Chapter 4

Isolation and identification of *Vibrio parahaemolyticus*

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4.1 Introduction

Vibrio parahaemolyticus is a zoonotic pathogen causes gastroenteritis in human and vibriosis in fish and shellfish. Shellfish are cultured in brackish or saltwater environment which is also the natural habitat for *V. parahaemolyticus* and other halophilic bacteria as well. Previous studies carried out by different workers have found that, *V. parahaemolyticus* is present in high frequency in environmental samples like water, sediment, fish and shellfish (Al-Othrubi et al., 2011 Khouadja et al., 2013; Yano et al., 2014; Lopatek et al., 2015) from different parts of world. Vibriosis is one of the major disease problems in aquaculture caused by *Vibrio* spp. (Chatterjee and Haldar, 2012). *V. parahaemolyticus* is one of the twelve known pathogenic *Vibrio* spp. species that is involved with vibriosis in finfish, shellfish and marine invertebrates. (Gopal et al., 2005; Khouadja et al., 2013 and Peng et al., 2016). *V. parahaemolyticus* is responsible for exophthalmia, ulcers, septicemias and corneal opaqueness in fish (Egidus, 1987) and Acute Hepatopancreatic Necrosis Disease (AHPND) in shrimp (Tran et al., 2013). Vibriosis is a common bacterial disease in finfish and shellfish which causes great economic loss in aquaculture industries. Shrimp is the most important brackish-water aquaculture species in India and mainly cultured in the coastal area of Andhra Pradesh, Tamil Nadu, Orissa and West Bengal in India. West Bengal and Andhra Pradesh is the largest producer of shrimp in India. The presence of this bacterium was also reported in shrimp, oyster from India by different workers (Deepanjali et al., 2005; Raghunath et al., 2008). Most of the environmental strain of *V. parahaemolyticus* is a nonpathogenic strain. The thermostable direct hemolysin (Tdh) encoded by *tdh* gene and thermostable

direct hemolysin-related hemolysin (Trh) synthesized by *trh* gene is the most important virulence factors in *V. parahaemolyticus* (Raghunath et al., 2015). The presence of *tdh*, *trh* or both the genes was considered as a pathogenic strain (Abbott et al., 1989).

Isolation of pure colonies of bacteria from environmental samples is a primary requirement of any bacteriological study. Different isolates of same species showing variation in the different biochemical tests such as sugar fermentation which makes it difficult for identification. In the present study, *V. parahaemolyticus* isolated from the environmental samples were identified using biochemical and molecular techniques.

4.2 Material and Methods

4.2.1 Sample collection

A total of 350 shrimp (*Litopenaeus vannamei*) samples were collected from aquaculture farms situated in 3 different states *viz.* Andhra Pradesh, West Bengal and Gujarat of India. The sampling was carried out in the months from February to October during 2014-2017. 150 samples were collected from three different districts *viz.* East Midnapur, North 24 Parganas and South 24 Parganas of West Bengal. 100 samples were collected from three different districts *viz*. East Godavari, Bhimavaram and Nellore of Andhra Pradesh. 100 samples were collected from three different districts *viz.* Surat, Navsari and Valsad of Gujarat. The samples were collected and stored in ice-cold condition for transportation to laboratory. Hepatopancreas and hemolymph were transferred aseptically into the tube containing sterile Alkaline Peptone Water (APW) and incubated at 37 °C for 24 hrs.

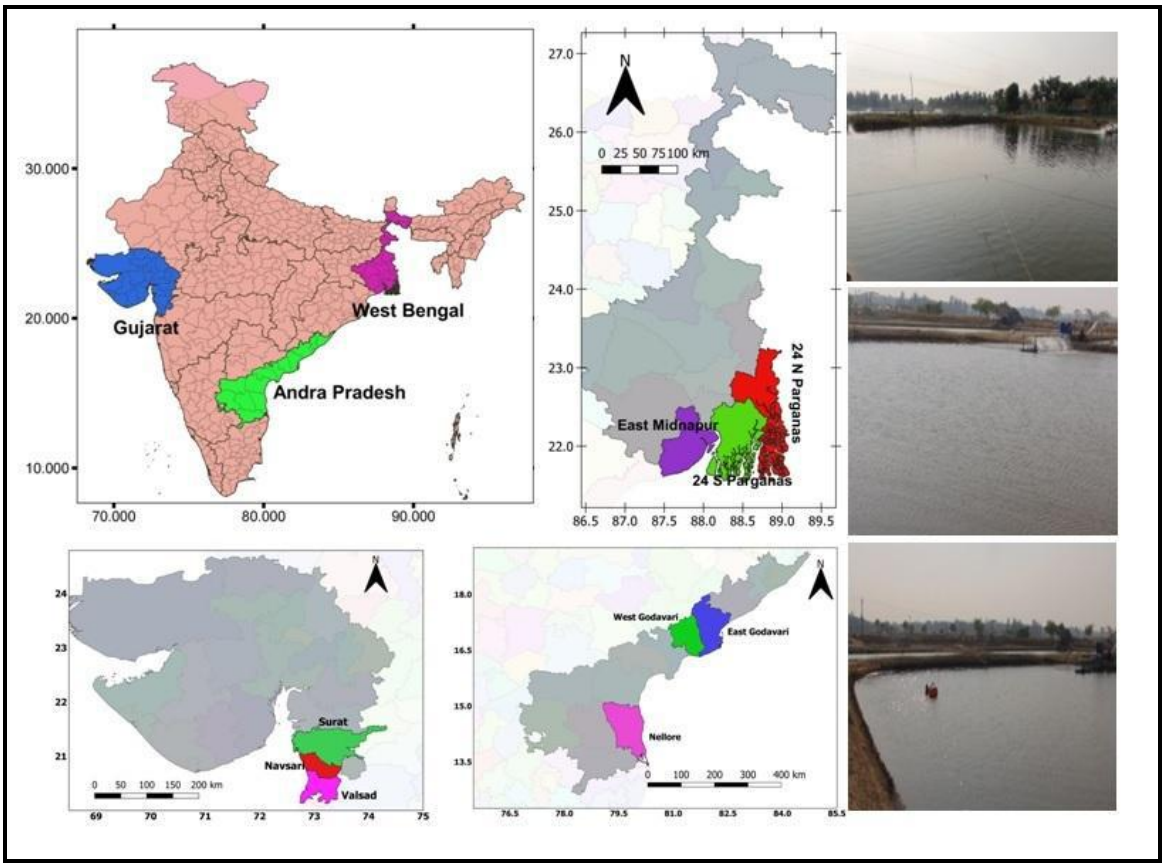

Figure 4.1 Map showing the geographical locations of sampling sites in the states of West Bengal Andhra Pradesh and Gujarat. Different color (Pink, Red and Green) within different states represented the different district from where sample had been collected

4.2.2 Bacterial isolates and cultivation

After 24 hrs of incubation, 1 ml of APW-enriched culture was serially diluted in autoclaved Phosphate-buffered saline (PBS) up to 10^{-4}. 100 µl of diluted bacterial culture were spread on Thiosulphate Citrate Bile salts Sucrose (TCBS) agar plate and were incubated at 37 °C for 24 hrs. *V. parahaemolyticus* colonies appeared as green or blue-green colonies were presumptively selected and then transferred aseptically to HiCrome Vibrio Agar (M1682, Himedia). *V. parahaemolyticus* appeared as a bluish-green color colony on the agar plates after incubation at 37 °C for 24 hrs. For further confirmation,

bluish-green colonies that appeared in the plate after incubation at 37 °C for 24 hrs were again transferred into CHROMagar Vibrio (CHROMagar Microbiology, Paris, France). Ultimately, the single pure colony was inoculated into sterile Tryptic Soya Broth (TSB) containing 2 % NaCl and maintained as glycerol stock at -20 °C.

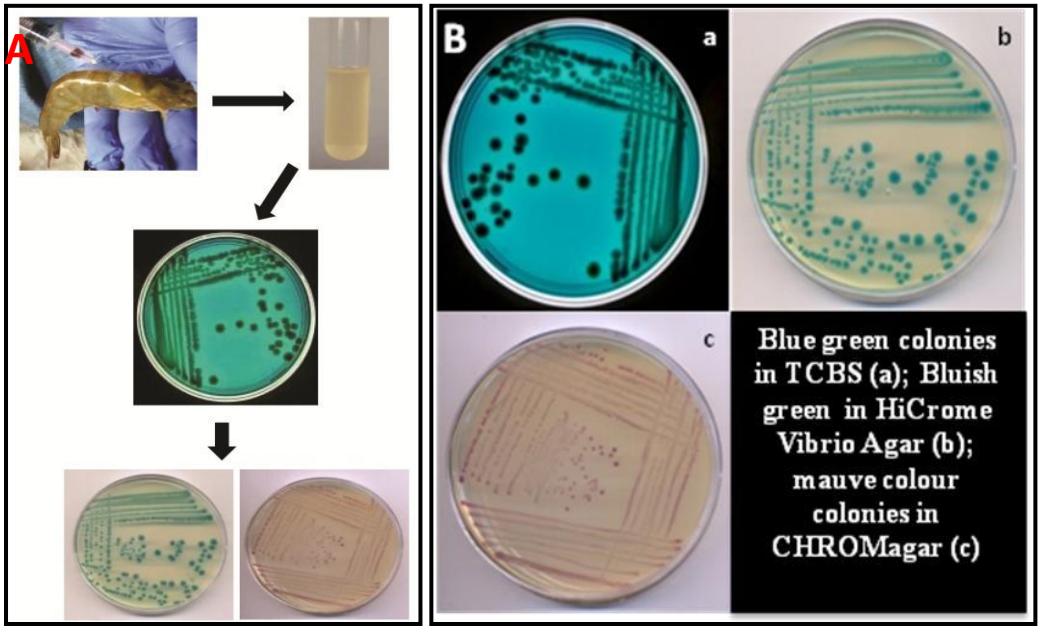

Figure 4.2. Schematic diagram of bacteria culture from shrimp and plating in TCBS plate (A). *Vibrio parahaemolyticus* stick in different media and different color colonies appear in different media (B).

4.2.3 Biochemical characterization

The isolates were primarily characterized by Gram-straining, urease, nitrate reduction, Voges Proskauer's (VP), lysine utilization, ONPG (β-galactosidase), malonate utilization, rhamnose, cellobiose, esculin hydrolysis, arabinose, xylose, adonitol, melibiose, saccharose, raffinose, trehalose, glucose, phenylalanine deamination, H_2S production, lactose oxidase, ornithine utilization, citrate utilization, methyl red and indole test were carried out (KB-003, HiMedia).

4.2.4 Hemolytic activity

Kanagawa test was carried out using Wagatsuma agar supplemented with 5 % human red blood cell. An isolated strain was strick in each plate and incubated for 24 hrs at 37 °C (Wagatsuma et al., 1968). The β hemolysis zone surrounding the bacterial growth on the blood agar plate, is an indication of positive reactions, were recorded.

4.2.5 Genomic DNA isolation

The pure culture of *V. parahaemolyticus* was grown in TSB containing 2 % NaCl under the aerobic condition at 37 °C overnight. 1 ml of culture was centrifugation at 5000 rpm for 5 min and the cells were pelleted down. The bacterial pellet was washed with autoclaved distilled water twice. The genomic DNA was extracted from isolated bacteria following the Sarkosyl method (Sambrook and Russel, 2001). The cells were resuspended in Tris-EDTA buffer (Sigma) and 40 µl of lysozyme (10 mg/ml). Again 10 µl of proteinase K (10 mg/ml) was added in the same tube and incubated at 37 °C for overnight. 80 µl Sarkosyl (10 %) was added and incubated at 65 °C for two hrs. DNA was extracted using Tris-saturated phenol (Sigma) and chloroform (Sigma). Finally, DNA was pellet down using ice-cold ethanol.The DNA concentrations were measured spectrophotometrically. The DNA quality was checked by ethidium bromide staining in 1 % agarose gel.

4.2.6 Molecular identification by 16S rRNA gene

16S rRNA gene was amplified using specific primers for the 16S rRNA gene with the thermal cycler Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA). The primers used for the amplification of the 16S rRNA gene were UFF2 5′-

GTTGATCATGGCTCAG-3′ as the forward primer and URF2 5′-GGTTCACTTGTTACGACTT-3′ as a reverse primer (Kumar et al., 2014). The PCR reaction mixture was prepared by mixing the 5 µl of 10X PCR buffer, 2 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP (Sigma, USA) 2 µl of 5 pmol of each primer, 1U Taq DNA polymerase (Sigma, USA) and 100 ng of isolated genomic DNA. The total volume of the reaction mixture will be 50 µl. The PCR program for amplification is denaturation for 2 min at 95 °C followed by denaturation at 94 °C for 30 s, annealing at 52 °C for 45 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.

4.2.7 Molecular identification by *toxR* gene

The thermal cycler Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA) was used for amplification of the *tox*R gene using specific primers. The primers used for the amplification of *toxR* gene were 5′-GTCTTCTGACGCAATCGTTG-3′ as the forward primer and 5′-ATACGAGTGGTTGCTGTCATG-3′ as a reverse primer (Kim et al., 1999). The PCR reaction mixture was prepared by mixing the 5 µl of 10X PCR buffer, 2 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP (Sigma, USA) 2 µl of 5 pmol of each primer, 1U Taq DNA polymerase (Sigma, USA) and 100 ng of isolated genomic DNA. The total volume of the reaction mixture will be 50 µl. The PCR program for amplification is denaturation for 2 min at 95 °C followed by denaturation at 94 °C for 30 s, annealing at 52 °C for 45 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.

4.2.8 Gene sequencing

ABI 3730xl capillary sequencer (Applied Biosystems, Foster City, CA) was used for sequencing of the amplified gene product in forward and reverse directions (Behera et al., 2017). 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 16S rRNA sequences of 1414 bp and *toxR* gene sequences of 368 bp were then compared in GenBank using the NCBI–BLAST program facility (http://www.ncbi.nlm.nih.gov\BLAST) with available sequences in the GenBank.

4.3 Results

4.3.1 Isolation of bacteria

A total of 350 shrimp samples (150 from West Bengal, 100 from Andhra and 100 from Gujarat) were collected from shrimp farms in three major shrimp producing states of India and were screened for *V. parahaemolyticus* using 3 different media. A total of 183 isolates (71 from West Bengal, 61 from Andhra Pradesh and 51 from Gujarat) were identified as *V. parahaemolyticus* and the highest prevalence of *V. parahaemolyticus* was recorded in the state of Andhra Pradesh followed by Gujarat and West Bengal.

Table 4.1 *V. parahaemolyticus* isolates from the aquaculture environments of India and the accession number of 16S rRNA gene submitted to the NCBI Genbank

Sampling Sites	Number of Sample collected	Number of Isolates	NCBI Accession Number
West Bengal (East Midnapur, North 24 Paraganas and South 24 Paraganas)	150	71	MG188672, MG762012, MG190871-MG190873, MG383934-MG383936, MG525090-MG525119 MG548337-MG548364
Andhra Pradesh (East Godavori, West Godavari and Nelore)	100	61	MG564725-MG564754, MG575435-MG575461
Gujarat (Surat, Navsari and Valsad)	100	51	MG593201-MG593230, MG970576-MG970596

4.3.2 Biochemical characterization

The bacterial strains with three different hemolysin genes were used for the biochemical characterization. The bacterial strains [S24P132 (*tdh* positive), AP429 (*trh* positive) and SPEM2 (*tlh*)] were found to be gram-negative and were positive for lysine, ornithine, urease, nitrate, arabinose, sorbitol, mannitol, sucrose tests. These strains showed negative for phenylalanine deamination, H_2S, VP, esculin hydrolase, xylose, adonitol, cellobiose, melibiose, saccharose, raffinose and lactose tests (Table 2). Variation in the result of biochemical test *viz*. adonitol, rhamnose, lactose, citrate, malonate, methyl red, ONPG, indole, and glucose were observed among the three different strains.

Table 4.2 Comparative biochemical analysis of three different isolates S24P132, AP429 and SPEM2 with published biochemical data of *V. parahaemolyticus*

Biochemical Test	***V. parahaemolytis* (S24P132)**	***V. parahaemolyticus* (AP429)**	***V. parahaemolyticus* (SPEM2)**	[*] ***V. parahaemolyticus***
ONPG (β-galactosidase)	+	-	-	V
Lysine	+	+	+	+
Ornithine	+	+	+	+
Urease	+	+	+	V
Phenylalanine deamination	-	-	-	
Nitrate	+	+	+	
H_2S	-	-	-	-
Citrate	+	-	+	-
V.P	-	-	-	-
Methyl red	-	-	+	
Indole	-	-	+	+
Oxidase	+	+	+	+
Catalase	+	+	+	
Malonate	+	-	+	
Esculin hydrolase	-	-	-	-
Anabinose	+	+	+	-
Xylose	-	-	-	-
Adonitol	-	-	-	-
Rhamnose	+	-	-	-
Cellobiose	-	-	-	ND
Mellibiose	-	-	-	ND
Saccharose	-	-	-	ND
Raffinose	-	-	-	ND
Trehalose		+	-	ND
Glucose	+	+	-	+
Lactose	-	-	-	ND
Sorbitol	+	+	+	-
Mannitol	+	+	+	+
Sucrose	+	+	-	+

[*]Phenotypic characteristics of *V. parahaemolyticus* observed earlier as described in Jones et al., 2012"+": positive; "−": negative, "ND" Not found

4.3.3 Hemolytic activity

Only six strains showed hemolytic activity in Wagatsuma agar (KP), which is the characteristic phenotype of Tdh positive *V. parahaemolyticus*. The Trh phenotype was not identified in the solid phase hemolysin test. The presence of the *tdh* and *trh* genes in the bacteria is considered as a pathogenic strain of *V. parahaemolyticus*.

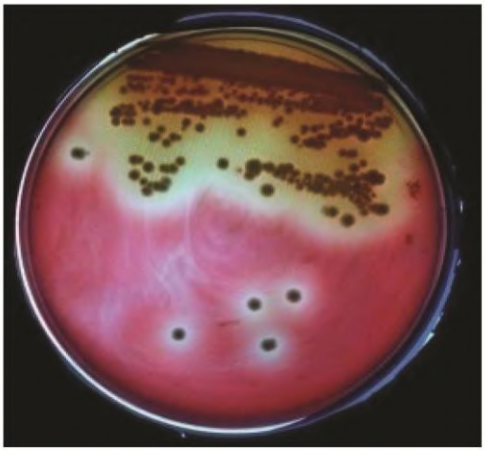

Figure 4.3 Solid Phase hemolysin assay using 5 % human blood

4.3.4 Molecular identification by 16S rRNA gene

1500 bp of PCR amplified 16S rRNA gene of all isolated bacteria was sequenced in both directions. The contig was prepared using both forward and reverse sequence. Further, the contig was examined by using NCBI-BLAST program. The BLAST results revealed that the 16S rRNA gene sequences of all the isolated bacteria showed 99 % identity with 16S rRNA gene sequences of *V. parahaemolyticus* in the NCBI database. All the 183 sequences of the 16S rRNA gene of *V. parahaemolyticus* were submitted to GenBank and the accession numbers are provided in Table 4.1.

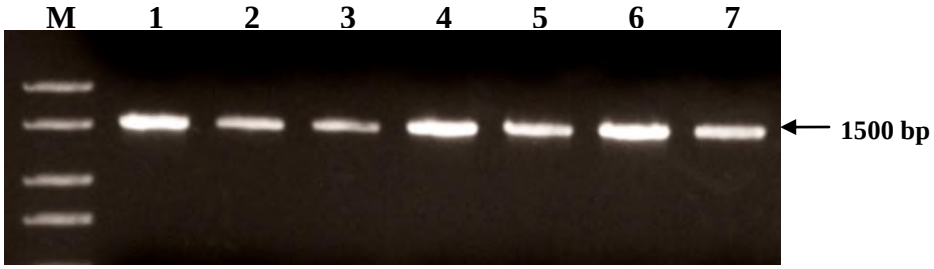

Figure 4.4 PCR amplified products of 16S rRNA gene of *V. parahaemolyticus.* Lane 1-7: Isolated *V. parahaemolyticus*; Lane M: Molecular weight marker (500 bp DNA Ladder).

4.3.5 Molecular identification by *toxR*

For further confirmation, the molecular identification of all the isolated bacteria was carried out by using another marker gene *toxR*. All the isolates were positive for *toxR* amplifying 368 bp fragments. The forward and reverse sequence was aligned by using DNA baser and the contig was analyzed by using NCBI-BLAST program. The sequence of the amplified product showed 100% identity with the *toxR* gene of *V. parahaemolyticus*, previously submitted in the NCBI database.

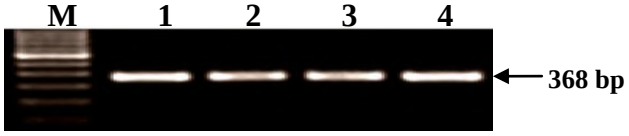

Figure 4.5 PCR amplified products of *toxR* of *V. parahaemolyticus.* Lane 1-4 Isolated *V. parahaemolyticus*; Lane M: Molecular weight marker (100 bp DNA Ladder).

4.4 Discussion

Vibrio parahaemolyticus is an important bacterium under *Vibrio* sp. responsible for the gastrointestinal infection in different parts of the world (Ghenem et al., 2017) and widely distributed in aquatic environments which include both pathogenic and

nonpathogenic strains (Julie et al., 2010). In the present study, all the bacterial strain isolated from aquaculture farms were identified as *V. parahaemolyticus* using three different selective media after 24 hrs of enrichment in APW. Alkaline pH of this media allows the growth of *Vibrio* sp. and inhibits the growth of other bacteria. A wide range of enrichment media was formulated for *Vibrio* sp. Alkaline peptone water (APW) was recommended as an enrichment medium for *V. parahaemolyticus* alike other media such as ST broth, Glucose Teepol (or sodium dodecyl sulphate) Salt Broth (GTSB) and Salt Polymyxin Broth (SPB) (Donovan and van Netten, 1995).

TCBS Agar developed by Kobayashi et al. (1963), was widely used for the isolation of *V. cholerae* and *V. parahaemolyticus* (Hara-Kudo et al., 2001) as most *vibrio* species grow in this media (Ottaviani et al., 2003). *V. parahaemolyticus* produces blue-green colonies in the TCBS Agar same as that of *V. mimicus* and *V. vulnificus* (Hara-Kudo et al., 2001). Non-*Vibrio* sp. like *Pseudomonas* sp, *Proteus mirabilis*, *Providencia rettgeri* and *Aeromonas* may also form blue-green colonies on TCBS Agar (MacFaddin, 1985). To avoid such condition, two different media had been used for the isolation of *V. parahaemolyticus*. HiCrome™ Vibrio Agar has been developed for identification of *Vibrio cholerae* and *V. parahaemolyticus* based on chromogenic differentiation and *V. parahaemolyticus* produce bluish-green colonies after 24 hrs of incubation at 37 °C. Recently, a chromogenic agar media (CHROMagar Vibrio, Paris, France) has been developed to distinguish *V. parahaemolyticus* from other *Vibrio* sp. *Vibrio cholera*, *V. mimicus*, *V. vulnificus* formed pale-blue whereas *V. alginolyticus* formed milk-white colonies on CHROMagar were as *V. parahaemolyticus* formed a mauve color colony.

Most of the biochemical test results of *V. parahaemolyticus* were found to be similar to the earlier workers (Shyne Anand et al., 2008; Jones et al., 2012). Slight variation in some of the biochemical tests like indol, sorbitol, glucose and rhamnose was observed in the present study. The variation in the biochemical test may be due to different geographical locations. Jones et al. (2012) also found variations in two biochemical tests like ONPG (β-galactosidase) and urease in *V. parahaemolyticus*.

The *toxR* is a regulatory gene of toxin operon and was 1st time identified in *Vibrio cholera*. *toxR* gene was identified in different *Vibrio* sp. like *V. cholera*, *V. parahaemolyticus* and *V. anguillarum* and is well conserved in *Vibrio* sp. (Kita-Tsukamoto et al., 1993; Lin et al., 1993). The identity between *toxR* gene of *V. parahaemolyticus* and *V. cholera* is much lower than the 16S rRNA gene sequence of both the species. The *toxR* gene is widely used by many workers for the identification of *V. parahaemolyticus* (Kim et al., 1999; Lo et al., 2008). The presence of the *toxR* gene in all the isolates reconfirms that it will be used as a marker to identify *V. parahaemolyticus*. For further conformation, 16S rRNA gene sequence analysis of all the isolates was performed.

16S rRNA gene is commonly used for identification and taxonomic classification of microbes (Drancourt et al., 2000; Kolbert and Persing, 1999). 16S rRNA gene sequence is approximately 1500 base pairs (bp) which include highly conserved and nine hypervariable regions (V1 – V9) (Patel et al., 2010). The presence of this gene in all the bacteria and the functional *conserverdness* of this gene over time have made it a dependable genetic marker for the identification of bacteria. Some bacterial genus like *Streptococcus* showed high sequence similarities in 16S rRNA gene difficult for

appropriate identification (Kalia et al., 2016). The comparison of 16S rRNA gene sequences of unknown strain is widely used for their identification. 95 %-98.65 % identity is recommended as a cutoff for identifying the new genus or species (Beye et al., 2018). 16S rRNA sequence similarity analysis between *V. parahaemolyticus* and other *Vibrio* sp. like *V. fluvialis*, *V. furnissii*, *V. hollisae*, *V. diazotrophicus*, *L. anguillarum* and *V. vulnijicus* showed 97.43 %- 92.47 % sequence similarity (Aznar et al., 1994). All the isolates showed 99-100 % sequence identity with 16S rRNA sequence of *V. parahaemolyticus* in the NCBI database.

2.5 Conclusion

V. parahaemolyticus was isolated from shrimp farms of India and was further confirmed using a biochemical and molecular technique like 16S rRNA gene and *toxR* gene as marker. The presence of *V. parahaemolyticus* was prevalent in brackish water culture system in India. Furthermore, *tdh* and *trh* positive strains *V. parahaemolyticus* were isolated from the environmental samples which are considered as a major virulent factor. The presence of hemolytic strain in the environmental sample increases the risk of the accidental spread of disease in aquatic animals as well as in human.