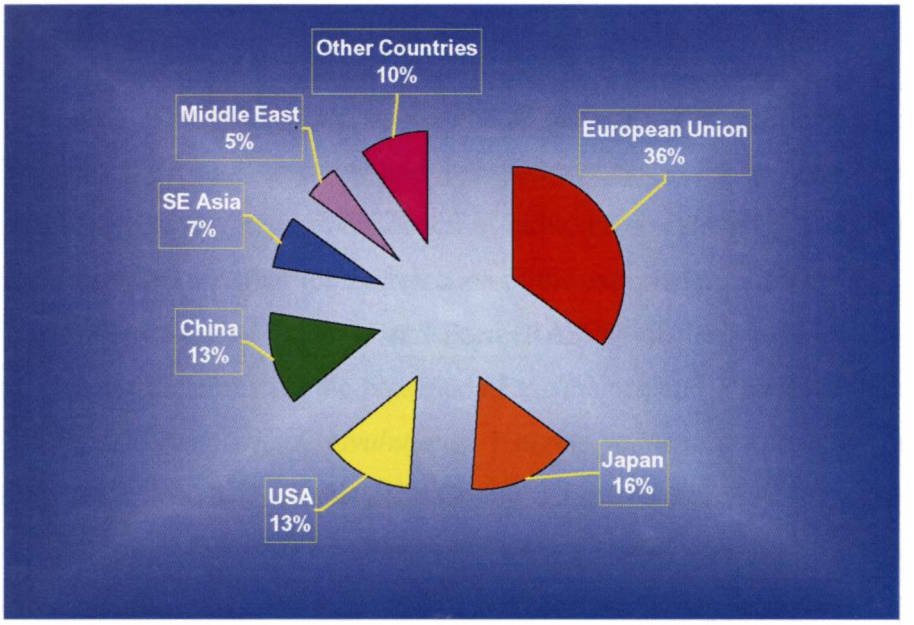


**Fig. 1.2a. Indian Marine Exports -2007-08 (US $ realization)**

Source : MPEDA, 2008

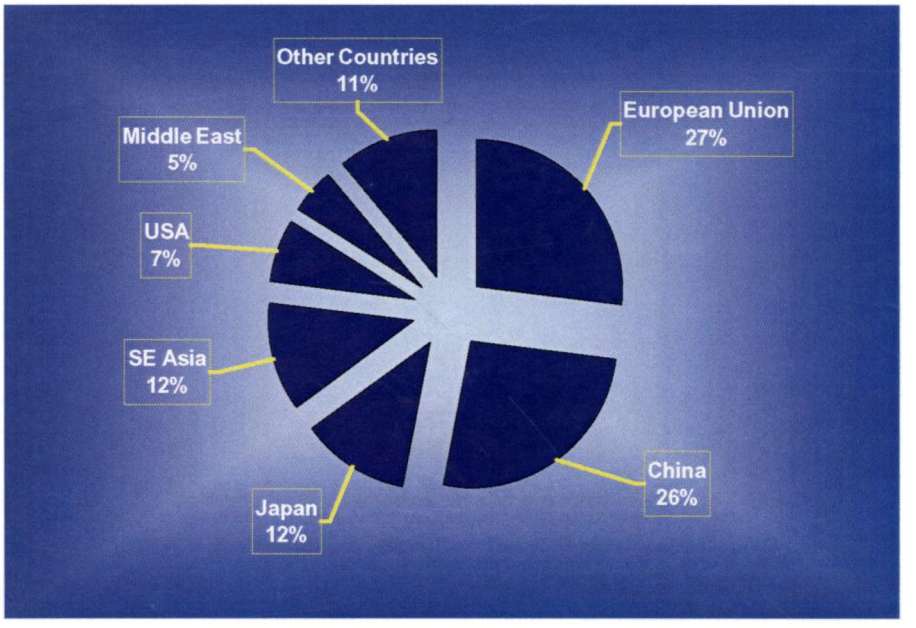


**Fig. 1.2b. Indian Marine Exports – 2007-08 (Quantity wise)**

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<br>30/03/2005 | 2005.ATL | France | *Vibrio parahaemolyticus* (presence of pathogenic strain) in frozen black tiger shrimps (*Penaeus monodon*) |
| 2008 | 29/05/2008<br>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<br>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<br>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<br>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<br>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<br>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.

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