Chapter 4

# VIRULENCE FEATURES OF *VIBRIO* FROM FOOD AND ENVIRONMENTAL SOURCES ALONG THE SOUTH WEST COAST OF INDIA

Contents



## 4.1 Introduction

The genus *Vibrio* is a normal inhabitant of the aquatic environments and plays an important role in maintaining the aquatic system. It contributes significantly to the nutrient recycling and decomposition of the organic matter in the aquatic environments. The nutrients and organic matter are used by *Vibrio* as their food source. The organism secretes various extracellular enzymes to degrade this organic matter. There are reports showing the presence of extracellular enzymes such as chitinase, caseinase, amylase, lipase, pectinase, cellulase, DNase, gelatinase, alginate lyase in vibrios (Raghul and Sarita, 2011). These enzymes also contribute to virulence of the organism. Apart from contributing to their virulence, they also help the organism to survive in various aquatic environments. Pathogenic bacteria have adopted a wide range of strategies to colonize and invade host cells. Secretion of extracellular

products, namely proteinases, lecithinases, gelatinase, lipases, DNase, hemolysins etc. are some of the strategies used by pathogenic bacteria to establish an infection. These enzymes are normally considered as virulence factors as they help the pathogen to damage the host tissues and make the host susceptible to infection (Edberg *et al.,* 1996; Pavlov *et al.,* 2004). Some authors report that the production of extracellular products help in the nutrition of the host (Balcazar *et al.,* 2006), whereas others suggest that the overproduction of these enzymes contribute to virulence of the pathogenic strains as such strains are reported to have high extracellular enzyme activity (John and Hatha, 2013). Thus, these virulence factors are used by vibrios as a means of survival, self-defence mechanism and establishment of pathogenicity.

*Vibrio parahaemolyticus* is a leading cause of seafood borne gastroenteritis in Asia and as well as in many other countries (Nair *et al.,* 2007; Matsuda *et al.,* 2012). The organism has also been reported as the causative agent of early mortality syndrome (EMS) in shrimps and caused large-scale economic losses in farmed shrimp production in India and other countries as well (Tran *et al.,* 2013; Krishna *et al.,* 2014). Many researchers have reported the abundance of *V. parahaemolyticus* throughout the year in tropical zones (Elhadi *et al.,* 2004). India being a tropical country has favourable conditions for the rapid dissemination of this pathogenic species. It thus increases the risk of disease outbreaks associated with the organism. The pathogenic potential of *V. parahaemolyticus* is not solely governed by a given virulence function (Klein *et al.,* 2014). A single factor alone does not contribute to the establishment of infection in the host cell. It is a complex process with different strains employing

different strategies. Thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) and type III secretion systems (T3SSs) are considered as major factors associated with pathogenicity of the organism (Honda *et al.*, 1987 a, b; Noriea *et al.*, 2010).

## 4.2 Review of literature

### 4.2.1 Extracellular enzymes

Enzyme studies are usually carried out to study the nutrient cycling in the aquatic environments (Mallet and Debroas, 1999). Heterotrophic microbes secrete various extracellular enzymes to hydrolyse recalcitrant organic compounds present in the environment (Arnosti, 2011). The organic compounds present in aquatic environments include high molecular weight proteins, starch, lipids, pectin, cellulose, chitin, nucleic acids, or lignin (Unanue *et al.*, 1999). Environmental bacteria utilise this organic matter as a source of carbon and nitrogen (Patel *et al.*, 2000). They also acquire energy for its various metabolic activities through hydrolysis of these compounds (Patel *et al.*, 2000).

Pathogenic *Vibrio* species secrete several extracellular enzymes, which are characterised as direct virulence factors causing skin damage (Miyoshi, 2013). Cell wall degrading enzymes play a major role in pathogenesis as they facilitate the bacterial penetration and tissue colonization (Prasannath, 2013). Proteases digest cell membranes and degrade host surface molecules. These hydrolytic enzymes are able to attack cells and molecules of the host immune system thus weakening host immune response. The presence of extracellular products has been used as an indicator of health risk associated with bacteria isolated from

clinical, environment and food sources (Lafisca *et al.,* 2008). These enzymes can help the pathogens to trigger infections in humans and aquatic animals under favourable conditions. Most of the extracellular enzymes like gelatinase, chitinase, DNase, amylase, lipase etc. are known to be associated with the pathogenicity of *V. parahaemolyticus* (Costa *et al.,* 2013). The urease and hemolysin production is typical for *V. parahaemolyticus* isolates from clinical samples, hence it is also considered as a virulence marker (Okuda *et al.,* 1997).

Experimental studies by previous researchers have already recognised the role of extracellular enzymes in virulence among vibrios (Rodrigues *et al.,* 1993; Costa *et al.,* 2013; Miyoshi, 2013). Raghul and Sarita (2011) have reported presence of hydrolytic enzymes in *Vibrio* from marine sediments of South coast of India. Bunpa *et al.* (2016) studied the production of extracellular enzymes by *Vibrio alginolyticus* isolated from environments and diseased aquatic animals in Thailand. There are reports suggesting the role of extracellular enzymes in virulence of many other bacterial pathogens such as *Aeromonas* (Bagyalakshmi *et al.,* 2009; John and Hatha, 2013), *Erwinia* (Py *et al.,* 1998), *Pseudomonas aeruginosa* (Jaeger, 1994), *Staphylococcus aureus* (Dinges *et al.,* 2000) etc.

### 4.2.2 Virulence related genes in *V. parahaemolyticus*

Pathogenicity of *V. parahaemolyticus* is not dependant solely on a given virulence function; rather, virulence is a complex process and different strains employ different strategies (Klein *et al.,* 2014). The virulence properties of *V. parahaemolyticus* have been usually found to be

associated with thermostable direct haemolysin (TDH) and TDH- related haemolysin (TRH) (Honda *et al.*, 1987 a, b). Several studies reported the absence of *tdh* and *trh* in clinical strains of *V. parahaemolyticus* (Li *et al.*, 2014; Pazhani *et al.*, 2014); thus suggesting that many other factors also contribute to pathogenicity of these strains. Recently, it was found that the type III secretion systems (T3SSs) also play a pivotal role in virulence machinery in *V. parahaemolyticus* (Ham and Orth, 2012).

#### 4.2.2.1 Thermostable direct hemolysin (*tdh*) and thermostable direct hemolysin-related hemolysin (*trh*) genes

*V. parahaemolyticus* is the causative agent of seafood borne gastroenteritis. It colonizes the human gut *via* the gastrointestinal route through consumption of contaminated raw or undercooked seafood. The most common symptoms include gastroenteritis, diarrhea, headache, nausea and vomiting. *V. parahaemolyticus* infections can be life threatening to the infants or immuno-compromised persons. The pathogenicity of *V. parahaemolyticus* is rather complex and involves multiple factors (Broberg *et al.*, 2011). Thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) are the most frequently used indicators of pathogenicity of the organism (Honda *et al.*, 1987 a, b; Nishibuchi and Kaper, 1995; Raghunath, 2015). Both the TDH and TRH are tetrameric proteins (Yanagihara *et al.*, 2010; Broberg *et al.*, 2011) and they share nearly 70% homology (Kishishita *et al.*, 1992). They act as porins and helps in the efflux of divalent cations and other solutes from and influx of water molecules into intestinal cells (Takahashi *et al.*, 2000; Ohnishi *et al.*, 2011). TDH forms large pores on erythrocyte membrane, allowing both water and ions to flow through the membrane (Matsuda *et al.*, 2012). TRH also

alters the ion flux by activating ion channels (Takahashi *et al.,* 2000). The change in the ion flux of the intestine results in diarrhea during infection.

Detection of *tdh* in *V. parahaemolyticus* is conventionally studied by beta haemolysis assay on a blood agar called Wagatsuma agar (Nishibuchi *et al.,* 1985) and the presence of *trh* gene in *V. parahaemolyticus* by urease phenotype (Cai and Ni, 1996). Previous reports state that most of the environmental *V. parahaemolyticus* strains are not pathogenic to humans, and only 1 to 2% of the environmental strains have shown to be positive for these genes while 90% of the clinical strains carry them (Nishibuchi and Kaper 1995; Hervio-Heath *et al.,* 2002; Robert-Pillot *et al.,* 2004). However, some investigators have reported a higher prevalence of *tdh* and *trh* genes in *V. parahaemolyticus* from environmental samples (DePaola *et al.,* 2003; Deepanjali *et al.,* 2005; Raghunath *et al.,* 2008; West *et al.,* 2013). Reports also reveal that these genes are not present in all the clinical strains of *V. parahaemolyticus* (Meador *et al.,* 2007). Hence, they alone are not responsible for *V. parahaemolyticus* pathogenicity (Makino *et al.,* 2003; Hiyoshi *et al.,* 2010). A study by Jones *et al.* (2012a) revealed that even in the absence of these genes *V. parahaemolyticus* strains exhibited pathogenicity. Thus, it was clear that other virulence factors also exist which contributes to the organism's virulence.

#### 4.2.2.2 Type III secretion systems in *V. parahaemolyticus*

The type III secretion systems (T3SSs) have been widely reported in many Gram-negative bacteria such as *Pseudomonas aeruginosa*, *Yersinia* sp., *Salmonella* sp., *Shigella* sp. and *Escherichia coli* (Ono *et al.,* 2006) and the bacteria use this to induce pathogenicity in the host cells

(Kumar *et al.,* 2014 a, b). Two non-identical and non-redundant T3SS clusters, T3SS1 and T3SS2, were identified during the genome mapping of *V. parahaemolyticus* RIMD2210633 (Makino *et al.,* 2003). Recently, it was found that toxins secreted by T3SS have a profound role in pathogenicity of *V. parahaemolyticus* (Noriea *et al.,* 2010; Broberg *et al.,* 2011; Karunasagar *et al.,* 2012). The organism carries two type III secretion systems namely T3SS1 and T3SS2. T3SS1 causes cytotoxicity and T3SS2 is mainly associated with enterotoxicity (Ham and Orth, 2012). They are needle-like apparatus that enable injection of bacterial effector proteins directly into host cells resulting in modulation of numerous host processes (Noriea *et al.,* 2010). The apparatus is made of about 20 proteins and is highly conserved among the bacteria (Ono *et al.,* 2006). Once the effector enters the host cells, it disrupts the host's immune response (Coburn *et al.,* 2007). These effectors modify the host signalling pathways and make it beneficial for the pathogen (Ham and Orth, 2012). The effectors secreted from T3SS1 include VopQ, VopS, VopR and VPA045 while those from T3SS2 include VopA/VopP, VopL, VopT, VopV and VopC (Ham and Orth, 2012; Matsuda *et al.,* 2012). All these effector proteins have a distinct role and they work in an orchestrated manner to help the pathogen in colonising the host cell and establishing an infection (Ham and Orth, 2012).

T3SS1 is located on chromosome 1 (Caburlotto *et al.,* 2009). T3SS2 genes are located on the pathogenicity island in Chromosome II of *V. parahaemolyticus* (Ham and Orth, 2012). This location of the gene on a pathogenicity island (Vp-PAI) may facilitate the transfer of this specific DNA fragment from a pathogenic strain to non-pathogenic autochthonous microorganism in the marine environment (Caburlotto *et al.,* 2009).

Noriea *et al.* (2010) studied the distribution of type III secretion systems in 130 *V. parahaemolyticus* isolates from the Gulf of Mexico. Kumar *et al.* (2014 a, b) reported the presence of T3SS2β genes in *trh*+ *V. parahaemolyticus* from seafood collected from Mangalore coast, India. Klein *et al.* (2014) found genes similar to the *V. parahaemolyticus* virulence-related genes to occur in other *Vibrionaceae* species that were isolated from a pristine estuary.

## 4.3 Objectives of the study

The specific objectives set for the present study are:-

1) To screen the *Vibrio* strains isolated from Cochin estuary, shrimp farm and seafood for extracellular virulence factors.
2) To detect the *tdh* and *trh* virulence genes in *V. parahaemolyticus* isolated from Cochin estuary, shrimp farm and seafood.
3) To detect the type III secretion system virulence genes in *V. parahaemolyticus* isolated from Cochin estuary, shrimp farm and seafood.

## 4.4 Materials and Methods

### 4.4.1 Screening of *Vibrio* strains for extracellular enzymes

#### 4.4.1.1 Bacterial strains used

A total of 276 *Vibrio* strains previously isolated from Cochin estuary (n=180), shrimp farm (n= 66) and seafood (n= 30) were used for the present study.

#### 4.4.1.2 Production of Lipase

Tributyrin is commonly used for studying lipolytic activities. Pure cultures of the isolates were spot inoculated on tributyrin agar plates (nutrient agar incorporated with 1% tributyrin). The plates were incubated at 37 $^{o}$C for 24-48 h. A positive result was indicated by zone of clearance around the colonies of lipolytic organisms, where the tributyrin has been hydrolyzed.

#### 4.4.1.3 Production of amylase

The isolates were spot inoculated onto starch agar plates (nutrient agar supplemented with 1% starch). The plates were incubated at 37 $^{o}$C for 24 to 48 h. A positive result was indicated by the presence of clear zones around the colonies on addition of Lugol's iodine solution.

#### 4.4.1.4 Production of gelatinase

The isolates were spot inoculated onto gelatin agar plates (nutrient agar supplemented with 2% w/v gelatin). The plates were incubated at 37 $^{o}$C for 24 to 48 h. Zone of clearance around the colonies after the plates were flooded with saturated solution of mercuric chloride indicated that gelatin has been hydrolyzed.

#### 4.4.1.5 Production of DNase

The isolates were spot inoculated onto DNA agar plates (nutrient agar containing 0.2% DNA). The plates were incubated at 37 $^{o}$C for 24 to 48 h. After incubation the plates were flooded with 1M HCl. Zone of clearance around the colonies indicated that bacteria have produced DNase and hydrolysed the DNA.

#### 4.4.1.6 Production of chitinase

The isolates were spot inoculated onto chitin agar (2% w/v colloidal chitin). The plates were incubated at 37 $^{o}$C for 7 days. Degradation of chitin was confirmed by clearance zone around the test colonies after incubation which indicated the production of chitinase enzyme by the bacteria.

#### 4.4.1.7 Production of phosphatase

To detect phosphatase production the isolates were spot inoculated onto phosphate agar (1% phenolphthalein diphosphate). The plates were incubated at 37 $^{o}$C for 24 to 48 h. After incubation, the colonies were exposed to ammonia vapour. Upon exposure, phosphatase producing colonies turn pink whereas the non-phosphatase producing colonies remained unchanged.

#### 4.4.1.8 Production of caseinase

The isolates were spot inoculated on skim milk agar plates. The plates were incubated at 37 $^{o}$C for 24 to 48 h. Caseinase production was detected by presence of clear zone around the colonies after incubation.

#### 4.4.1.9 Detection of hemolytic activity

Hemolytic activity was analysed on blood agar containing 5% human blood. The isolates were spot inoculated onto blood agar plates. The hemolytic activity was checked after incubation at 37 °C for 24 h.

#### 4.4.1.10 Statistical analysis

Any significant difference in the prevalence of extracellular enzymes among *Vibrio* from different sources was analysed using Chi- Square test in SPSS statistical software. Significance level was set at $\alpha = 0.05$.

### 4.4.2 Screening for virulence genes in *V. parahaemolyticus* from Cochin estuary, shrimp farm and seafood

*V. parahaemolyticus* was the most predominant *Vibrio* species observed in our study areas. Hence, we planned to undertake a detailed study on the virulence potential of the *V. parahaemolyticus* strains.

#### 4.4.2.1 Bacterial strains used

A total of 85 *V. parahaemolyticus* strains were used in the present study. This included isolates from various stations of the Cochin estuary (n=45), from a traditional pokkali cum shrimp farm (n=25) situated at Edavanakkad, adjoining the Cochin estuary and seafoods (n=15) collected from retail markets situated in and around Cochin.

#### 4.4.2.2 DNA isolation

Genomic DNA was extracted from the *V. parahaemolyticus* strains using the method described in the previous section 2.4.7.1.

#### 4.4.2.3 Detection of virulence genes *tdh* and *trh* by multiplex PCR

The detection of *tdh* and *trh* genes were performed using previously described primers (Bej *et al.*, 1999). The details of the primer pairs are given in Table 4.1. PCR amplification was performed in a total reaction volume of 25 µl consisting of sterile Milli Q water (15.5 µl), 10X PCR buffer (2 µl), primer (1 µl each), dntp mix (1 µl, 200 mM), template (4 µl), and Taq DNA polymerase (0.5 µl). The PCR conditions included an initial denaturation of 94 $^{o}$C for 3 min, followed by 30 cycles of denaturation 94 $^{o}$C for 1 min), primer annealing (58 $^{o}$C for 1 min), primer extension (72 $^{o}$C for 1 min) followed by a final extension (72 $^{o}$C for 5 min).

#### 4.4.2.4 Detection of type III secretion system genes

For detection of T3SS1 gene, the primer VP1669 previously described by Noriea *et al.* (2010) was used. To detect T3SS2α and T3SS2β genes, the primers VPA1346 and VPA1376 (Caburlotto *et al.*, 2009) respectively were used. The details of the primer sequences used are given in the Table 4.1. PCR amplification was optimized in a total reaction volume of 25 µl consisting of sterile Milli Q water (15.5 µl), 10X PCR buffer (2 µl), primer (1 µl each), dntpmix (1 µl, 200 mM), template (4 µl), and Taq DNA polymerase (0.5 µl). The PCR conditions included an initial denaturation at 95 ºC for 5 min, followed by 33 cycles of amplification consisting of denaturation at 95 ºC for 45 sec, annealing at 60 ºC for 40 sec (for T3SS1 and T3SS2 α)/ 50 ºC for 45 sec (for T3SS2 β), extension at 72 ºC for 45 sec, followed by final extension at 72 ºC for 3 min.

**Table 4.1** Details of the primer sequences used for the study

| Gene | Forward primer | Reverse primer |
|---|---|---|
| *tdh/trh* | *tdh* -F<br>5' ccatctgtccctttcctgcc 3'<br>*trh*- F<br>5' ttggcttcgatattttcagtatct 3' | *tdh*-R<br>5 'ccactaccactctcatatgc 3'<br>*trhR*<br>5'cataacaaacatatgcccatttccg 3' |
| T3SS1 | VP1669<br>5' taccgagttgccaacgtg 3' | VP1669<br>5' gattgttccgcgatttcttg 3' |
| T3SS2α | VPA1346<br>5' ggctctgatcttcgtgaa 3' | VPA1346<br>5′gatgtttcaggcaactctc 3' |
| T3SS2β | VPA1376<br>5' gctctccttggtaccaatcac 3' | VPA1376<br>5' ctgggatcttgatgtcaaggt 3' |

#### 4.4.2.5 Gel documentation and image analysis

Refer section 3.4.3.5

## 4.5 Results

### 4.5.1 Prevalence of extracellular virulence factors in *Vibrio* from Cochin estuary

Results revealed that gelatinolytic *Vibrios* dominated in the Cochin estuary. DNase and Phosphatase producers were the second and third dominant groups respectively in the estuary. Figure 4.1 shows the percentage distribution of extracellular enzymes among *Vibrio* from Cochin estuary. Out of the 180 strains screened, 83.8% (n=151) produced gelatinase, 83.3% (n=150) produced DNase, 80% (n=144) produced phosphatase, 76% (n=137) produced lipase and 67.7% (n=122) were amylase producers. Comparatively lesser number of chitinase (n=94), caseinase (n=79) and hemolysin (n=39) producers were detected in the estuary.

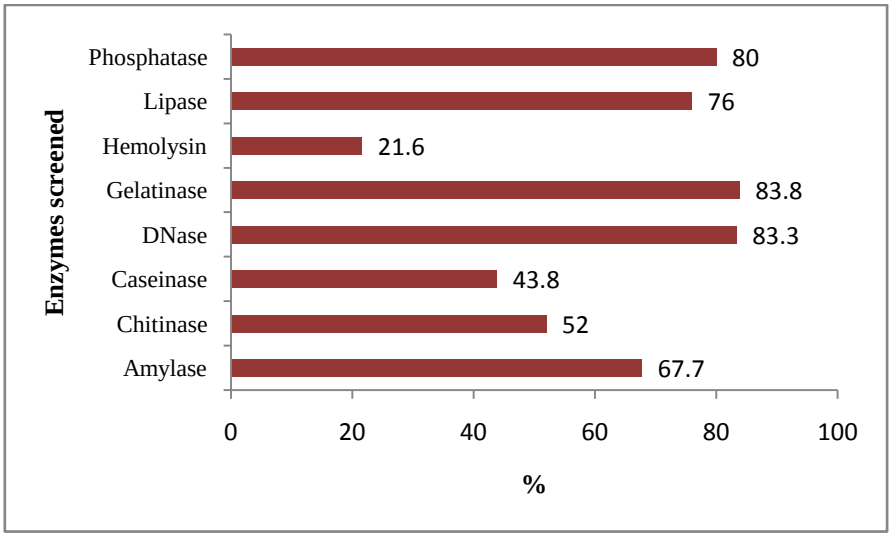


**Figure 4.1** Percentage distribution of extracellular enzymes among *Vibrio* from Cochin estuary

### 4.5.2 Prevalence of extracellular virulence factors in *Vibrio* from shrimp farm

A total of 66 *Vibrio* strains from shrimp farm were screened for production of extracellular enzymes. Figure 4.2 shows the percentage distribution of extracellular enzymes among *Vibrio* from shrimp farm. The gelatinolytic vibrios dominated followed by lipase producers. Out of the 66 strains screened, gelatinase was produced by 89.3% (n=59) strains, lipase by 80.3% (n=53), DNase by 75.7% (n=50), phosphatase by 72.7% (n=48), cellulase by 68% (n= 45), caseinase by 51.5% (n=34) and amylase by 42.4% (n=28) strains. Chitinase producers constituted only 25.7% (n=17). Hemolysin was produced by only 10.6% (n=7) of the strains.

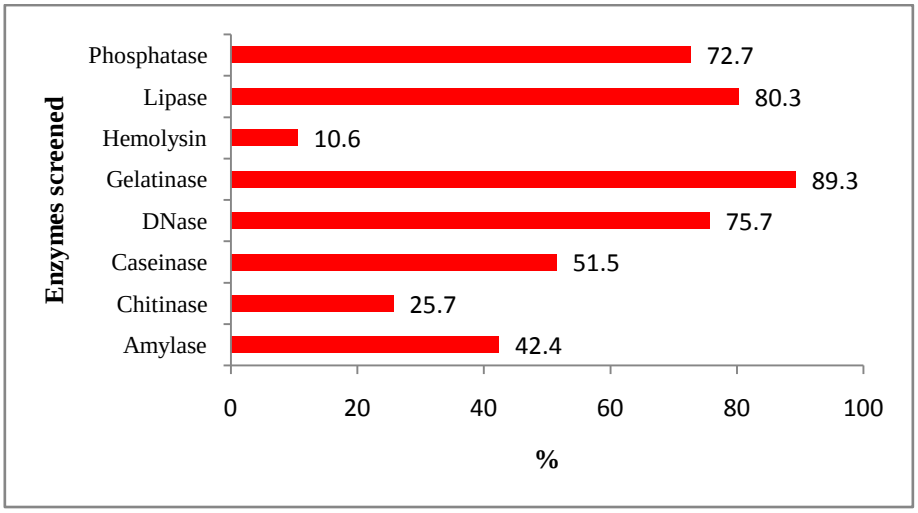


**Figure 4.2** Percentage distribution of extracellular enzymes among *Vibrio* from shrimp farm

### 4.5.3 Prevalence of extracellular virulence factors in *Vibrio* from seafood

A total of 30 *Vibrio* strains from seafood were screened. Figure 4.3 gives the percentage distribution of extracellular enzymes among *Vibrio*

from seafood. Gelatinase and DNase producers were the dominant groups. All the strains produced gelatinase and DNase. About 27 strains (90%) were phosphatase and amylase producers while 80% (n=24) of the strains produced lipase and amylase. Only a few strains were positive for chitinase, caseinase and hemolysin.

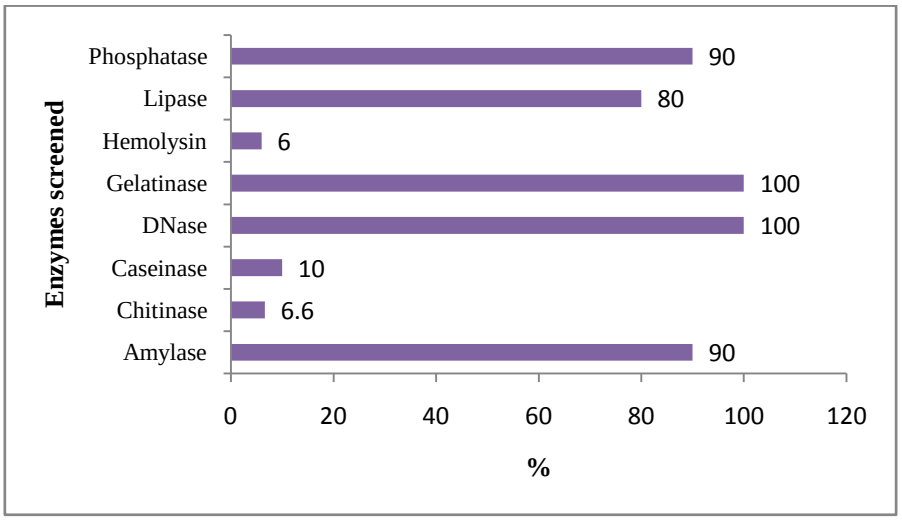


**Figure 4.3** Percentage distribution of extracellular enzymes among *Vibrio* from seafood

### 4.5.4 Relative prevalence of extracellular virulence factors among *Vibrio* from Cochin estuary, shrimp farm and seafood

There was significant variation in the prevalence of DNase, gelatinase, phosphatase, amylase, chitinase, caseinase and hemolysin among *Vibrio* from the three sources ($p<0.01$). However, there was no significant difference in the distribution of lipase among *Vibrio* from the three sources ($p>0.05$). Almost 80% of the *Vibrio* from all the three sources produced lipase. The relative prevalence of extracellular virulence factors among *Vibrio* from Cochin estuary, shrimp farm and

seafood is represented in Figure 4.4. DNase and gelatinase producers were the dominant groups from Cochin estuary and seafood. Gelatinase producing *Vibrio* dominated in shrimp farm followed by lipolytic vibrios. The extracellular enzyme profile of the *Vibrio* from Cochin estuary showed the following dominance pattern: DNase= gelatinase> phosphatase>lipase>amylase>chitinase>caseinase>hemolysin producers. *Vibrio* from shrimp farm revealed the following extracellular enzyme profile: Gelatinase> lipase> DNase> phosphatase> caseinase> amylase> chitinase> hemolysin producers. Among the *Vibrio* from seafood the following pattern was revealed; DNase= gelatinase > phosphatase, amylase>lipase>caseinase>chitinase>hemolysin producers. Five enzymes namely gelatinase, DNase, amylase and phosphatase were frequently expressed in *Vibrio* from seafood. Hemolysin was the least expressed enzyme in all the three sources.

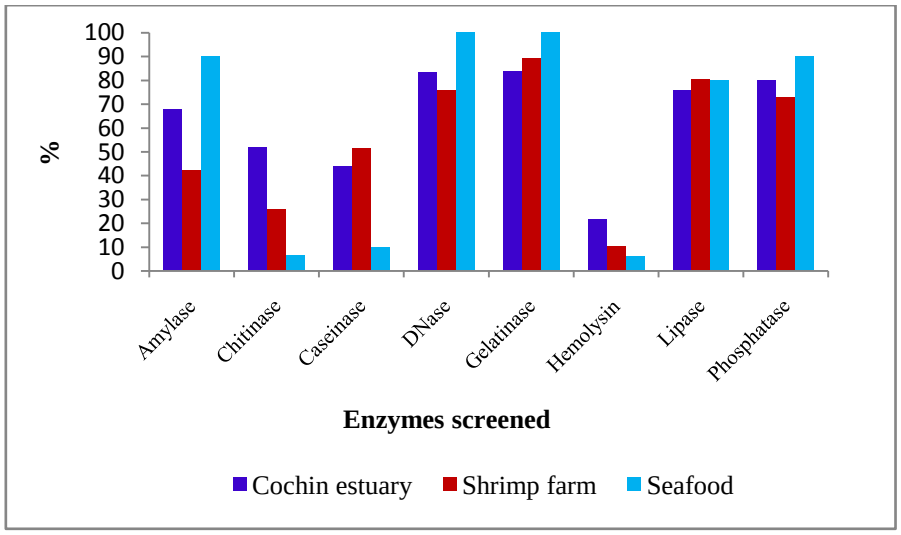


**Figure 4.4** Relative prevalence of extracellular virulence factors among *Vibrio* from Cochin estuary, shrimp farm and seafood

### 4.5.5 Prevalence of *tdh* and *trh* genes among *V. parahaemolyticus* from Cochin estuary, shrimp farm and seafood

A total of 85 *V. parahaemolyticus* strains from three different environmental sources were screened for the presence of *tdh* and *trh* genes. Results revealed very low prevalence of the genes among the strains.

By multiplex PCR, the *tdh* and *trh* positive strains produced 270 bp and 500 bp amplicons respectively (Figure 4.5). Among the 45 strains screened from Cochin estuary, 2 strains revealed the presence of *tdh* gene and 7 strains revealed the presence of *trh* gene. None of the strains from shrimp farm and seafood were positive for *tdh* and *trh* genes.

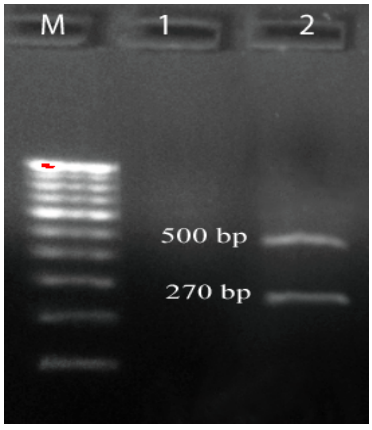


**Figure 4.5** Agarose gel showing PCR amplified *tdh* and *trh* genes. Lane M: 100 bp DNA ladder; lane 1: negative control; lane 2: *V. parahaemolyticus* strain carrying *tdh* and *trh* genes

### 4.5.6 Prevalence of type III secretion system genes among *V. parahaemolyticus* from Cochin estuary, shrimp farm and seafood

A total of 85 *V. parahaemolyticus* strains from three different sources were screened for the presence of type III secretion system genes (T3SS) genes.

This included 45 strains from Cochin estuary, 25 strains from shrimp farm and 15 strains from seafood collected from retail market in Cochin. All the strains showed the presence of T3SS1 gene and yielded a 300 bp PCR product (Figure 4.6).

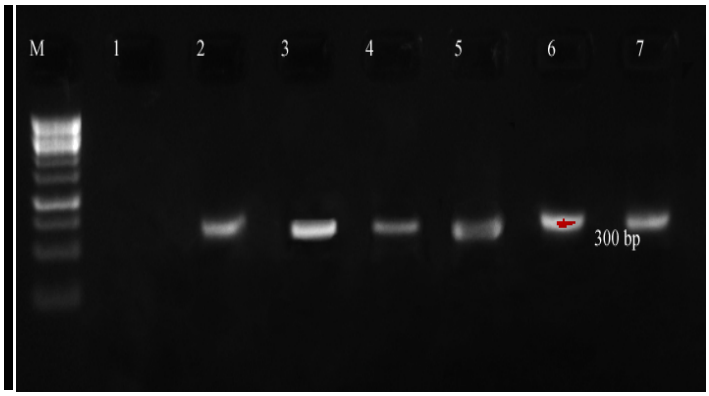


**Figure 4.6** Agarose gel showing PCR amplified T3SS1 gene. Lane M: 100 bp DNA ladder; lane 1: negative control; lanes 2 to 7: *V. parahaemolyticus* strains

Among the T3SS2 genes, T3SS2$\alpha$ gene was not detected in any of the strains from the three sources. The T3SS2β positive strains produced a 1067 bp PCR product (Figure 4.7). T3SS2β gene was present in 30 out of the 45 strains from Cochin estuary. Among the 15 strains isolated from fishes, 9 were positive for the T3SS2β gene. Among the 25 strains from shrimp farm, 7 revealed the presence of T3SS2β gene.

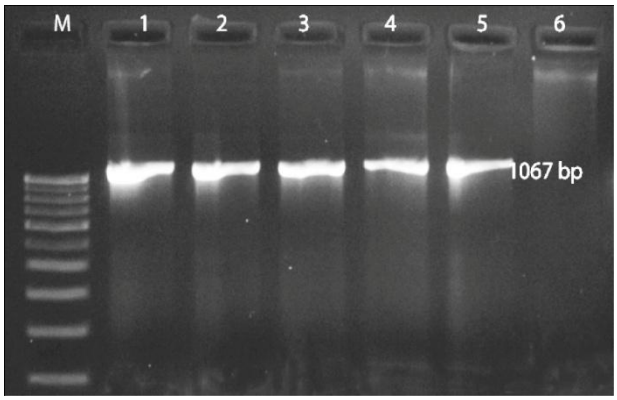


**Figure 4.7** Agarose gel showing PCR amplified T3SS2β gene. Lane M: 100 bp DNA ladder; lane 1-5: *V. parahaemolyticus* strains; lane 6: negative control

### 4.5.7 Relative distribution of virulence genes among *V. parahaemolyticus* from Cochin estuary, shrimp farm and sea food

The distribution of virulence genes in *V. parahaemolyticus* isolated from the three sources was compared. *V. parahaemolyticus* strains from Cochin estuary were comparatively more virulent than those from shrimp farm and seafood.

Figure 4.8 shows the relative distribution of the virulence genes among *V. parahaemolyticus* from various sources. The *tdh* and *trh* genes were detected only in strains from Cochin estuary. T3SS1 gene was detected in all the *V. parahaemolyticus* strains isolated from the three sources. T3SS2α gene was not present in any of the strains screened. T3SS2β gene was most frequently observed in Cochin estuary isolates (66.6%), followed by seafood isolates (60%) and it was least frequent in the shrimp farm isolates (28%).

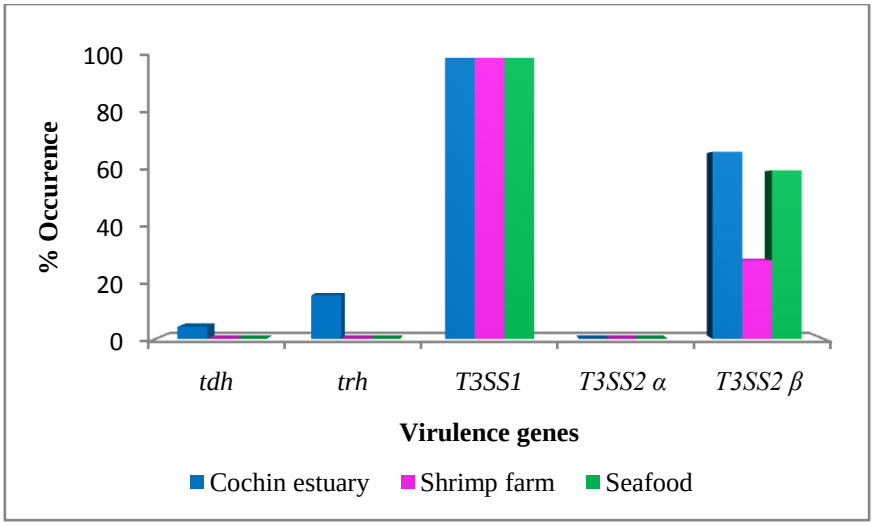


**Figure 4.8** Relative distribution of virulence genes among *V. parahaemolyticus* from various sources

## 4.6 Discussion

### 4.6.1 Screening of *Vibrio* from Cochin estuary, shrimp farm and seafood for extracellular enzymes

Enzyme assays can be used for understanding the organic matter degradation and nutrient cycling in the aquatic systems. The mineralisation process in the marine environments is carried out mainly by the extracellular enzymes secreted by heterotrophic bacteria (Belanger *et al.*, 1997). Presence of hydrolytic enzymes in *Vibrio* from marine sediments of South west coast of India has been reported by Raghul and Sarita (2011). Gelatinolytic activity observed by these authors among marine vibrios (80.1%) was comparable to our results (84-89%). *Vibrio* from our study areas showed much higher cellulolytic and chitinolytic activity when compared to those from marine environment (Raghul and Sarita, 2011). In contrary to our results, phosphatase producers were not detected in the

previous study (Raghul and Sarita, 2011), while in our study they constituted one among the dominant groups. Though Cochin estuary and adjoining pokkali-cum-shrimp farms are physically linked to the south east Arabian Sea, the nutrient dynamics in these environments seems to be quite different, which in turn might have affected the microbial communities and their functionalities.

The screening of extracellular microbial enzymes is also important to understand their role in pathogenesis. Most of the *Vibrio* in our study belonged to pathogenic species such as *V. parahaemolyticus, V. cholerae, V. vulnificus, V. fischeri, V. mimicus, V. alginolyticus* etc. The enzymes produced by pathogenic species occasionally act as virulence factors (Hase and Finkelstein, 1993; Harrington, 1996). They help the organism to establish an infection in the host. Pathogenic *Vibrio* species produce and secrete several enzymes that are characterised as direct virulence factors causing skin damage (Miyoshi, 2013). The production of urease and hemolysin are considered as typical virulence markers for *V. parahaemolyticus* isolates from clinical samples (Okuda *et al.,* 1997; Miyoshi, 2013). Extracellular DNases acts as endonucleases and cause DNA hydrolysis. Gelatinase production has already been recognized as a virulence factor in bacteremia cases in humans (Vergis *et al.,* 2002). Bacterial lipase activity is mainly involved in nutrient acquisition from the host by degrading the membrane lipids. Phospholipases are also associated with virulence in bacteria and may act as haemolysins (Costa *et al.,* 2013).

Previous studies have already recognised the role of amylase, chitinase and caseinase in the virulence of *Vibrio* (Rodrigues *et al.,* 1993). Previous reports suggest that proteolytic enzymes of fish pathogens such as *Aeromonas* and *Vibrio* have a major role in causing massive tissue damage in the host (Rodrigues *et al.,* 1993; John and Hatha, 2013). Previous report on multi-enzymatic profile of *V. parahaemolyticus* from oysters supported our findings (Costa *et al.,* 2013). They also reported that gelatinase and DNase producers were the dominant groups and caseinase producers were the least (Costa *et al.,* 2013). Shrimp mortality due to viral/bacterial infections was frequent in the farms chosen for present study, for which the aforementioned virulence factors might have contributed significantly.

Our previous studies (Selvam *et al.,* 2012) revealed considerable environmental stress existing in these farms. Under such circumstances, environmental vibrios with potential virulence features could act as opportunistic pathogens, playing major role in disease outbreaks in the estuarine and shrimp farm systems.

Apart from that, we have also observed the presence of enzymes in *Vibrio* isolated from seafood samples, which further evokes the problem. Thus, the presence of these extracellular virulence factors in the *Vibrio* isolated from our study areas may play a substantial role in triggering infections in humans and aquatic animals under favourable conditions.

### 4.6.2 Screening for virulence genes in *V. parahaemolyticus* from Cochin estuary, shrimp farm and seafood

The *tdh* and *trh* genes are considered as major virulence associated genes in *V. parahaemolyticus* (Ceccarelli *et al.*, 2013). In our study, the virulence genes *tdh* could be detected in 4.4% and *trh* in 15.5% strains isolated from Cochin estuary. A previous study revealed much higher prevalence of *tdh* (8.4%) and *trh* (25.3%) genes in *V. parahaemolyticus* isolated from seafoods harvested from the south west coast of India (Raghunath *et al.,* 2008). In another study, the *tdh* and *trh* genes were detected in 4.3% and 0.3% of environmental *V. parahaemolyticus* strains from South Carolina and Georgia coasts (Baker- Austin *et al.,* 2008) while none of the strains from seafood and shrimp were positive for these genes. Previous reports state that most of the environmental strains are not pathogenic to humans, and only 1 to 2% of the environmental strains have shown to be positive for *tdh* and *trh* genes (Hervio-Heath *et al.,* 2002; Robert-Pillot *et al.,* 2004). However, in a recent study, *tdh* and *trh* genes were recovered from 48% and 8.3% of *V. parahaemolyticus* respectively (West *et al*., 2013).

In the present study, all the *V. parahaemolyticus* strains isolated from Cochin estuary, shrimp farm and seafood collected from retail markets were positive for the T3SS1 gene. This was in agreement with previous studies stating that T3SS1 are ubiquitous in *V. parahaemolyticus* (Park *et al.,* 2004 a; Noriea *et al.,* 2010). T3SS1 gene induces cytotoxicity in the host cells (Park *et al.,* 2004 a).

T3SS2 genes are involved in enterotoxicity (Park *et al.,* 2004 a) and helps in the environmental fitness of strains (Hiyoshi *et al.,* 2010; Matz *et al.,* 2011). Among the two T3SS2 genes screened, T3SS2α was not detected in any of the strains from the three sources. T3SS2β was detected in 66.6% strains from Cochin estuary, 28% strains from shrimp farm and 60% strains from seafood. A previous study reported the presence of T3SS2α and T3SS2β genes in *V. parahaemolyticus* isolated from Gulf of Mexico (Noriea *et al.,* 2010). T3SS2 genes are located on the pathogenicity island in Chromosome II of *V. parahaemolyticus* (Ham and Orth, 2012). This location of the gene on a pathogenicity island (Vp-PAI) may facilitate the transfer of this specific DNA fragment from a pathogenic strain to non-pathogenic autochthonous microorganism in the marine environment (Caburlotto *et al.,* 2009). The presence of T3SS genes in our strains suggests the ability of these pathogens to cause infection in humans and marine animals.

Previous studies reported the existence of a correlation of *tdh* with T3SS2α and *trh* with T3SS2β (Park *et al.,* 2004 a; Noriea *et al.,* 2010). However, in the present study we could not observe any such correlation between the occurrence of these genes. Our results were supported by a previous report stating the presence of T3SS2 in *tdh* and *trh* negative environmental strains of *V. parahaemolyticus (*Paranjpye *et al.,* 2012*)*. The current study thus revealed the pathogenic potential of *V. parahaemolyticus* strains from our study areas which enable them to act as opportunistic pathogens and play major role in disease outbreaks in this system. This increases the risk of disease outbreak in Cochin estuary and adjacent shrimp farms.

Hence, the present study emphasises the necessity to implement continuous surveillance of water bodies in Cochin estuary by regulatory agencies for human pathogens, in order to ensure the seafood and public health safety of the study area. The study also highlights the urgent need for adoption of proper water quality management in the aquaculture systems surrounding the estuary.

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