Chapter 5

Identification of virulent genes of *Vibrio parahaemolyticusolecular*

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5.1 Introduction

Molecular characterization means characterizing the organism at the genetic level without any interference of environmental factors. Normally, the biochemical characterization of bacteria was carried out based on carbon and nitrogen utilization, cellular metabolic enzymatic activity like hydrolysis, catalysis, oxidization and reduction which provides phenotypical traits of the organism (Uzoma, 2016). Bacterial characterization using biochemical tests varies in a different strain of same species depending upon geographical location and growth condition mutation (Uzoma, 2016). Therefore, molecular techniques have been developed for bacterial characterization using both DNA and protein-based markers (Emerson et al., 2008). In the environment, both pathogenic and nonpathogenic strains of bacteria exist. The pathogenic strain of bacteria emerged from nonpathogenic bacteria after the acquisition of genes from the environments. So, virulent genes are generally included in the study to characterize both pathogenic and nonpathogenic strain.

V. parahaemolyticus is a halophilic bacteria. Therefore, the brackish and marine water ecosystem plays a natural habitat for this bacterium. Both pathogenic and nonpathogenic bacteria share the same ecological niche and the acquisition of virulence gene or their homologous gene might have taken place in the aquatic environment. The presence of virulent genes or their homologoues genes were identified in the environmental strains (Sechi et al., 2000; Xie et al., 2005; Caburlotto et al., 2009), highlighting the potential threat of environmental strains to human health (Turner et al., 2013). Understanding the distribution of virulence genes in environmental isolates is very

important for monitoring the emergence of new pathogenic strain. *V. parahaemolyticus* is widely distributed in the environment but only the strain carrying *tdh*, *trh* or both were considered as a pathogenic strain. The environmental strains are more diversified than the pathogenic strain. So, only biochemical characterization is not enough to differentiate the different strains of *V. parahaemolyticus*. Thus, molecular characterization of *V. parahaemolyticus* isolated from the shrimp farms of Andhra Pradesh, West Bengal and Gujarat (3 major shrimp producing states of India) was done by using virulence-associated genes under T3SS1 (*vcrD1*, *vp1680*, *vopD*), T3SS2 (*vcrD2*, *vopD2*, *vopB2*, *vopC*) and three hemolysin genes *tlh*, *tdh*, *trh*.

5.2 Material and Methods

5.2.1 Genomic DNA isolation and Virulence-related gene detection

Bacterial culture and genomic DNA isolation methodologies are described in Chapter 4. PCR was carried out with the genomic DNA of each isolate to determine the presence of the thermolabile hemolysin (*tlh*), thermo directed hemolysin (*tdh*), thermo directed related hemolysin (*trh*) and representative genes of T3SS1 (*vcrD1*, *vp1680*, *vopD*) and T3SS2α (*vcrD2*, *vopD2*, *vopB2*, *vopC* and *vopT*). All the primers used in this study were given in Table 5.1. The PCR reaction mixture was prepared by mixing reagents described in Chapter 4. The PCR program for amplification is denaturation for 2 min at 95 °C followed by denaturation at 94 °C for 30 s, annealing temperature for 30 s and extension at 72 °C for 45 s for 35 cycles with final extension for 3 min. at 72 °C. PCR product was visualized on 1.8 % agarose gel stained with ethidium bromide.

Table 5.1 List of primers used in this study

Target (Locus)	Sequence	Annealing Temperature	Amplicon (bp)	Source
vcrD1 (*VP1662*)	5′ CTGCTGGTCTTGTTCGCTCT 3′	53.8	439	Tsai et al. (2013)
	5′ TCTGGTCGCTTCCTTCTGTG 3′			
VP1680	5′ AATTTTGGAAGTGGTGAGCCTA 3′	51.1	502	Tsai et al. (2013)
	5′ TTCTTTTGCTATCGGCGTAACT 3′			
vopD (*VP1656*)	5′ GCGACACTATCAAAACAACCAA 3′	51.1	515	Tsai et al. (2013)
	5′ TGCCATCTCGGTCTTAATTTCT 3′			
vcrD2 (*VPA1355*)	5′ GTTGGTGCTCGCTTCTCTCT 3′	53.8	300	Tsai et al. (2013)
	5′ CCCATCCCCTACTGTCAAGA 3′			
vopD2 (*VPA1361*)	5′ TTCTGTAAATCTAGCGCAACCA 3′	51.1	476	Tsai et al. (2013)
	5′ TGTATTTGGCAGTACGACCTTG 3′			
vopB2 (*VPA1362*)	5′ GGGGGCAAGCTAATAAAGAGAT 3′	53	527	Tsai et al. (2013)
	5′ GTTAAAGCTGAGCAACATCGTG 3′			
vopC (*VPA1321*)	5′ AGTCAAGGGACTAATTTAGCAA 3′	47.4	576	Tsai et al. (2013)
	5′ TACCATTATTCAACGAATCAGA 3′			
tdh	5′ GTAAAGGTCTCTGACTTTTGGAC 3′	58.0	269	Bej et al. (1999)
	5′ TGGAATAGAACCTTCATCTTCACC 3′			
trh	5′ TTGGCTTCGATATTTTCAGTATCT 3′	58.0	500	Bej et al. (1999)
	5′ CATAACAAACATATGCCCATTTCCG 3′			
tlh	5′ AAAGCGGATTATGCAGAAGCACTG 3′	58.0	450	Bej et al. (1999)
	5′ GCTACTTTCTAGCATTTTCTCTGC 3′			
toxR	5′ GTCTTCTGACGCAATCGTTG 3′	63.0	368	Kim et al. (1999)
	5′ ATACGAGTGGTTGCTGTCATG 3′			
AHPND	5′ ATGAGTAACAATATAAAACATGAAAC 3′	55	1269	Sritunyalucksana et al. (2007)
	5′ ACGATTTCGACGTTCCCCAA 3′			
	5′ TTGAGAATACGGGACGTGGG 3′	55	230	
	5′ GTTAGTCATGTGAGCACCTTC 3′			

5.2.2 Liquid-phase hemolysis assays

For liquid hemolysis assay, *tdh*, *trh* and *tlh* positive isolates were cultured in Brain Heart Infusion broth. The bacterial cells were pelleted down by centrifugation at 5000 rpm for 10 min and after washing twice with Phosphate-Buffer Saline (PBS), the bacterial cells pellet was resuspended in PBS. Goat blood and human blood were centrifuged at 2000 rpm for 10 min, washed thrice with equal volume of PBS, and finally resuspended in PBS. 400 µl of bacterial suspension was added to 400 µl of 2 % (final concentration) suspension of erythrocytes (RBC) in microcentrifuge tube, centrifuged at

1200 rpm for 30 min and then incubated at 37 °C. In case of sheep blood, every 6 hrs interval and in case of human blood every 2 hrs interval the tubes were centrifuged at 4 °C at 2000 rpm for 10 min. The supernatant was carefully transferred to a microcentrifuge tube and the hemolytic activity was determined by measuring the released hemoglobin spectrophotometrically at 540 nm (Evans et al., 2013). The hemolysis percentage was calculated by using the formula as described previously (Lai et al., 2003).

5.2.3 Cytotoxicity test

Overnight culture of *tdh*, *trh*, *tlh* positive strains of *V. parahaemolyticus* were pelleted down separately and washed twice with PBS. Finally, the pellets were suspended in PBS. The HEK cells were grown on coverslips and were covered with bacterial suspension. After 5 hrs of incubation, the coverslips were washed with ice-cold PBS and dipped in PBS containing 25 μg/ml ethidium bromide (EtBr) and 5 μg/ml acridine orange (AO) (Kodama et al., 2008; Zhou et al., 2010). The coverslips were rinsed with PBS and observed under a Zeiss confocal microscope using fluorescein isothiocyanate (543 nm) and rhodamine (488 nm) filters.

5.2.4 Gene sequencing

The amplified gene product was sequenced using an ABI 3730xl capillary sequencer (Applied Biosystems, Foster City, CA) in forward and reverse directions (Behera et al., 2014). The contig was prepared by aligning the forward and reverse sequences using DNA baser 7.0.0. software. The reverse sequence was used to proofread the forward sequence during contig preparation. The assembled gene sequences were

then compared in GenBank using the NCBI-BLAST program facility (http://www.ncbi.nlm.nih.gov\BLAST) with available sequences in the GenBank.

5.3 Results

5.3.1 Hemolytic activity

The *tdh* and *trh* positive strains were considered as a pathogenic strain. Only *tdh* positive strain produces hemolytic zone in blood agar plate. Six strains (3%) out of 183 showed hemolytic activity in Wagatsuma agar (KP). This was validated by amplification of 251-bp *tdh* gene fragment in these 6 strains.

5.3.2 Virulence-related gene detection

All the isolates were subjected to PCR for identifying virulence genes like *vcrD1*, *vopD* and *vp1680* under T3SS1 and *vcrD2, vopB2, vopC,* and *vopD2* under T3SS2. The *vcrD1, vopD* and *vp1680* genes were identified from *V. parahaemolyticus* strains isolated in the present study, which clearly indicated that these genes are well conserved in both pathogenic and nonpathogenic strains. Earlier studies by Ono et al., 2006 and Shimohata et al., 2011 revealed that, *vcrD1* (*vp1662*) gene synthesizes the inner membrane protein of TTSS1 while the other two genes, *vopD* (*vp1656*) and *vp1680* synthesize effector proteins. Again all the isolates were screened for four different virulent genes under T3SS2. A significant variation was observed in the presence of four different genes in all the isolates. Of total isolates examined, five number of isolates were found positive for *vrcD2*, *vopD2*, *vopB2* and *vopC2*. The *vrcD2* encodes an inner membrane protein while *vopD2* encodes a translocon protein responsible for enterotoxicity and *vopC* encodes a protein helping in the invasion of *V. parahaemolyticus* into the cells. The pathogenic

strain of *V. parahaemolyticus* was identified based on the presence of two virulent genes, *tdh* and *trh.*

Only six of the 183 strains (3 %), amplified the 251 bp *tdh* gene fragment and 14 strains of *V. parahaemolyticus* amplified the 488 bp *trh* gene fragment. All isolates were positive for *tlh* amplifying 451 bp fragment. only five isolates carry the entire 10 virulent genes used in the present study.

Table 5.2 Identification of virulent genes from *V. parahaemolyticus* isolates from the aquaculture environments of India

Gene	Environmental Isolates		
	West Bengal n = 71	**Andhra n = 61**	**Gujarat n = 51**
Hemolysin			
tlh	71	61	51
tdh	2	3	1
trh	6	5	3
T3SS1			
vcrD1	71	61	51
vp1680	71	61	51
vopD	71	61	51
T3SS2			
vcrD2	8	8	4
vopD2	2	3	1
vopB2	2	3	1
vopC2	2	3	1
Others			
toxR	71	61	51
AHPND	0	0	0

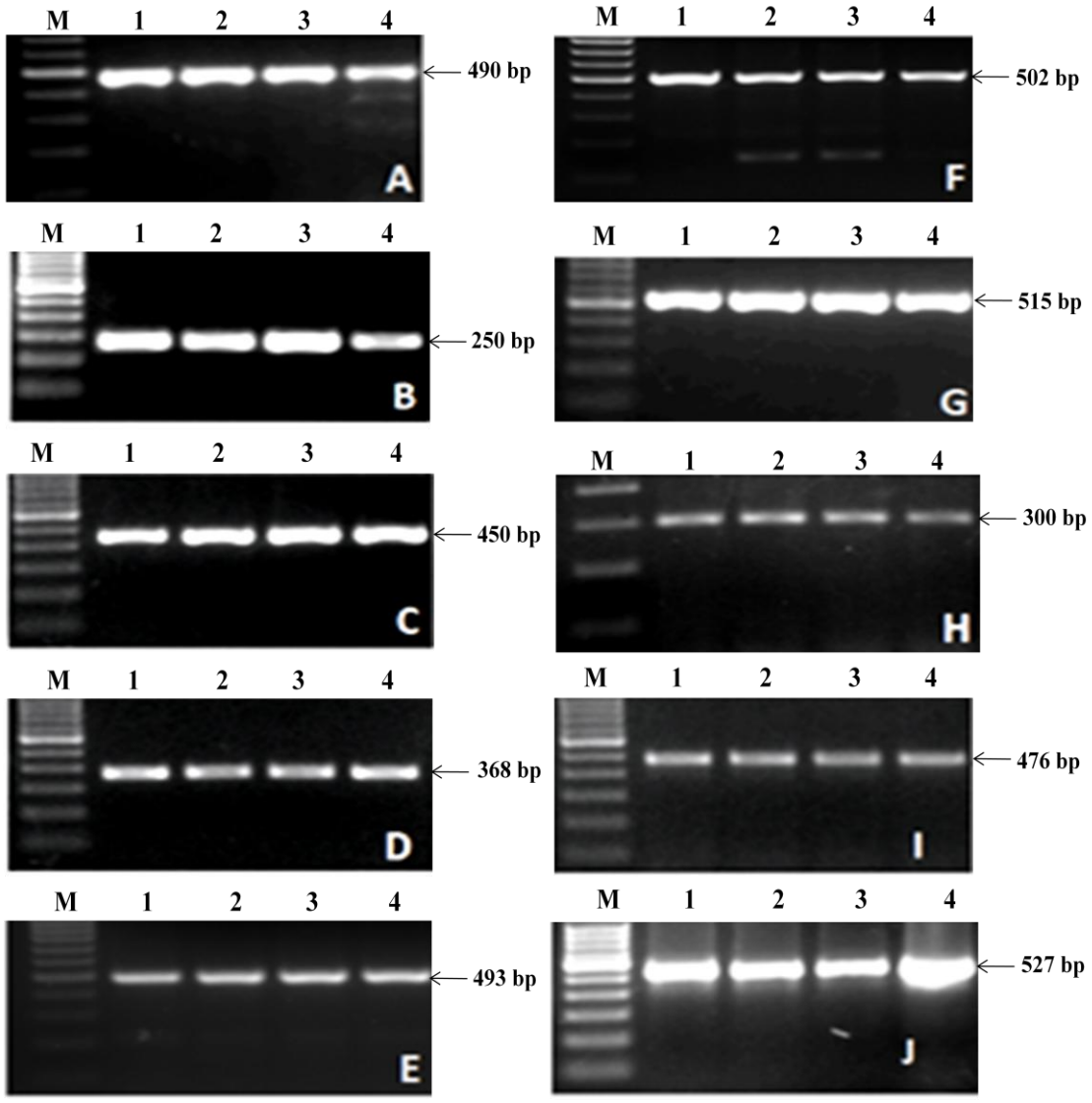

Figure 5.1 Agarose gel electrophoresis of the PCR products of different virulent genes amplified from *V. parahaemolyticus*. *trh* gene (~490 bp) (A); *tdh* gene (~250 bp) (B); *tlh* gene (~450 bp) (C); *toxR* (~368 bp) (D); *vcrD1* (~493 bp) (E); *vp1680* (~502 bp) (F); *vopD1*gene (~515 bp) (G); *vcrD2* (~300 bp) (H); vopD2 (~476 bp) (I); vopB2 (~527 bp) (J). M: Molecular weight marker (100 bp DNA Ladder); Lane 1-4 isolated *V. parahaemolyticus*

5.3.3 Liquid-phase hemolysis assays

To understand the kinetics of haemolytic activity of three different stain of *V. parahaemolyticus viz*. *tdh*, *trh* and *tlh* positive strain, liquid hemolysin assay were carried out with two different types of RBC (goat and human). Within 48 hrs of the experiment, very little hemoglobin was release in solution from goat blood by *tdh* and *trh* positive strain. However, 90 % hemolysin was recorded in goat RBC by *tlh* after 30 hrs of incubation. The hemolysis of human RBCs were quite faster and 100 % of RBCs were hemolysed within 14 hrs by *tdh* and *trh* positive strain of *V. parahaemolyticus*. Whereas, 40 % hemolysin was recorded in solution with human RBCs by *tlh* positive strain of *V. parahaemolyticus*, after 14 hrs of interval (Figure 5.2).

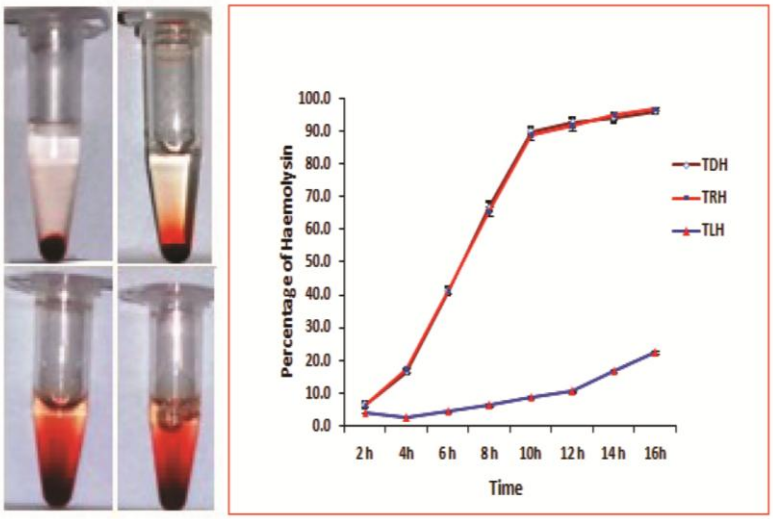

Figure 5.2 The kinetics of human RBC haemolysis by three different strains of *V. parahaemolyticus* containing *tdh*, *trh* and *tlh* genes in liquid-phase hemolysin assay.

5.3.4 Cytotoxicity test

The control and infected cells were stained with Ethidium Bromide (EtBr) and Acridine Orange (AO). The control cells showed green fluorescence at 543 nm under microscopic observation whereas only a few cells showed red fluorescence at 488 nm. The infected cells with three different strain of *V. parahaemolyticus* (*tdh*, *trh* and *tlh* positive strain) exhibited yellow and orange fluorescence at 543 nm after 5 hrs of incubation, whereas, most of the cells infected with *tdh* and *trh* positive strain showed red fluorescence at 488 nm (Figure 5.3). Few cells showed red fluorescence at 488 nm and orange fluorescence at 543 nm after 5 hrs incubation with tlh positive strain of *V. parahaemolyticus*.

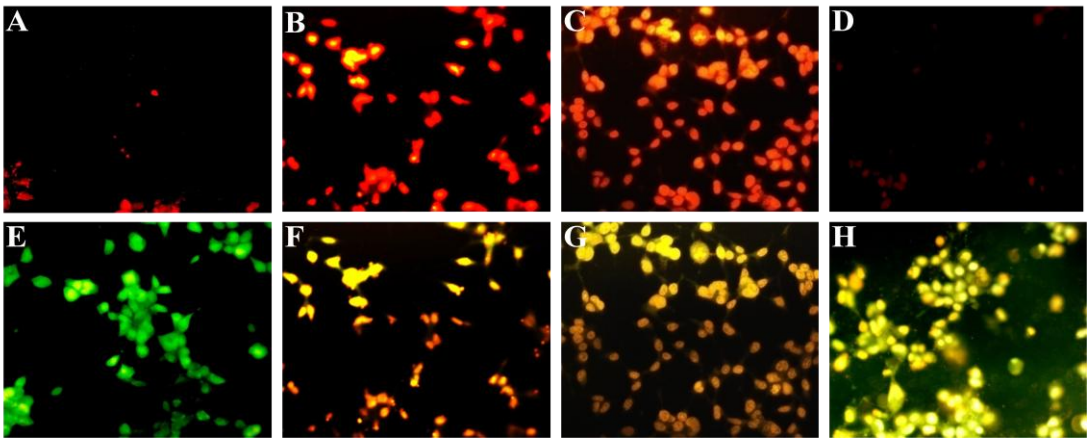

Figure 5.3. (A to H) HEK cells stained with EtBr and Acridine Orange to show the cytotoxicity after 5 h of infection with *V. parahaemolyticus*. (A and E) Un-infected HEK cell control; (B and F) HEK cell infected with the *tdh*-positive stain (S24P132); (C and G) HEK cell infected with *trh*-positive strain (AP429); (D and H) HEK cell infected with the *tlh* positive strain stain (SPEM2).

5.4 Discussion

V. parahaemolyticus is an important bacteria under *Vibrio* sp. responsible for gastrointestinal infection in different parts of the world (Wang et al., 2017) and widely distributed in aquatic environments which include both pathogenic and nonpathogenic stains (Gennari et al., 2012). *V. parahaemolyticus* was isolated from infected tissue collected from different aquaculture pond after 24 hrs of enrichment in APW using three different types of specific media. All the isolates that identfied as a *V. parahaemolyticus* in primary analysis were further confirmed using *toxR* and *tlh*. All the isolates of *V. parahaemolyticus* showed positive for *tlh* gene which reconfirm that it can be used as a molecular marker (Johnes et al., 2012). Both pathogenic and non-pathogenic strains showed the presence of *tlh* gene (Bej et al., 1999) and is responsible for the lysis of red blood cells (Shinoda et al., 1991; McCarthy et al., 1999 and Wang et al., 2013). The expression of *tlh* gene is considerably upregulated under simulated intestinal infection conditions Gotoh et al. (2010). The environmental strains generally lack virulence genes that are generally found in pathogenic isolates. However, after whole genome sequencing and analysis, it was found that, virulence genes, or their homologous, could also be present in environmental strains. The acquisition of different genes might have taken place in the aquatic environment (Xie et al., 2005 and Caburlotto et al., 2009) by horizontal gene transfer or by the acquisition of foreign DNA. The acquisition of foreign DNA may increase their fitness and virulence potential.

Hemolysis of RBC is considered as a major virulent property in pathogenic bacteria. To date, three different types of hemolysin genes were reported in *V. parahaemolyticus viz. tdh, trh* and *tlh.* However, *tdh* and *trh* are the major virulence

factor responsible for hemolysis of erythrocytes, cardiotoxicity and enterotoxicity (Raghunath, 2015).

The presence of different hemolysin gene is also reported in other *Vibrio* sp. like *V. anguillarum*, *V. cholera*, *V. fluvialis*, *V. harveyi*, *V. hollisae*, *V. mimicus*, *V. tubiashii* and *V. vulnificus*, etc (Zhang and Austin, 2005). In the present study, it was obseved that, human RBCs were completely lysed by *tdh* and *trh* positive strains within 14 hrs of incubation, however, little hemolysis was recorded in goat RBCs by both the strains. In contrast to *tdh* and *trh* positive strain, *tlh* positive strain showed 40 % hemolysis after 14 hrs of incubation in the case of human RBCs. The hemolytic effect by bacteria depends on the type of RBCs from different blood cell types within the same species and different species. A study carried out by Rajkumar et al. (2016) using six different types of bacteria showed that the hemolysis of RBCs by the same bacteria varies on different human blood cell types.

After incubation of HEK cells with the *tdh* and *trh* positive strains, most of the cells in the combination with AO and EtBr exhibited yellow and orange fluorescence, which confirmed the cytotoxic activities of the bacteria as reported by Roy et al. (2004). Only a few cells showed yellow and orange fluorescence in the combination of both AO and EtBr, after incubation with *tlh* strain.

T3SSs of *V. parahaemolyticus* secrets many effectors proteins that create a pore in the membrane. Zhou et al. (2010) found that Vp1686 secreted from T3SS1 is responsible for cytotoxicity in the host cell. In a separated experiment carried out by Kodama et al. (2008) identified two effector proteins VopD2 and VopB2 under T3SS2, responsible for cytotoxicity and pore formation in infected cells. The presence of *vopD2*

and *vopB2* genes were identified in *tdh* positive strains. In spite of different effector proteins under T3SS1 and T3SS2, the *tdh* and *trh* gene also have cytotoxic effects in different cell types (Raimondi et al., 2000). The *tlh* positive strain showed less toxicity in HEK cell after 5 hrs of incubation, confirms the less cytotoxicity in comparison to *tdh* and *trh*. Wang et al. (2012) observed the cytotoxicity in three different cell types after 24 hrs of incubation with purified *tlh* protein.

The *V. parahaemolyticus* isolated from fish, shrimp, mollusks, water, soil and sediment samples were positive to *tdh* and *trh* genes were reported in earlier studies from different parts of the world (Khouadja et al., 2013; Alipour et al., 2014; Chonsin et al., 2016 and Rahman et al., 2017). The presence of virulent *V. parahaemolyticus* in the environmental samples had also been reported from India (Raghunath et al., 2009; Deepanjali et al., 2005 and Kumar et al., 2014). Only 1-10 % of environmental isolates of *V. parahaemolyticus* are positive to *tdh* and *trh* gene (DePaola et al., 2000; Cook et al., 2002 and Johnson et al., 2009). The low frequency of *tdh* and *trh* positive strains has been reported from Japan and Chile (Fuenzalida et al., 2006 and Alam et al., 2003). The present study recorded the presence of *tdh* gene only in 3 % of the isolates (3 isolates out of 61 isolates from Andhra Pradesh, 1 isolates out of 51 isolates from Gujarat and 2 isolates out of 71 isolates from West Bengal). Deepanjali et al. (2005) showed a high prevalence of *trh* positive *V. parahaemolyticus* (59.3 %) in the oyster. A study conducted by DePaola et al. (1988) and Deepanjali et al. (2005) showed a high frequent occurrence of *tdh* positive strain of *V. parahaemolyticus* in the oyster, which might be due to the preferential selection of *tdh* positive strains.

A study carried out by Jones et al. (2012) showed that 27 % of clinical *V. parahaemolyticus* strains were negative for *tdh* and *trh* though they were pathogenic which indicates the presence of other virulence factor (s). In a separate experiment carried out by Mahoney et al. (2010) found that environmental isolates of *V. parahaemolyticus* produced putative virulence factors like siderophore, biofilm and extracellular proteases which is cytotoxic to human gastrointestinal cells though *tdh* and/or *trh* gene absent in their genome. Similarly, Park et al. (2004) did not found any change in the cytotoxicity effect to HeLa cells after deletion of both copies of *tdh* from *V. parahaemolyticus*. Ming et al. (1994) found that partial but clear accumulation of fluid in the small intestine ligated rabbit after deletion *trh* gene. These results clearly indicate that only *tdh* and *trh* are not responsible for cytotoxicity and enterotoxicity. There may be some unknown virulence factor(s) present in *V. parahaemolyticus* responsible for pathogenicity (Caburlotto et al., 2010).

In the last two decades, the extensive study on the virulence gene of *V. parahaemolyticus* revealed the presence of three pathogenic islands (T3SS1, T3SS2 and T3SS6) that is responsible for secretion of many different effector proteins. These proteins have functions like, modification and suppression of host defense system (Matlawska-Wasowska et al., 2010), dysregulation of actin network (Zhou et al., 2010), helps in pathogen survival, replication and colonization inside the host cell (Zhang et al., 2012).

A non-pathogenic strain can be transformed into a pathogenic strain by acquiring these virulence genes, or their homologous, in the aquatic environments. Thus, previous studies have included testing of some pandemic markers and genes belonging to the

secretion system along with *tdh*, *trh* (Caburlotto et al., 2009, Caburlotto et al., 2010). Keeping this backdrop in mind, the virulent genes under T3SS1 (*vcrD1*, *vopD*, *vp1680*) and T3SS2 (*vcrD2*, *vopB*, *vopD2*, *vopC*) were tested to understand distribution of these virulent genes within the environmental isolates. The presence of *vcrD1*, *vopD* and *vp1680* genes in all the isolates is an indication that these genes are well conserved in *V. parahaemolyticus*. The presences of these genes were also reported in both environmental and clinical isolates (Noriea et al., 2010 and Tey et al., 2015). We found that the presence of *tdh* and *trh* gene in the environmental isolates were quite less 3 % and 8 %, respectively. All the *tdh* positive *V.parahaemolyticus* showed the presence of virulent genes under T3SS2 as reported by other workers (Noriea et al., 2010 and Jones et al., 2012). However, all isolates containing *trh* in this study were negative for the entire virulent gene under T3SS2 that have been included in the present study as reported by (Noriea et al., 2010 and Jones et al., 2012). Noria et al. (2010) found all the environmental isolates were negative for *vopB* gene contrary to this report Paranjpye et al. (2013) found the presence of the *vopB* gene in all the isolates. The absence of *vopB* gene also reported in the *tdh* / *trh* positive stains (Noria et al., 2010). All the isolates showed negative for AHPND, however positive for other virulence factor genes used in the present study.

5.5 Conclusion

The dataset presented here is a part of the study on the distribution of virulence genes under the T3SS of *V. parahaemolyticus* isolated from environmental samples in India. The result of the present study also supported the fact that T3SS1 is well conserved in all *V. parahaemolyticus* strains. But, the genes under T3SS2 is sporadic that it is

present only in *tdh* positive *V. parahaemolyticus*. The *tdh* and *trh* positive bacteria strains of *V. parahaemolyticus*, hemolysis human RBCs quite faster in comparison to *tlh* positive strain. Both the *tdh* and *trh* positive isolates showed high cytotoxicity in HEK cell line in comparison to *tlh* positive strain. The experimental challenge in *L. rohita* confirmed the pathogenic potential of *tdh* and *trh* positive strain of *V . parahaemolyticus.* The presence of virulent genes under T3SS2 in *tdh* negative stains reconfirms the statement of acquisition of virulent genes in aquatic environment by horizontal gene transfer which might be leading to the emergence of new pathogenic strain in the environment, is a serious concern for human health.