# Molecular identification and characterization of *Vibrio parahaemolyticus* causing Acute Hepatopancreatic Necrosis Disease in cultured Shrimp

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Master of Science (MS) in Fisheries

**Submitted By**

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**Dhaka-1000, Bangladesh**

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# Declaration

I hereby declare that the dissertation entitled **"Molecular identification and characterization of *Vibrio parahaemolyticus* causing Acute Hepatopancreatic Necrosis Disease in cultured Shrimp"**submitted to the Department of Fisheries, University of Dhaka for the degree of Masters of Science (MS) is based on self-investigation, carried out under the supervision of **Dr Mohammad Shamsur Rahman,** Department of Fisheries and **Dr Mihir Lal Saha,** Department of Botany**,** University of Dhaka, Dhaka-1000, Bangladesh.

I also declare that this or any part of this work has not been submitted for any other degree anywhere. All sources of knowledge used have been duly acknowledged.

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# Certificate

This is to certify that the research work embodied in this thesis entitled **"Molecular identification and characterization of *Vibrio parahaemolyticus* causing Acute Hepatopancreatic Necrosis Disease in cultured Shrimp"**submitted by **Md. Mostavi Enan Eshik**(Examination Roll No. Curzon 808, Reg. No. 2011-512-765, Session. 2015-2016) was carried out under our supervision. This is further to certify that it is an original work and suitable for the partial fulfillment of the degree of Master of Science (MS) in Fisheries from the Department of Fisheries, University of Dhaka.

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*Acknowledgements*

# Abstract

Acute Hepatopancreatic Necrosis Disease (AHPND), also called Early Mortality Syndrome (EMS), is a recently emergent shrimp bacterial disease caused by strains of *Vibrio parahaemolyticus* that contain a unique virulent plasmid, resulted in substantial economic losses since 2009.AHPND has caused severe mortalities up to100% in farmed populations of marine shrimp *Penaeus monodon*. The purpose of this thesis work was to identify and characterize the pathogenic strain of *V. parahaemolyticus* causing EMS in cultured shrimp using classical and molecular techniques.

To conduct this work, shrimp samples were collected from three different locations of south-west shrimp farming region of Bangladesh *viz.*Sadar Upazilla of Satkhira district, Mongla and Morrelganj Upazilla under Bagerhat district. The shrimp samples were processed for microbial load count and to isolate*V. parahaemolyticus* strains. Four types of bacteriological culture media were used to enumerate the Total Bacterial count (TBC), Total *Vibrio* count (TVC). Besides Classical microbiology, molecular approaches (16S rRNAgene sequencing, pathogenic gene PCR using AP3 and AP4 primers) wereperformed to identify the pathogenic strains of *V. parahaemolyticus* causing AHPND in cultured shrimp.

Shrimp suffering from AHPND showed significantly atrophy of hepatopancreas (HP), pale to white hepatopancreas due to pigment loss in connective tissue capsules and guts with discontinuous or no contents. In this study, TBC, TVC on TCBS and on HiChrome showed little variation of AHPND affected shrimp. TBC was found highest($6.37 \times 10^8$cfu/g) in shrimp of Satkhira Sadar Upazilla, whereas highest number of Total *Vibrio* on TCBS agar ($2.40 \times 10^7$ cfu/g) was found in shrimp of Mongla Upazilla.

In this study, among 46 isolates, representative eighteen isolates were checked for the species-specificdetection of *V. parahaemolyticus* using *ldh* primers; and *tdh*primerswere used for the detection of human pathogenicity of *V. parahaemolyticus*. Detection of *ldh* gene fragment in the isolates showed positive result for *ldh*but isolates were negative for human toxigenic gene *tdh*. The representative isolates were also subjected to 16S rRNA gene sequencing and were identified as *V. parahaemolyticus*. Multiple sequence alignment was performed to find out the polymorphic sites among the sequenced strains with considering 1386 bp nucleotides where 1.15 % dissimilarities were observed.Six strains (Vp2, Vp4, Vp21, Vp27, Vp35 and Vp39) were highly similar to each other but

dissimilar to only two sites (34, 35 bp) on their polymorphic region. Phylogenetic analysis also confirmed the taxonomic positionof the isolates as *V. parahaemolyticus*.

On the other hand, 18 *V. parahaemolyticus* isolates were further characterized to check the AHPND positivity using AP3 and AP4 primer. Twelve isolates showed positive result for AP3 and fourteen isolates for AP4 primer that indicate those isolates were AHPND positive which caused EMS in cultured shrimp.This study also reported that all the representative strainsof AHPND positive *V. parahaemolyticus*were resistant to Gentamycin whereas all the strain showed sensitivity to Chloramphenicol, Nalidixic Acid,Nitrofurantion and Tetracycline.

This report also agreed with the mortalities of shrimp that occurred within 30 days after stocking in shrimp farms of three different south-west regions of Bangladesh which caused by AHPND positive *V. parahaemolyticus*;and to the best of my knowledge, this is the first report of this shrimp pathogen in Bangladesh.

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# Chapter 1

# Introduction

## 1.1 Background

The shrimp aquaculture industry is rapidly expanding and accounts for 15% of the internationally traded seafood products (FAO, 2013). Farmed shrimp account for 57% of the total shrimp consumed globally (7.9 million tons), with white leg shrimp *Penaeusvannamei*(Boone 1931) being the main cultured species (74% of total cultured shrimp) (FAO,2013). The major producers are China, Vietnam, Thailand and Bangladesh (FAO,2013).Although global shrimp production has increased from approximately one million tons in 2000 to four million tons in 2011, the 2015 level is expected to be 15% below that of 2011 due to disease outbreaks causing significant production losses (FAO, 2015). With intensification of penaeid aquaculture industry, occurrence of diseases has increased and is a major constraint to the shrimp aquaculture resulting in significant socio-economic losses in affected areas. Diseases of shrimp have contributed to billions of dollars of economic loss in the aquaculture industry.

Asian shrimp farming industry has experienced massive production losses due to a disease caused by toxins of *Vibrio* bacteria, known as early mortality syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND) for the last 5 years (Boonyawiwat et al., 2016). It is estimated that approximately 60% of disease losses in shrimp aquaculture have been caused by viral pathogens and 20% by bacterial pathogens. Vibrio species are the most frequent bacterial pathogens detected in the cultivated shrimp (Zhou et al., 2012). Tran et al. (2013) showed that certain strains of *V. parahaemolyticus* belonging to the Harveyi clade are responsible for pathological changes in the hepatopancreas of EMS/AHPND affected animals and are negative for genes encoding the thermostable direct hemolysin (TDH) and TDH-related hemolysin (TRH) that are associated with human pathogenic strains. As aquatic animals, shrimps are quite different from terrestrial one and the factors accounting for AHPND may be varied and complex. Biological factors, such as toxic algae, bacteria, viruses, and parasites, and water chemical factors, such as nitrite and ammonia, can cause the same clinical signs. Secondary viral infections, such as YHV, can also lead to AHPND pathology. Some cases of early mortality were related to environment factors, such as nitrite, ammonia, salinity, and pH. The acute toxicity of nitrite in*P. vannamei* juveniles was demonstrated to be related to salinity. When exposed to higher salinity, the LC50 values of nitrite on *P. vannamei*

were higher (Chen, 1990). The toxicity of ammonia in *P. vannamei* is similar to that of nitrite (Chen, 1988). The early mortality observed under high concentrations of nitrite and ammonia shows similar clinical signs to covert mortality (Zhang et al., 2004).

Both pathogens and environmental factors cause clinical signs including atrophic hepatopancreas, empty stomach and gut, soft shell, and slow growth, which are similar to AHPND to some degree; however, when compared with D.V. Lightner's definition described above, it is clear that none of them are the cause of AHPND. *V.parahaemolyticus* is a halophilic Gram-negative bacterium that is a normal inhabitant of marine environments. Most of the strains of *V. parahaemolyticus* isolated from the marine environments are non-pathogenic strains. A few human pathogenic strains are detected in the environments, but they are mostly isolated from clinical samples and harbour a thermo stable direct haemolysin (TDH) or the TDH related haemolysin (TRH) encoded by the *tdh* or *trh* genes, respectively (Vuddhakul et al., 2000). However, any association of these genes with the *V. parahaemolyticus* isolates causing AHPND has not been detected. Professor Lightner from the University of Arizona who described a unique histopathology of shrimp (both*P. monodon* and *P. vannamei*) taken from many cultivation ponds reported early mortality in China and Vietnam (Lightner et al., 2012; NACA, 2014).

The histopathology consisted of massive sloughing of hepatopancreatic tubule epithelial cells beginning in the center of the hepatopancreas and progressing outward to the embryonic (E-cell) region and is called acute hepatopancreatic necrosis syndrome (AHPNS). This sloughing occurred in the absence of any associated pathogen that might be the cause. The sloughing is preceded by a lack of differentiation in the tubule epithelial cells and hemocyte aggregation in the HP prior to sloughing, and it is followed by hepatopancreatic, bacterial septicemia by secondary bacterial invasion. Lightner's case definition of AHPNS was adopted by a meeting of specialists in Bangkok in August 2012 (NACA, 2012) and is available as a disease card at the website of the Network of Aquaculture Centre's in Asia-Pacific (NACA) (NACA, 2014). In the first NACA report, the early stage of AHPNS was characterized by lack of HP tubule epithelial cell differentiation and the late stage was characterized by bacterial sepsis of the HP.

Soto-Rodriguez et al.(2015) identified bacterial isolates from shrimp hepatopancreas as *Vibrio parahaemolyticus* by the presence of the *tlh* gene. The *tlh* positive *V. parahaemolyticus* strains were further characterized by repetitive extragenic palindromic

element-PCR and primers AP1, AP2, AP3 and AP and an ems2IQ2000 detection kit were used in the diagnostics test for AHPND and found that the different *V. parahaemolyticus* strains have different virulence's; some of the less virulent strains do not induce 100% mortality and mortality rates also rise more slowly than they do for the more virulent strains. Kongrueng et al.(2014) isolated *V. parahaemolyticus* from shrimp of five farms located in the pattani and songkhla provinces of southern Thailand and used a PCR method targeted to the unique DNA sequences derived from the plasmid (AP2 primer) and the toxin gene (AP3 primer) and found that a total 33 of 108 isolates were AHPND positive. Joshi et al. (2014) concluded that isolates of *V. parahaemolyticus*that may cause EMS in shrimp and histological examination of the collected shrimp confirmed the presence of histopathological characteristics of AHPND. AHPND Positive shrimp or feeds should be suitably destroyed to prevent dispersal of the target pathogen andAP3 method gave 100% specificity and sensitivity for detection of *V. parahaemolyticus* responsible for AHPNDP (Soto-Rodriguezet al., 2015). Thitamadee et al. (2016) identified that as unique isolates of *V. parahaemolyticus* (VPAHPND) that colonized the shrimp Stomach was causative agent of AHPNS and founding that a unique plasmid (pVA1) of approximately 69 kbp was present in all of the VP AHPND isolates but not in non-AHPND isolates of *V. parahaemolyticus.* Nunan et al. (2014) concluded that bacterial plaques within the stomach chambersin some of the affected shrimp and also distinguished between pathogenic AHPND causing *V. parahaemolyticus* isolates and non-pathogenic isolates by PCR assay. Kumar et al.(2014) demonstrated that *V. parahaemolyticus* strains are probably opportunistic pathogen of immunocompromised cultured *L. vannamei* under unfavorable environmental conditions and also observed no external parasites in the moribund and healthy shrimps on microscopic examination. Han et al. (2015) showed that low quantities of pathogenic bacteria, could act as vectors capable of spreading the disease. Soto -Rodriguez et al.(2015) Identified three stages of AHPND (initial , acute and terminal) in affected shrimp using histological analysis and bacterial density count and also showed that a low presence of bacteria in the hem lymph (HL) and HP and a high load of *Vibrio* bacteria in the stomach indicating by microbiological analysis. Lai et al.(2015) found that AHPND-causing strains of *V. parahaemolyticus* secrete the plasmid encoded binary toxin PirABVp into the culture medium and also showed that while some antibiotics, such as Ciprofloxacin, Oxyfloxacin, still seem to be universally effective, there also seem to be universal resistant to ABPC, SM, SMZ, FOM and BCM. The presence of virulence

factors is associated with the pathogenesis of *V. parahaemolyticus*. Studies using *tdh* or *trh* or both gene probes on *V. parahaemolyticus* strains showed a strong correlation between clinically significant strains and presence of either of these genes, implying that both *tdh* and *trh* genes are virulent factors in *V. parahaemolyticus* (Shirai et al., 1990).

Acute hepatopancreatic necrosis disease (AHPND) has subsequently been proposed as a novel disease criterion, based on the specific histopathology of affected shrimp hepatopancreas (HP) to differentiate from other EMS cases (NACA, 2012; FAO, 2013; Tran et al., 2013). AHPND-suspected shrimps show gross signs of abnormal HP with significant atrophy and discoloration when compared with the normal shrimps (NACA, 2012). The histopathology of AHPND is characterized by massive cell sloughing of HP tubule epithelial cells together with the dysfunction of B, F, R and E cells of HP of affected shrimps (Flegel, 2012; NACA, 2012; Tran et al,. 2013; Joshi et al., 2014). The causative agent of AHPND in Thai and Vietnamese cultivated shrimps is identified as *V. parahaemolyticus* (Tran et al., 2013; Joshi et al., 2014).

**1.2 *Penaeus monodon*, the study specimen**



**Fig1.1.Photograph showing Black Tiger Shrimp (*Penaeus monodon*) collected from shrimp Gher of Satkhira Sadar Upazilla.**

The giant tiger shrimp, *P. monodon*, the largest and most commercially important species among penaeid reaching 270 mm in body length or 260 g in weight, is suitable for culture in ponds and offers high market price. Recently in Southeast Asian countries, enthusiasm for natural and artificial propagation of both fry and adult giant tiger prawn has been growing rapidly among government and private agriculturists due to strong demand with higher prices in the national and international markets. On the other hand, their habitats such as shore areas and mangrove waters are under destruction in several areas. *P. monodon* is distributed from 30°E to 155°E longitude and from 35°N to 35°S latitude. However, the main fishing grounds are mostly located in tropical countries, particularly in Indonesia, Malaysia and the Philippines. The fry, juveniles and adolescents inhabit surface waters such as shore areas and mangrove estuaries, while most of the adults inhabit waters down to about 160 m.

**1.3 Early Mortality Syndrome (EMS) of Shrimp**



**Fig1.2.Geographical distribution of (EMS/AHPND) in the world**

**(source:Zorriehzahra and Banaederakhshan, 2015)**

 By comparison, losses to fungi and parasites have been relatively small. For bacterial pathogens, *Vibrio* species are the most important while for viral pathogens importance has been changed since 2003 when domesticated and genetically selected stocks of the American white leg shrimp *L. vannamei* (Boone 1931) replaced the formerly dominant giant tiger or black tiger shrimp *P. monodon* (Fabricius 1798) as the dominant cultivated species. While most of the recent reports on large scale mortalities have been due to viral diseases and bacterial diseases have recently gained importance following the identification of certain strains of *V. parahaemolyticus* as the causative agent of early mortality syndrome (EMS) that caused large scale losses in farmed shrimp production in China, Vietnam, Thailand and Malaysia (FAO, 2013; Tran et al., 2013).Acute hepatopancreatic necrosis disease (AHPND, also known as early mortality syndrome, (EMS) has caused severe mortalities (up to100%) in farmed populations of marine shrimp *P. vannamei* and *P. monodon*. The disease has led to significant production and economic losses to shrimp farms, and generally to the aquaculture industry in general, in affected regions (Flegel et al., 2012; Leano et al., 2012; Lightener et al., 2012). It most frequently occurs within the first 30 days after stocking a post larvae shrimp in pond. The EMS/AHPND disease typically affects shrimp post larvae within 20–30 days after stocking and frequently causes up to 100% mortality.

**1.4 *Vibrio parahaemolyticus*: the causative agent of EMS**
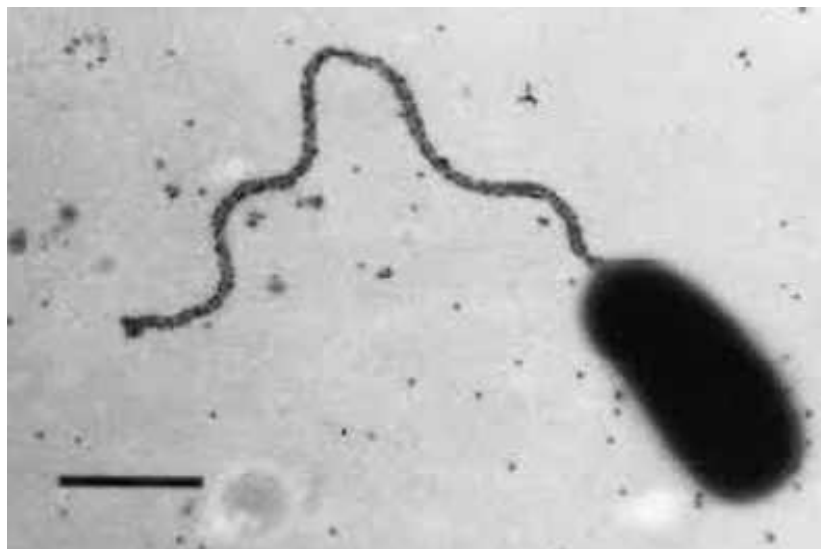


**Fig. 1.3.Photomicrograph showing *Vibrio parahaemolyticus***
**(http://www.ppdictionary.com/bacteria/gnbac/parahemolyticus_cell_flag.gif)**

*Vibrio parahaemolyticus* is one of the several causative agents of shrimp disease that has become prevalent with the surge in global shrimp demand and production. The causative agent of EMS/AHPND has been reported to be a bacterium—more specifically a pathogenic *Vibrio* belonging to the Harveyi clade, presumably *V. parahaemolyticus*.This pathogen has gained much attention due to its high pathogenicity that results in severe production losses upon being introduced to shrimp production facilities and ponds (FAO, 2014; Kongrueng, 2014; Vuddhakul, 2014). *V. parahaemolyticus* is a bacterium in the same family as those that cause cholera. It lives in brackish water and causes gastrointestinal illness in humans. It is a halophile, or salt-requiring organism. Most people become infected by eating raw or undercooked shellfish, particularly oyster and cockles. At least 12 *Vibrio* sp. are classified as pathogenic strains and become major factor for foodborne diseases. *V.parahaemolyticus* caused about 25% of total foodborne diseases in comparison to other *Vibrio* species (Feldhusen et al*.,* 2000). Three species of *Vibrio*(among 28 species) that are often associated with *V. parahaemolyticus* in aquatic environmental and seafood are *V.vulnificus*, *V. alginolyticus* and *V. cholerae.*Clinical signs of the EMS disease in infected shrimp include an empty gastrointestinal tract, milky appearance of the stomach, whitish atrophied hepatopancreas, lethargy, and a soft exoskeleton. The causative agent was determined to be the unique strains of the bacterium *V. parahaemolyticus* (Tran et al., 2013).*V. parahaemolyticus* is the leading causal agent of human acute gastroenteritis following the consumption of raw, undercooked, or mishandled marine products. In rare cases *V. parahaemolyticus* causes wound infection, ear infection or septicemia in individuals with preexisting medical conditions.

**1.5 Pathogenicity of *Vibrio parahaemolyticus***
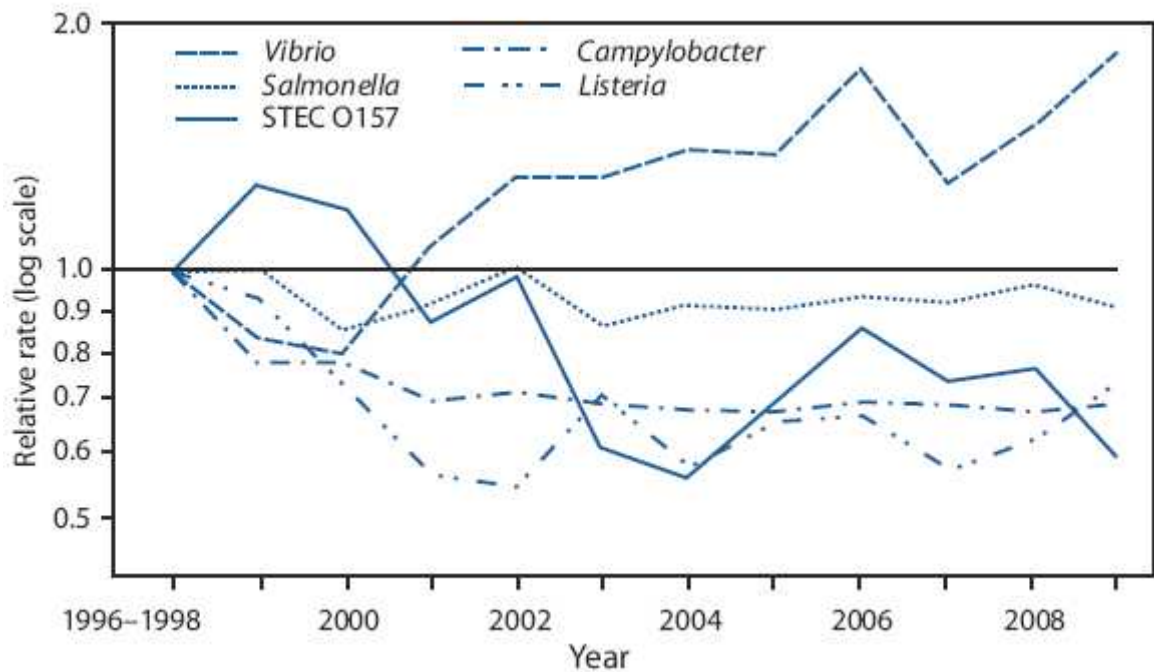


**Fig 1.4.Among common foodborne pathogens in the United States, incidence of *Vibrio* (mostly *V. parahaemolyticus*) associated infection shows an upward trend (reprint from CDC 2007). The relative ratio is determined using the average number of illnesses in 1996-1998 as the denominator.**

*V. parahaemolyticus* has two hemolysin virulence factors that are thermos table direct hemolysin (*tdh*)-adore. Forming protein that contributes to the invasiveness of the bacterium in humans, and TDH-related *trh*which plays a similar role as *tdh* in the disease epathogenesis.In addition, the bacterium is also encodes for adhesion and typeIII secretion systems (T3SS1andT3SS2) to ensure its survival in the environment. Almost all of human pathogenic strains produce thermostable direct hemolysin (TDH) and/or TDH-related hemolysin (TRH), which are encoded by *tdh* and *trh*, respectively (Okada et al., 2009). T3SS1 is present in all *V. parahaemolyticus* strains whereas T3SS2 is present only in human clinical strains. The T3SS2 is divided into two types, T3SS2$\alpha$ and T3SS2$\beta$. The presence of T3SS2$\alpha$ is correlated with the presence of *tdh* while T3SS2$\beta$ is present in *trh*-positive strains (Okada et al., 2009).

Besides T3SSs and TDH genes, *V. parahaemolyticus* have two different types of flagella with distinct functions for swimming and swarming, as well as the ability to produce a capsule. Both these factors are likely to help in the strains survival in the environment and also in colonization of a human host (Broberg et al., 2011).

**1.6 Transmission process of *V. parahaemolyticus* into shrimp**
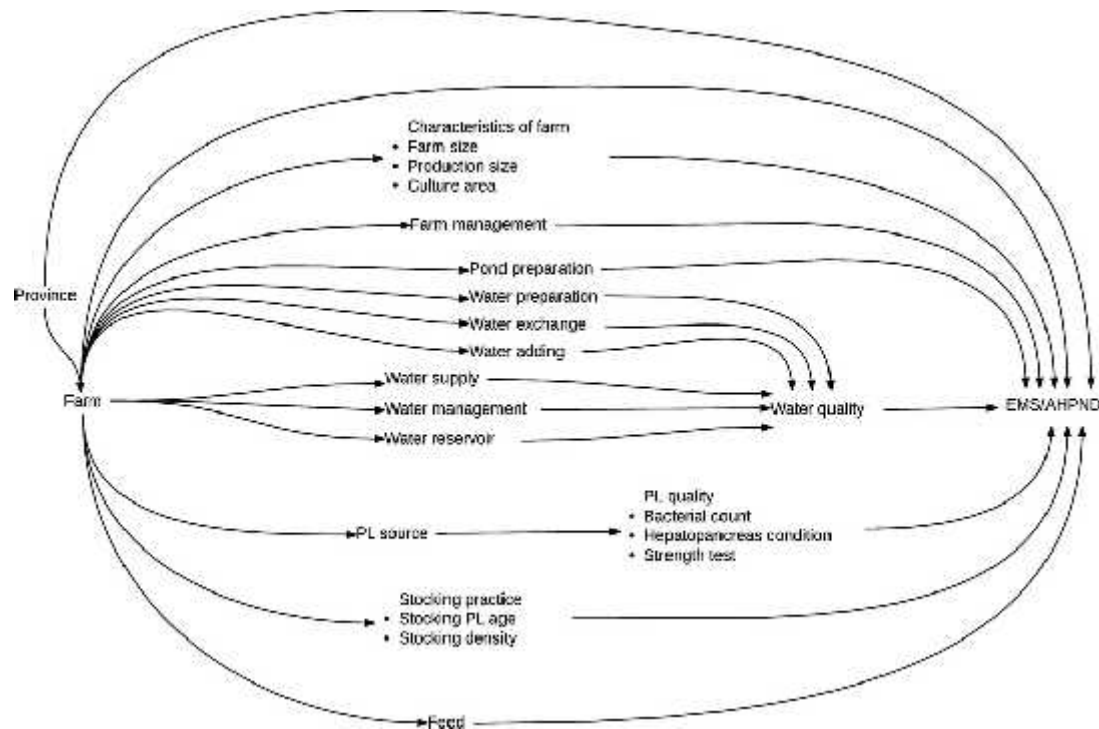


**Fig 1.5.A causal diagram of factors associated with early mortality syndrome/acute hepatopancreatic necrosis disease (EMS/AHPND) occurrence in shrimp ponds. PL refers to post-larvae shrimp. (Source: Boonyawiwat et al., 2016)**

Outbreaks due to *V.parahaemolyticus* in many countries over the last several decades; none of them reported a pathology similar to EMS or AHPND. The disease was first reported in China several years ago and has subsequently spread. If in fact a bacterial virus is the cause (in the sense that it encodes for the toxin) it can readily be spreaded by movement through aquatic ecosystems via natural mechanisms. There are some very interesting observations that suggest that this may not be the whole story. The first one is that there are anecdotes that co-culturing shrimp with Tilapia eliminates the problem. There are also reports that when shrimp are stocked into cages above the bottoms of the pond they do not get EMS while shrimp that forage on the pond bottoms do. Many different *Vibrio* species can attach to algae and *V. parahaemolyticus* is among them. It is

likely that there are going to be some *V.parahaemolyticus* strains attached to some of the PLs that are stocked. So it is part of the normal flora.

**1.7 Molecular identification of causative agent of EMS**

The conventional phenotyping and biochemical identification techniques of *V. parahaemolyticus* are complicated when the strains are isolated from seafood and aquatic environment (Nishibuchi, 2006). As a result, PCR based assay has become a popular molecular technique for identification and detection of *V. parahaemolyticus* (Drake et al., 2007). The genetic composition of *Vibrio* species is extremely variable thus the genes present inside a targeted strain of *Vibrio* can be used to distinguish the genus from other bacteria and are obvious candidates for the development of DNA based methods for identification of *Vibrio* species (Foley et al., 2009). In fact, a number of researchers have studied pandemic isolates to carry bacteriophage sequences that non pandemic strains do not, and they have exploited these differences to develop pandemic strain–specific detection methods (Bisha et al., 2012). To increase the output and lessen the reagent costs, PCR primers can be multiplexed in a single reaction or tailored for the real-time PCR analysis to provide more rapid results (Grant et al., 2006). Polymerase chain reaction is a method with high sensitivity and specificity for detection and identification of pathogenic bacteria from clinical, environmental or seafood samples (Nelapati et al., 2012). PCR method was developed to identify *V. parahaemolyticus* strains at the species level by targeting *toxR* gene. The *toxR* gene stimulates the expression of *tdh* gene and it is present in either pathogenic or non-pathogenic *V. parahaemolyticus* isolates (Sujeewa et al., 2009). Alternatively, the thermo labile hemolysin (*tlh*) in *V. parahaemolyticus* is another gene that was used to develop a multiplex PCR procedure for simultaneous detection of total and virulent *V. parahaemolyticus* (Yi et al., 2014) Although, the *tlh* gene is not considered a virulence factor of *V. parahaemolyticus*, the gene is reported to be a reliable marker for the bacteria (Su and Liu, 2007). Bej et al. (1999) reported a multiplex PCR protocol for amplification of *tlh*, *tdh*, and *trh*, which could be employed for detecting total and virulent *V. parahaemolyticus* in shellfish.All of the suspected colonies were analyzed in the presence of the molecular markers of *V. parahaemolyticus*, i.e., the thermolabile hemolysin (*tlh*), thermostable direct hemolysin (*tdh*), and thermostable direct hemolysin related (*trh*) genes.

**Polymerase chain reaction (PCR) for detection of specific gene**

PCR is an in vitromethod for system of nucleic acid in which a particular segment of DNA can be specifically amplified. When kary Mullis and co-workers developed PCR as a technique, it could be used to generate large amounts of single copy genes from genetic DNA (Mulllis et al., 1986). The result of PCR is an exponential accumulation of specific target DNA fragment approximately up to 2n, when n is the number the cycles of amplification performed (Saiki et al.,1988).



**Fig1.6.PCR Machine (Gene Atlas, G2, Astec, Japan)**

Typically, enough amplified product is generated after 20 to 30 cycles of PCR so that it can be visualized on an ethidium bromide stained gel. The reaction is composed of several components e.g. the template, primers, reaction buffer, Magnesium chloride, dNTPs mixture, thermo-stable polymerase. The template must be sufficient to be able to visualize PCR products using ethidium bromide. A primer should be 20 to 30 bases in length.The most frequently used thermo-stable polymerase is *Taq* DNA polymerase. Taq DNA polymerase permitted the use of higher temperatures for annealing and extension that improved the stringency of primer template hybridization and this specify of the products. A very important property of *Taq* DNA polymerase has its own error rate, which was initially estimated at 2x10/4 nucleotides/cycle (Saiki et al., 1988). The

purified enzyme lacks a proofreading 3′⇒5′-exonuclease activity, which lowers error rates than other enzymes.

The magnesium ion concentration has an effect on enzyme activity primer annealing, melting temperature of the template and the PCR product, fidelity and primer dimer formation (Innis et al., 1990). Reaction buffer supports the reaction condition.

**1.8 Primers used for molecular study**

*ldh* and*tdh* **primer**

Detection of pathogenic *V. parahaemolyticus* isolates is typically based on molecular biological analysis that amplify *ldh* and *tdh* sequences. These 2 genesrespectively, have been implicated in *V. parahaemolyticus* virulence .However, a recent study showed that pathogenesis of *V. parahaemolyticus* does not appear to rely solely on a given virulence function; rather, virulence is a complex trait and different strains may employ somewhat different strategies (Xu et al., 2016). Species specific thermostable direct hemolysin *(tdh)* gene is used to detect for the presence of total and pathogenic *V. parahaemolyticus* strains, respectively.On the other hand *ldh* gene is used to detect the species specific pathogenicity of *V. parahaemolyticus*that are able to cause AHPND in cultured shrimp.

**16S rRNA gene**

The use of 16S rRNA gene sequences to study bacterial phylogeny and taxonomy has been by far the most common housekeeping genetic marker used for a number of reasons. These reasons include (i) its presence in almost all bacteria, often existing as a multigene family, or operons; (ii) the function of the 16S rRNA gene over time has not changed, suggesting that random sequence changes are a more accurate measure of time (evolution); and (iii) the 16S rRNA gene (1,500 bp) is large enough for informatics purposes .

**AP3 Primer**

The AP3 method for detection of the small AHPND toxin gene (AHPND ToxA) released at the NACA website in June 2014 (A new and improved PCR method for detection of AHPND bacteria) has proven effective for detection of bacteria that cause acute hepatopancreatic necrosis disease (AHPND), but it has the disadvantage in being a 1-step PCR method that lacks the sensitivity to detect very low levels of AHPND bacteria. It cannot be adapted into an effective nested PCR method. Thus, the method required a

bacterial pre-enrichment culture step prior to DNA extraction and PCR testing for such samples.

**AP4 primer**

The AP4 target sequence consists of a chimeric DNA fragment comprising a major part of the whole ToxA and ToxB region (1665 bp) on the 69 kbp pVA plasmid of VP$_{AHPND}$ isolates. The AP4-F1 primer is equal to the AP3-F primer of the AP3 method. For the second-step PCR reaction, a portion of the final solution from the first-step PCR reaction is used as the template with the inner (nested) primers AP4-F2 and AP4-R2. The target is a 230 bp portion of the sequence that includes 209 bp of the *ToxA* gene sequence plus the 12 bp spacer sequence plus 9 bp of the succeeding *ToxB* gene sequence. At high concentrations of target DNA, additional, bands for amplicons may occur as the product of residual primer AP4-F1 working with AP4-R2 (357 bp) or AP4-F2 with AP4- R1 (1142 bp) in the nested step. The advantage of the AP4 method over the AP3 method is that it has 100 times higher sensitivity than the AP3 method. Because of its higher sensitivity, the bacterial culture enrichment step is needed when using the AP3 with low levels of AHPND bacteria may be omitted. However, the AP4 method should not be considered as a replacement for the AP3 method but simply as an alternative choice for the users to choose should they need a more sensitive detection method.

**1.9 16S rRNA sequencing**

16S ribosomal RNA (rRNA) sequencing is a common amplicon sequencing method used to identify and compare bacteria present within a given sample. 16S rRNA gene sequencing is a well-established method for studying phylogeny and taxonomy of samples from complex micro biomes or environments that are difficult or impossible to study. The genes for ribosomal RNA have changed little over millions of years as organisms evolved. The slight changes that have occurred which provide clues as how closely or distantly various organisms are related. Because the 16S rRNA gene is very short, just 1,542 nucleotide bases, it can be quickly and cheaply copied and sequenced. So, when a scientist has a test tube full of pond water or dirt from an arid mountainside, he must first pull out the rRNA that's mixed up with all the other RNA, DNA and other stuff in that tube. To do this, she cleans and purifies the sample first, getting rid of unwanted debris. The 16S rRNA gene is the most conserved (least variable) DNA in all

cells. Portions of the rDNA sequence from distantly related organisms are remarkably similar. This means that sequences from distantly related organisms can be precisely aligned, making the true differences easy to measure. For this reason, genes that encode the rRNA (rDNA) have been used extensively to determine taxonomy, phylogeny (evolutionary relationships), and to estimate rates of species divergence among bacteria. Thus the comparison of 16S rDNA sequence can show evolutionary relatedness among microorganisms. The 16S rDNA sequence has hyper variable regions, where sequences have diverged over evolutionary time. Strongly conserved regions often flank these hyper variable regions. Primers are designed to bind to conserved regions and amplify variable regions. The DNA sequence of the16S rDNA gene has been determined for an extremely large number of species. In fact, there is no other gene that has been as well characterized in as many species.

**1.10 Phylogenetic Analysis**
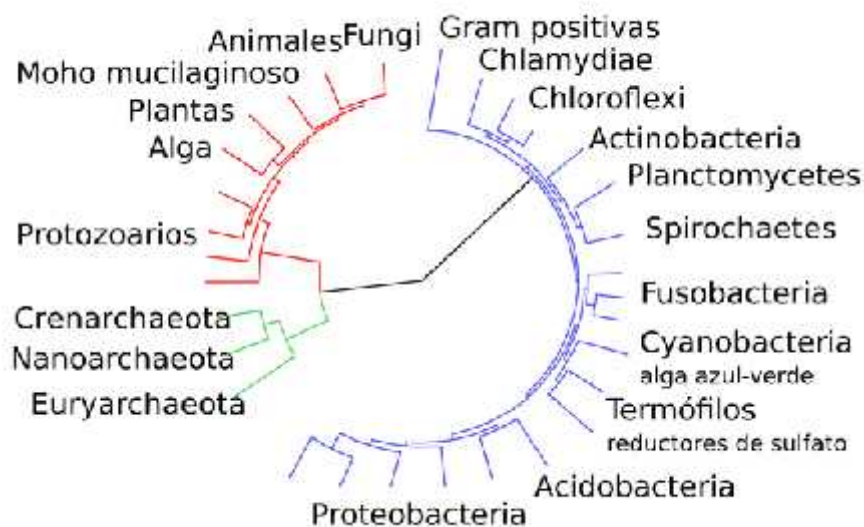


**Fig 1.7.A highly resolved Tree of Life, based on comparatively sequenced genomes. Eukaryotes are colored red, Archaea green and Bacteria blue (Ciccarelli, 2006).**

Phylogenetic analysis is the means of inferringor estimating these relationships. The evolutionary history inferred from phylogenetic analysis is usually depicted as branching, treelike diagrams that represent an estimated pedigree of the inherited relationships among molecules ('gene trees'), organisms, or both.Phylogenetic reconstruction is an attempt to discern the ancestral relationship of a set of sequences. It

involves the construction of a tree, where the nodes indicate separate evolutionary paths, and the lengths of the branches give an estimate of how distantly related the sequences represented by those branches are. Roughly speaking high sequence identify suggest that the sequences in question have a comparatively young most recent ancestor  low identity suggest that the divergence is more ancient.

## 1.11 Antibiotic susceptibility test

Certain bacteria can display resistance to one or more antibiotics. Determining bacterial antibiotic resistance – whether a bacterium can survive in the presence of an antibiotic - is a critically important part of the management of infectious diseases in both human and others animal. The Kirby-Bauer (K-B) disk diffusion test is the most common method for antibiotic resistance/susceptibility testing. The Kirby-Bauer (K-B) test utilizes small filter disks impregnated with a known concentration of antibiotic. The disks are placed on a Mueller-Hinton agar plate that is inoculated with the test microorganism. Upon incubation, antibiotic diffuses from the disk into the surrounding agar. If susceptible to the antibiotic, the test organism will be unable to grow in the area immediately surrounding the disk, displaying a zone of inhibition. The size of this zone is dependent on a number of factors, including the sensitivity of the microbe to the antibiotic, the rate of diffusion of the antibiotic through the agar, and the depth of the agar. Microorganisms that are resistant to an antibiotic will not show a zone of inhibition (Hudzicki, 2009).

## 1.12 Rationale of this thesis work

EMS/AHPND has become the most serious disease which is being faced by the tropical shrimp farming. AHPND has been described to specific *V. parahaemolyticus* strains. EMS is causing serious production losses in affected areas and is also impacting employment, social welfare and international markets. EMS affecting both Pacific white shrimp *P. vannamei* and black tiger shrimp *P. monodon*, the disease has caused significant losses in Southeast Asian shrimp farms such as Bangladesh. Within the first 20- 30 days after a pond is stocked with post larvae, AHPND can cause up to 100% mortality. An atrophied pale hepatopancreas is the most commonly observed sign of AHPND in diseased shrimp. In Bangladesh most of the people in southeast region are involved in shrimp farming for their livelihood. But every year they are facing serious problem due to diseases such as EMS. They loss huge amount of money due to this problem. To relief from this loss, necessary steps should be taken.

**1.13 Research Gap**

Though many works have been done in the world to detect the causative agent of early mortality syndrome of shrimp but in Bangladesh no work has been done yet. Through this study, I have tried to detect the causative agent of early mortality syndrome of shrimp from different shrimp farming areas in south-west region of Bangladesh. For this work, moribund shrimp affected by acute hepatopancreatic necrosis disease (AHPND) from different shrimp farm werecollected for further work.Thenbacterial isolates were identified as *Vibrio parahaemolyticus* by the presence of the *ldh*. Then *ldh* positive *V. parahaemolyticus* were further characterized by PCR and primers, *tdh* AP3, AP4, 16S rRNA gene sequencing.

**1.14 Research Needs**

Shrimp farming and related activities contribute significantly to the national economy of Bangladesh. The main areas of contribution are export earnings and employment generation for on and off farm activities. At present shrimp has emerged as one of the largest export earners of Bangladesh. The shrimp farming industry in Bangladesh has suffered severe problems, mainly due to disease outbreaks caused by Early Mortality Syndrome (EMS), White Spot Syndrome Virus (WSSV) and other diseases. Among these Early Mortality Syndrome causes serious damage in shrimp farming. Every year shrimp farmers face huge loss of money these are investing their farming. To improve this situation necessary step should be taken. For this knowledge about this diseases source, symptoms and control should be provided among farmers. This study provide proper knowledge about the source, symptoms, control of Early Mortality Syndrome of shrimp in Bangladesh. By gaining this knowledge disappointed farmers become interested in   shrimp culture at large scale that will help to increase our export earnings.

**1.15 Objectives**

The overall objective of the thesis work was to identify and characterize the causative agent of acute hepatopancreatic necrosis disease (AHPND) in shrimp.

The specific objectives were

1.To isolate and identify*V. parahaemolyticus* in shrimp by using *ldh* gene, 16S rRNA sequencing and reconstruction of phylogenetic tree

2. To identify and characterize AHPND positive *V. parahaemolyticus* strains causing EMS in cultured shrimp using molecular techniques (AP3 and AP4 primers)

3. To check human pathogenicity of AHPND positive *V. parahaemolyticus* in shrimp by using *tdh* gene

4. To test the antibiotic susceptibility of AHPND positive *V. parahaemolyticus* isolates using available antibiotic discs.

**Chapter 2**

**Materials and Methods**

South-west region of Bangladesh are very much prominent for shrimp farming especially Satkhira, Bagerhat and Khulna. The shrimp samples for this study were collected from Sadar Upazilla of Satkhira and two Upazilla (Morrelganj and Mongla) of Bagerhat district.

**2.1 Sampling location**

Satkhira Sadar is located at 22.7167°N 89.0750°E . It has 61839 households and total area 400.82 km².Satkhira Sadar Upazilla is bounded by Kalaroa Upazilla on the north, Tala Upazilla on the east, Debhata and Assasuni Upazilla on the south.Most of the peoples of southern part of Satkhira depends on pisciculture,locally called gher.3046fisheries, 3650 shrimp farms, 66hatcheries are located in Satkhira District. Shrimp is the main export product from Satkhira District. Shrimp culture is the main occupation for people of Satkhira sadar Upazilla in Satkhira District (https://en.wikipedia.org/wiki/Satkhira District).



A. **Geographical map Satkhira Sadar Upazilla, Satkhira**

B. **Representative Gher of Satkhira Sadar Upazilla, Satkhira**

**Fig 2.1. Geographical map (A) Satkhira Sadar Upazilla and (B) representative gher from which shrimp was collected.**

| Geographic map of   Morrelganj Upazilla | Representative Gher of Morrelganj Upazilla |

**Fig2.2. (A) Geographical map of Morrelganj Upazilla of Bagerhat district and (B) representative gher from which shrimp was collected.**

Bagerhat district has a total area of 3959.11 square kilometers.Morrelganj is an Upazillaof Bagerhat District in the Division of Khulna, Bangladesh. Morrelganj (Town) consists of 9 wards and 12 mahallas. The area of the town is 15.36 km$^2$. The main rivers are Pasur and Mongla.The main occupations are Agriculture, fishing, agricultural laborer, commerce, transport service. Some people depend on shrimp culture for their livelihood.

Mongla Upazilla with an area of 1461.22 km$^2$, borders Rampal Upazilla on the north, the Bay of Bengal on the south, Morrelganj and Sarankhola Upazilla on the east and Dacope Upazilla on the west. The main rivers are Pasur and Mongla. Mongla (Town) stands on the river Pashur. It is the second biggest seaport of the country. It consists of 9 wards and 13 mahallas. Total no of shrimp gher is about 2,365 that cover 9787 ha area.(https://en.wikipedia.org/wiki/Bagerhat District)

**A. Geographical map of Mongla**



**B. Representative Gher of Mongla**

**Fig2.3. Geographical map of (A) Mongla Upazilla of Bagerhat district and (B) representative gher from which shrimp was collected.**

## 2.2 Experimental Species

The shrimp inhabits the coasts of Australia, South East Asia, South Asia and East Africa. Similar to all penaeid shrimp, the rostrum is well developed and toothed dorsally and ventrally. Carapace is without longitudinal or transverse sutures. Cervical and orbito-antennal sulci and antennal carinae always present. Hepatic and antennal spines are pronounced. Pterygostomain angle round. Stylocerite at first antennule segment. Basal spines on first and second pereiopods and exopods on the first to fourth pereiopods are usually present.

**Taxonomic Study of giant tiger shrimp**

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Order: Decapoda

Family: Penaeidae

Genus: *Penaeus*

Species: *Penaeus monodon* (Fabricius 1798)



**Fig 2.4. Photograph showing Black Tiger Shrimp (*Penaeus monodon*) collected from shrimp Gher of Satkhira Sadar Upazilla.**

**2.3 Collection of Samples**

Shrimp samples were collected and immediately transported to the laboratory. Ice was placed around the bags to lower the temperature (4°C) during transportation. For detecting EMS causative agent Shrimp PL (15-30 days) was selected from different shrimp farming areas. In this present study samples were collected from Satkhira District and Bagerhat District.

**2.4 Laboratories of Investigation**

All investigation were being carried out in the Aquatic Laboratory of Department of Fisheries, University of Dhaka with the help from Microbiology laboratory of Department of Botany University of Dhaka. The sequencing was done in the First BASE Laboratories Sdn Bhd, Malaysia.

**2.5External examination for disease signs**



**Fig 2.5.A pale and shrunken hepatopancreas, and an empty stomach, of a juvenile** *P. vannamei***, indicative of AHPND (source: Lightner et al., 2012).**

At first, the collected shrimps were examined externally to check any signs of EMS/AHPND. Most important clinical symptoms consist of lethargy, low, speed in growth rate, spiral swimmingas well as empty or interrupted gut. Also infected shrimps constantly reveal an abnormal hepatopancreas (Lightner et al., 2012). Therefore, some of

the behavioral changes such as lethargy, swimming sluggishly along the dikes, spiral swimming and reduced preening and feeding are also observed in EMS.

## 2.6 Processing of shrimp samples

The shrimp sample was processed within 12 hours of collection following aseptic techniques. For removing sand, detritus and microorganisms attached to the surface of shrimp, the samples washed with sterile phosphate buffer saline (PBS) or normal saline. Then the hepatopancreas were collected aseptically following the method of APHA (1998). The collected sample was homogenized with PBS solution using homogenizer. Then the homogenized sample was used for microbial load count and to isolate*V. parahaemolyticus* strains.

## 2.7 Media used for the enumeration of bacteria

### 2.7.1Enrichment Media

Several enrichment media have been used for the selection of one group or species of *Vibrio*. Specialeffort has been devoted to enhance the presence of potential pathogenic species in contaminated food or in clinical samples. For enrichment of *V. cholerae, V. parahaemolyticus*, and *V. vulnificus,* the most widely used medium and the one recommended by the U.S. Food and Drug Administration (FDA) is alkaline peptone water(APW).

### A. Alkaline peptone water

Alkaline peptone water (APW) is not only the preferred enrichment medium for vibrios, especially devised for *V. cholerae*but also used for *V. parahaemolyticus* and other species. The high pH of the medium (pH closeto 9) and NaCl concentration inhibit many other bacteria and favor vibrios. Peptone concentration can range between 1 and 2%, with the latter being moreappropriate for marine species. NaCl can be omitted to favor the growth of *V. cholerae and*V. mimicus*. Electrolyte supplements (NaCl, $MgCl_2$and KCl) may be included to stimulate the growth of *V. cholerae* and other pathogenic vibrios. Enrichment can be accomplished in 6 to 8 h at 35 to 36°C and then again at 16 to 24 h.

**Flow chart1. Overview of processing of shrimp, identification and characterization of bacterial isolates**

```
                    ┌─────────────────────────────┐
                    │     Collection of Shrimp     │
                    └─────────────────────────────┘
                                  │
                                  ▼
                    ┌─────────────────────────────┐
                    │ Separation of shrimp hepatopancreas │
                    └─────────────────────────────┘
                                  │
                                  ▼
                    ┌─────────────────────────────┐
                    │        Homogenization        │
                    │   1 g sample + 9 ml saline   │
                    └─────────────────────────────┘
```

| Quantitative analysis of Bacterial load | Qualitative analysis using Enrichment 1 ml homogenized sample + 9 ml APW Incubation 37°C, 6 hours |
|---|---|

| TBC | Inoculation on TCBS, Hi chrome, ChromAgar *Vibrio*. Incubation at 37°c for 18-24 hrs & also inoculation in APW , Incubation at 37°c for 6 &18h |
|---|---|

| Total *Vibrio* like colonies on Hi Chrome | Single colony inoculated on TSA, Chrome agar plate, Incubated at 37°c for 24 hrs |
|---|---|

| Total *Vibrio* like colonies on TCBS | Colonies of suspected*V. parahaemolyticus*stored at −20°C for furthercharacterization by molecular techniques |
|---|---|

| | Antibiotic susceptibility test of*V. parahaemolyticus* isolates |
|---|---|

**2.7.2 Selective Media for *Vibrio* spp.**

**A. TCBS agar**



| A | B |
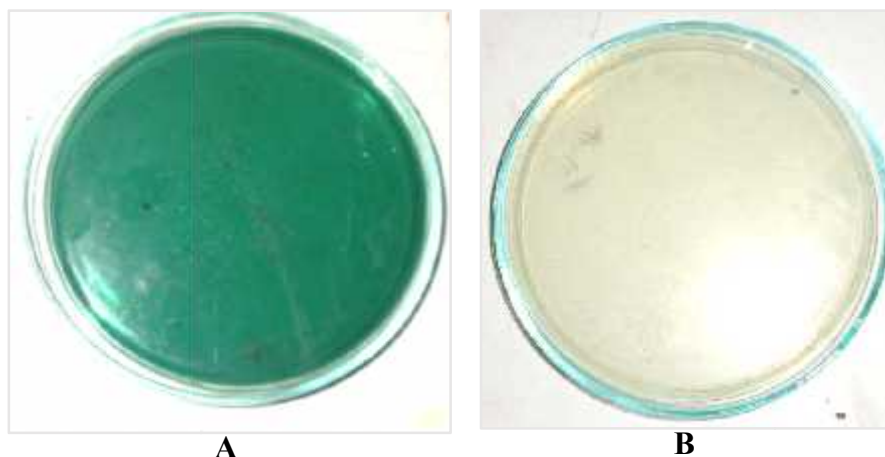
**Fig 2.6.(A) Fresh TCBS Plate and (B) TSA plate**

TCBS (Oxoid) is an ideal medium for the selective isolation and purification of vibrios.Although this medium was originally designed for the isolation of *V. cholerae* and *V. parahaemolyticus*, most vibrios grow to healthy large colonies with many different colonial morphologies. Gram-positive and coliform bacteria are strongly inhibited owing to the presence of bile salts. Vibrios that are able to use sucrose will form yellow colonies, while sucrose-negative strains will form green colonies. Care should be taken, since, in older cultures (more than 48 h), refrigerated plates, or heavily grown plates, the color of the colonies may change; therefore, colonial color has to be registered only in recent and well-isolated colonies. Since TCBS is composed of many ingredients not easily acquired, we recommend obtaining it from commercial sources. Marked variations have been registered between brands. It is not necessary to add extra salt (NaCl), since TCBS has an adequate amountfor the growth of the majority of vibrios.

**B. Tryptone soya medium**

TSA and TSB (for the agar and broth, respectively) are perhaps the most useful media for after sample analysis of marine bacteria, provided an adequate concentration of salt (NaCl) is added. It is necessary to obtain a final concentration of NaCl between 1.5 and 2.5%; TSA and TSB already have 0.5% NaCl. Vibrios grow as big creamy colonies after just 24 h at temperatures between 15 and 30°C, depending on the strain under analysis.

### C. Identification on CHROMagar *Vibrio* media



**Fig 2.7. CHROMagar *Vibrio* Plate**

*V. parahaemolyticus*, *V. vulnificus* & *V. cholerae* are pathogenic bacteria which can cause serious seafood poisoning. For the detection of those bacteria, traditional methods (TCBS) are long, require heavy workload and are not very sensitive. On the contrary, ChromAgar *Vibrio* medium helps to easily differentiate *V. parahaemolyticus*, *V. vulnificus* and *V. cholerae* from other *Vibrio* directly at the isolation step by colony color with a sensitivity higher than conventional methods.

### 2.7.3 Isolation of suspected *V. parahaemolyticus* from Shrimp

Sampling of the hepatopancreas should be done carefully to avoid contamination with intestinal bacteria, and serial dilutions can be done in sterile saline solution. Nutrient agar, TCBS agar, Hi Chrome agar were used for culture of bacteria.1g of sample (shrimp hepatopancreas and whole shrimp) and 9 g physiological saline were mixed first. Then 100μl sample was spread in TCBS and Hi chrome agar plate and plates were incubated at 37°C for 24 hours and finally counted bacteria and single colony picked up. On the other hand 1ml sample and 9ml alkaline peptone water were mixed first and it was spreaded in TCBS and Hi Chrome agar plate. Then plates were incubated at 37°C for 18 h and 6h and colony counted and single colony picked up. For the first approach thestomach, HP and mid-gut from the EMS affected shrimp was removed, minced and separately inoculated into flasks containing TSB with added sodium chloride. The flasks was incubated at 37° C for 18 to 24 h to obtain mixture culture. All mixed culture was subjected to sub- culture in order to obtain individual colonies on the TCBS, ChromAgar *Vibrio*.From the broth culture, a loop full of culture was streaked on

theChromAgar*Vibrio* surface and later sealed with parafilm before incubated for overnight. Pure purple colonies were selected and were streaked onto a new ChromAgar*Vibrio* surface for purity and then were grown in Luria-Bertani (LB) broth and later on LB agar (as a stock). All single isolates were preserved in LB with 30% added glycerol and frozen at -80°C for further studies.

**2.8 Morphological observation of isolates**

For the identification of selected isolates, following morphology characters were studied and recorded. Colony characters such as form, elevation, margin, shape, color, surface and arrangement of cells were observed under a phase contrast microscopic from 18-24 hours old culture grown on TCBS, HI chrome, ChromAgar*Vibrio*.

**Flow chart 2. Molecular identification of bacterial isolates**

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┌─────────────────────────────────────────────────────┐
│  Subculture of 18 suspected colonies in TSA plate    │
│           from -80°C stored samples                   │
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                          ▼
┌─────────────────────────────────────────────────────┐
│   Isolation of genomic DNA by Maxwell DNA extraction kit │
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                          │
                          ▼
┌─────────────────────────────────────────────────────┐
│      PCR amplification by using 16S rRNA gene primer  │
└─────────────────────────────────────────────────────┘
                          │
                          ▼
┌─────────────────────────────────────────────────────┐
│              Gel Electrophoresis (1%)                 │
└─────────────────────────────────────────────────────┘
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┌─────────────────────────────────────────────────────┐
│              PCR product purification                 │
└─────────────────────────────────────────────────────┘
                          │
                          ▼
┌─────────────────────────────────────────────────────┐
│          Nucleotide sequencing by ABI                 │
│            genetic analyzer 3100                       │
└─────────────────────────────────────────────────────┘
                          │
                          ▼
┌─────────────────────────────────────────────────────┐
│   Identification of V. parahaemolyticus through       │
│              nucleotide BLAST                          │
└─────────────────────────────────────────────────────┘
                          │
                          ▼
┌─────────────────────────────────────────────────────┐
│      Multiple and pairwise Sequence Alignment         │
└─────────────────────────────────────────────────────┘
                          │
                          ▼
┌─────────────────────────────────────────────────────┐
│          Construction of Phylogenetic Tree            │
└─────────────────────────────────────────────────────┘
```

**Flowchart 3: DNA extraction by heat extraction method**

```
┌─────────────────────────┐
│                         │
└─────────────────────────┘
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┌─────────────────────────┐
│                         │
└─────────────────────────┘
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            ▼
     ┌──────────────┐
     │              │
     └──────────────┘
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     └──────────────┘
```
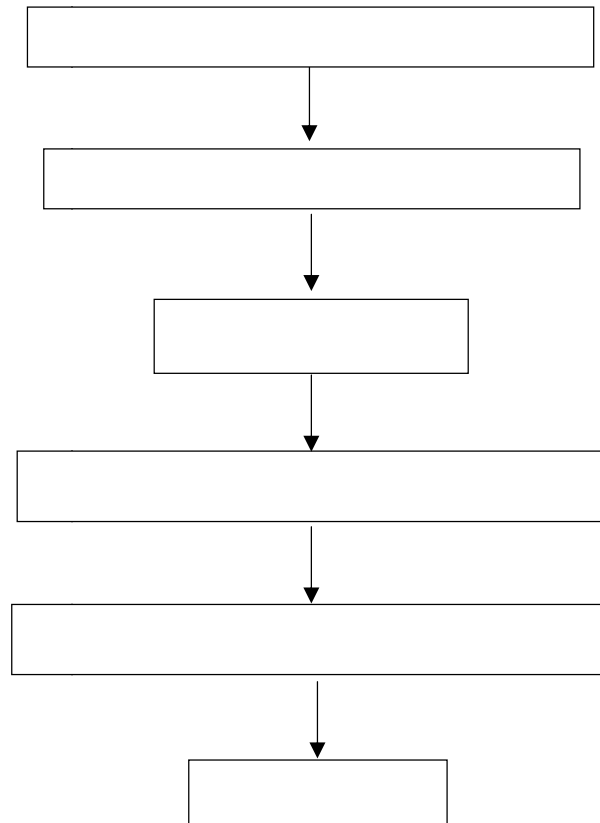
**2.9 Molecular identification and characterization of the isolates**

**2.9.1 Isolation of genomic DNA**



**Fig2.8. Photograph Automated DNA extraction machine (Maxwell 16 kit)**

A loop of fresh overnight grown colony of bacterial isolates incubated into 5 ml LB broth in incubator at 37°C for overnight. 300µl of the overnight grown culture was taken

into the DNA kits with plungers. After that, 300µl of elution buffer was taken into the elution tube. For washing 35 mins DNA kits were placed into the Automatic nucleic acid purification system (Maxwell 16 MDx Research Instrument, Promega, USA).

## 2.9.2 Purity of DNA

Absorbance of purified DNA by Nano Drop spectrophotometer (Nano Drop 2000 UV – Vis spectrophotometer, Thermo Fisher Inc., USA ) was checked at 260 nm to determine the purity of DNA of bacterial isolates which was around 1: 8 Ratio of less than 1.8 indicate that the preparation was contaminated , either with protein, RNA or with phenol.

## 2.9.3 Polymerase chain reaction

Amplication of washed DNA samples were performed by Polymerase Chain Reaction (PCR) for further analysis.

## A) **Preparation of reaction Mixture**

The reaction mixture for PCR was prepared by mixing the specific volume of the components in an appropriate sized tube provided in the following table-

- 12.5µl of Hot start colorless Master Mix containing – dNTPs, Buffer, $MgCl_2$, Taq Polymerase (Cat: M7432, Origin: Promega, USA) without template DNA was prepared and aliquoted into PCR tubes.
- Specified template was added into a properly labeled PCR tube. The PCR tube containing reaction mixture and template DNA was capped properly followed by vortex and centrifuge briefly to mix the mixture gently and collect all components to the bottom of the tube respectably.
- The total mixture was then recollected, sealed and placed in a thermal cycler and the cycling was started immediately. PCR amplification was done in an oil free thermal cycler (Applied Bio systems 2720 Thermal cycle)

**Table 2.1.PCR condition for *ldh & tdh***

| SL no. | Step | *tdh* | *ldh* |
|--------|------|-------|-------|
| 1 | Initial denaturation | 94°C/10 min | 94°C/10min |
| 2 | Final denaturation | 94°C/1min | 94°C/1min |
| 3 | Annealing | 55°C/1.5min | 55°C/1.5min |
| 4 | Initial extension | 72°C/1.5min | 72°C/1.5 min |
| 5 | Final extension | 72°C/10min | 72°C/10min |

**Table 2.2.Components and Volume of Reaction Mixture for PCR (10 reactions)**

| Reaction components | Volume (μL) | Total Volume (μL) |
|---------------------|-------------|-------------------|
| Master mix | 12.5 | 12.5×10 = 125 |
| Forward primer | 1 | 1× 10 =10 |
| Reverse primer | 1 | 1× 10 = 10 |
| Template DNA | 1 | 1× 10 = 10 |
| Nuclease free water | 9.5 | 9.5 × 10 = 95 |
| Total reaction volume | 25 | 250 |

For the partial amplification of 16S rDNA gene the following primer pairs were used in PCR showed in table 2.3

**Table 2.3.Primers 16S rRNA sequence used in PCR**

| Primer | Type | Sequences | Reference |
|--------|------|-----------|-----------|
| 27 F | Forward | GCCTAACACATGCAAGTCGA | Lane., 1991 |
| 1492 R | Reverse | GACTACCAGGGTATCTAATCC | Frank et al., 2008 |

The reaction mixtures containing PCR tubes were preheated at 95°C for 5 minutes in the thermal cycle to ensure the denaturation of all DNA templates. The PCR reaction was then continued according to the following table

**Table 2.4.Amplification cycle, temperature and required time for PCR**

| Number of cycle | Step | Temperature | Time |
|---|---|---|---|
| 1 | Pre Heat | 95°C | 5 min |
| 32cycles | Denaturation | 95°C | 30 sec |
| | Annealing | 48°C | 30 sec |
| | Extension | 72°C | 1min 30 sec |
| 1 | Final Extension | 72°C | 5 min |
| 1 | Hold | 4°C | Overnight |

**Table 2.5. List of primers to identify AHPND positive *V. parahaemolyticus***

| SL | Name of primer | Sequence | Purpose | Ref. |
|---|---|---|---|---|
| 1 | AP4-F1 AP4-R1 | 5`-ATGAGTAACATATAAACATGAAC-3` 3`-ACGATTTCGACGTTCCCCAA-5` | To detect TOXA gene | Dagtip et al. (2015) |
| 2 | AP4-F2 AP4-R2 | 5`-TTGAGAATACGGGACGTGGG-3` 3`-GTTAGTCATGTGAGCACCTTC-5` | To detect TOXB gene | Same |
| 6 | *ldh* (lecithin dependent hemolysin) | 5`-AAAGCGGATTATGCAGAAGCACTG-3` 3`-GCTACTTCTAGCATTTTCTCTGC-5` | To detect *V.parahaem olyticus* haemolysin gene | Joshi et al., (2014) |
| 7. | *tdh* (thermosta ble direct hemolysin) | 5`-GTACCGATATTTTGCAAA-3` 3`-ATGTTGAAGCTGTACTTGA-5` | Same | Same |

**PCR reaction condition for AP3**

The sequence of the AP3 primer target plus the primers and PCR protocol are given freely below for application in detecting AHPND bacteria.

**Table 2.6. Primer sets for AP3**

| AP3 | 5`-3` | Length | %GC | Tm | Ta | Expected amplicon |
|---|---|---|---|---|---|---|
| F | ATGAGTAACAATATAAAACATGAAAC | 26 | 23.08 | 57.63 | 53 | |
| R | GTGGTAATAGATTGTACAGAA | 21 | 33.33 | 55.46 | | 336 bp |

*Tm= Melting temperature, Ta= Annealing temperature

**Table 2.7. AP3 primer based PCR reaction condition**

| Components | Amount (µl) |
|---|---|
| 10X PCR Mix | 2.5 |
| 50Mm $MgCl_2$ | 0.7 |
| 10mM dNTPs | 0.4 |
| 10µM $F_1$ | 0.5 |
| 10 µM $R_1$ | 0.5 |
| Taq DNA polymerase | 0.2 |
| Total | 25 |

**Table 2.8. Amplification cycle, temperature and time for AP3 primer based PCR**

| Protocol | Temperature & Time |
|---|---|
| Denature   30 cycles | 94° C, 5 min |
| Denature | 94°C , 30 sec |
| Annealing | 53°C , 30 sec |
| Extension | 72°C , 40 sec |
| Final | 72°C , 5 min |

**Table 2.9. PCR condition for AP4 primers**

| AP4 | 5`-3` | Length | %GC | Tm | Ta | Expected amplicon |
|------|-------|--------|-----|-----|-----|-------------------|
| **AP4-F1** | ATGAGTAACAATATAAAACATGAAAC | 26 | 23 | 49 | | |
| **AP4-R1** | ACGATTTCGACGTTCCCCAA | 20 | 50 | 54 | 55 | 1269bp |
| **AP4-F2** | TTGAGAATACGGGACGTGGG | 20 | 55 | 52 | | |
| **AP4-R2** | GTTAGTCATGTGAGCACCTTC | 21 | 48 | 54 | 55 | 230bp |

### A.  1st step AP4 PCR reaction

| Component | µl | Final concentration | Protocol |
|-----------|-----|---------------------|----------|
| 10X PCR mix | 2.5 | 1X | Denature 94°C, 2 min 30 cycles |
| 50 mM MgCl$_2$ | 1.5 | 3mM | Denature 94°C , 30 sec |
| 10 mM dNTPs | 0.5 | 0.2 mM | Annealing 55 °C , 30 sec |
| 10µM AP4 F1 | 0.5 | 0.2µM | Extension 72°C , 90 sec |
| 10µM AP4 F2 | 0.5 | 0.2µM | Final Extension 72°C, 2min |
| Taq DNA polymerase | 0.3 | 1.5 U | |
| DNA template | 2.0 | 100ng/ µl | |
| Sterile water | 17.2 | | |
| Total | 25 | | |

### B.  2nd step  AP4 PCR reaction

| Component | µl | Final concentration | Protocol |
|-----------|-----|---------------------|----------|
| 10X PCR mix | 2.5 | 1X | Denature 94°C, 2 min 25 cycles |
| 50 mM MgCl$_2$ | 1.5 | 3mM | Denature 94°C , 20 sec |
| 10 mM dNTPs | 0.5 | 0.2 mM | Annealing 55 °C , 20 sec |
| 10µMAP4 F1 | 0.375 | 0.15µM | Extension 72°C , 20 sec |
| 10µMAP4 F2 | 0.375 | 0.15µM | Final Extension 72°C, 2min |
| TaqDNA polymerase | 0.3 | 1.5 U | |
| 1st PCR product | 2.0 | | |
| Sterile water | 17.2 | | |
| total | 25 | | |

**2.10 Gel Electrophoresis of the amplified products and documentation**



**Fig. 2.9. Alphalmager MINI gel-documentation system**

The successful amplification of the desired genes was visualized by resolving the PCR products in 1% agarose gel depending on the size of amplicon.

- The gel was prepared using 0.32g 1% Agarose powder (V3125, Promega, USA) and 40 ml 1X TAE buffer (V4251, Promega, USA)
- The mixer was heated in a hot plate and magnetic stirrer (VS-130HS, Vision scientific Inc., LTD, Korea) for about 3 minutes to dissolve the agarose
- The boiled mixture was allowed to cool to about 45°C and 2µl of Ethidium Bromide was added.
- The gel was poured onto gel casing present with well former (comb) and allowed to set on a flat surface for about 15 minutes.
- After solidification of the gel, the comb was removed and buffer (1X TAE) was poured into tank to submerge the solidified gel.
- Samples were prepared by mixing 5µl PCR product with 1µl loading dye and each 6µl prepared PCR product was loaded into the wells formed in the gel.
- Electrophoresis was conducted in 1X TAE buffer at 100 volts for 60 minutes.
- 1 kb DNA ladder was also electrophoresed along the side of the amplified sample DNA.
- DNA bands were observed and photographed by Alphalmager MINI gel-documentation system

## 2.11 Purification of PCR products

The PCR products were resolved by agarose gel electrophoresis to confirm successful amplification of the desired sequence. The PCR products of specific genes were purified with the Wizard PCR SV gel and PCR Clean-up System Kit (Promega, USA) according to the manufacturer's instruction prior to sequencing. The steps of purification are given below in brief.

**a) Processing of PCR products**: An equal volume of Membrane Binding Solution was added to the PCR amplification. Binding of DNA A SV Minicolumn was inserted into collection tube. The prepared PCR product was transferred to the Minicolumn assembly and incubated at room temperature for 1 minute. The preparation was centrifuged at 16000X g for 1 minute using the centrifuge (Sigma, USA). The flow through was discarded and the Minicolumn was reinserted into collection tube.

**b) Washing**: 700µl Membrane Wash Solution was added into the Minicolumn and centrifuge at 16000Xg for 1 minute. The flow through was discarded and the Mini column was reinserted into Collection Tube. The previous step was repeated with 500µl Membrane Wash Solution and centrifuged at 16000 X g for 5minutes. The Collection Tube was emptied and the column assembly was re-centrifuged for 1 minute with the micro-centrifuge lid open to allow evaporation of any residual ethanol.

**c) Elution:** The Minicolumn was carefully transferred to a clean 1.5 mL micro centrifuge tube. 50 µL Nuclease-Free Water was added to the Minicolumn and incubated at room temperature for 1minute followed by centrifugation at 16000 X g for 1 minute. The Minicolumn was discarded and DNA was stored at 4°C or -20°C.

## 2.12 16S rRNA sequencing

The PCR products were sent to First Base Laboratories Snd Bhd (Malaysia) where cycle sequencing was performed using BigDye Terminator v 3.1 Cycle Sequencing Kit according to manufacturer's instruction and extension product was purified followed by capillary electrophoresis using ABI Genetic Analyzer (Applied Biosystem, USA). Bidirectional sequences were done for all the 18 representative isolates. The basic sequencing protocol involved amplification of target sequence by PCR and purification of desired amplicon followed by cycle sequencing reaction, cycle sequencing product purification and capillary electrophoresis.

**Flowchart 4. 16S rRNA sequencing process**

```
┌─────────────────────────────┐
│   Isolation of the bacteria  │
└─────────────────────────────┘
               │
               ▼
┌─────────────────────────────┐
│  Extraction of bacterial DNA │
└─────────────────────────────┘
               │
               ▼
┌─────────────────────────────┐
│ Amplification of the 16S rRNA gene │
└─────────────────────────────┘
               │
               ▼
┌─────────────────────────────────────┐
│ Sequencing a portion of the 16S rRNA gene │
└─────────────────────────────────────┘
               │
               ▼
┌──────────────────────────────────────┐
│ Comparing the sequenced gene with GenBank to │
│          obtain a match                │
└──────────────────────────────────────┘
```
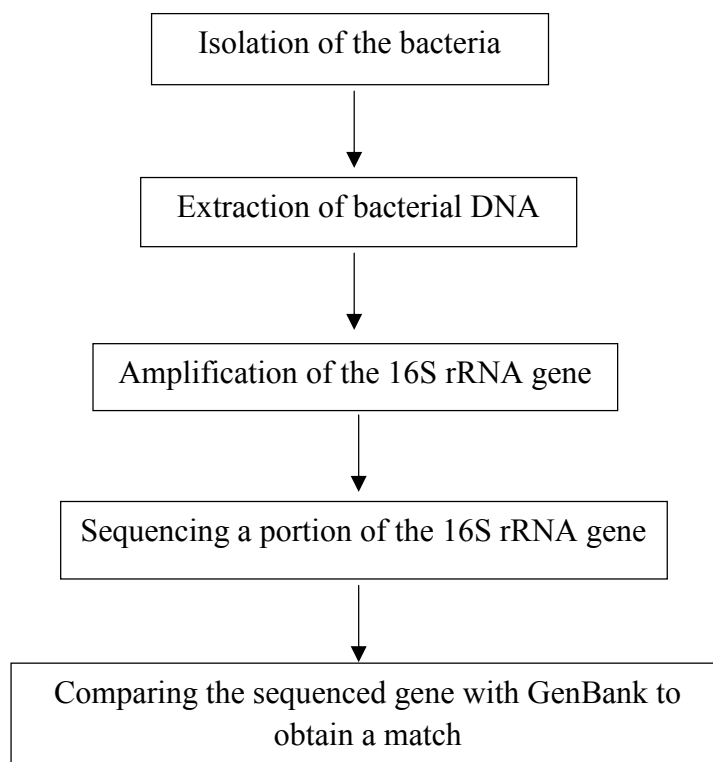
**2.13 Bioinformatics tools**

Different bioinformatics tools were used to analyze the sequences. These tools are given below-

**Finch TV version 1.4**

To view DNA sequence Geospizas Finch TV version 1.4 was used. And it leads the way with raw data views, BLAST searching and the ability to reverse complement sequences and traces.

**Nucleotide BLAST**

BLAST for Basic Local Alignment Search Tool was used for comparing primary sequence information. The statistical significance of matches was used to infer functional and evolutionary relationships between sequences as well as helps to identify members of gene families.

**CLUSTALW**

CLUSTALW a program which was used for multiple sequence alignment (MSA). This helps to find out the similarity, dissimilarity or identify between different sequences.

**Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 software**

MEGA was used for the comparative analysis of molecular sequence data was used for reconstructing the evolutionary histories of species and inferring the nature and extent of selective forces shaping the evolution of genes and species.

**2.14 Antibiotic susceptibility test**

Antimicrobials are commonly used in the treatment of infectious diseases in the aquaculture industry; however, the extensive use of antimicrobials has led to the development of antimicrobial resistance among pathogens in aquatic products and has rendered many known antimicrobials ineffective. *V. parahaemolyticus* has been reported to have resistance to ampicillin, streptomycin, kanamycin, tetracycline, and ciprofloxacin. Antimicrobial resistance, particularly multi-drug resistance, is among the most important public health concerns because it is directly related to disease management and control. Therefore, it is necessary to establish a monitoring system for the objective evaluation of the antimicrobial-resistance profile (Xu et al., 2016). The development of antimicrobial resistance among pathogenic bacteria has emerged as a major public health concern, which has led to an intensification of discussion about the prudent use of antimicrobial agents, especially in veterinary medicine, nutrition and agriculture. Antimicrobial agents have been applied to the shrimp feed and water in large quantities primarily to treat and prevent diseases in farmed shrimps. Consequently, antimicrobial agents persist in sediment and aquatic environments, leading to deteriorated environmental conditions and conferringantimicrobial resistance to the sediment bacteria (Hossain et al., 2012). The Kirby-Bauer disc diffusion technique was performed to determine the sensitivity or resistance of pathogenic bacteria to 14 antibacterial compounds. 18 presumptively identified isolates were inoculated on LB broth and inoculated for 16 hours. The test the antibiogram of AHPND positive *V. Parahaemolyticus* isolates Muller-Hinton broth and Muller- Hinton agar were used. The detail procedure of antibiogram are given as below.

**Flowchart 5. Antibiotic Disc Diffusion Testing**

```
┌─────────────────────────┐
│    Selection colonies    │
└─────────────────────────┘
             │
             ▼
┌─────────────────────────────┐
│ Preparationinoculum suspension │
└─────────────────────────────┘
             │
             ▼
┌──────────────────────────────────┐
│ Standardization inoculum suspension │
└──────────────────────────────────┘
             │
             ▼
┌─────────────────────────┐
│     Inoculation Plate    │
└─────────────────────────┘
             │
             ▼
┌─────────────────────────────┐
│  Addition antimicrobial disks │
└─────────────────────────────┘
             │
             ▼
┌─────────────────────────┐
│     Incubation plate     │
└─────────────────────────┘
             │
             ▼
┌─────────────────────────────┐
│  Measuring inhibition zones  │
└─────────────────────────────┘
             │
             ▼
┌─────────────────────────┐
│   Interpretation results │
└─────────────────────────┘
```
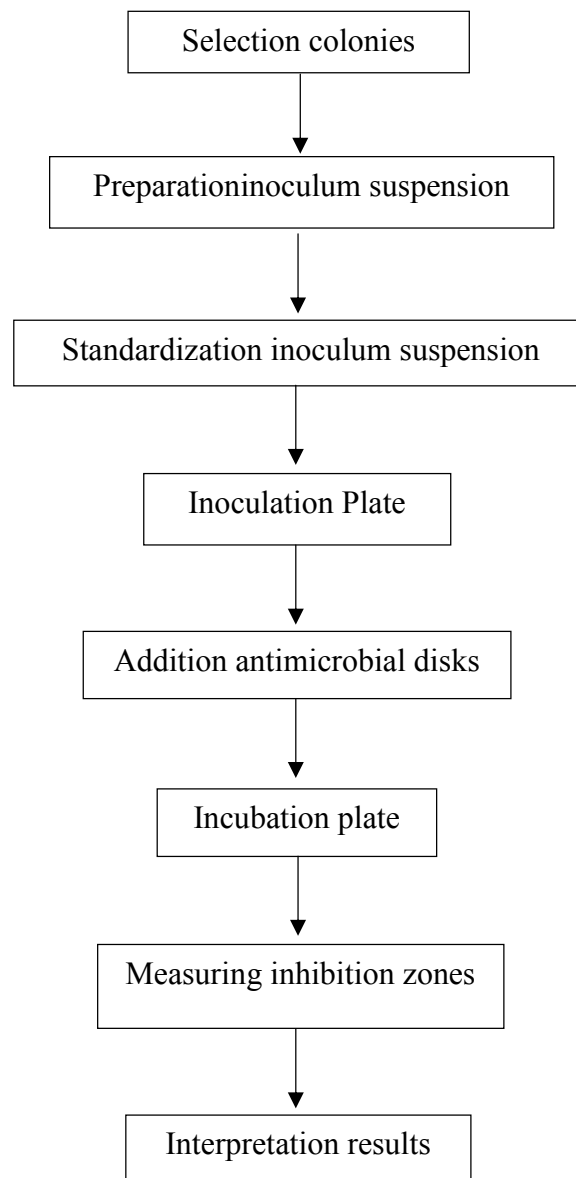
**Procedure of antibiotic susceptibility test**

1. Dipped asterile swab into the broth and express any excess moisture by pressing the swab against the side of the tube.

2. Swabbed the surface of theMueller-Hintonagar plate completely.

3. After completely swabbing the plate, turned it 90 degrees and repeat the swabbing process.

4. Allowed the surface to dry for about 5 minutes before placing antibiotic disks on the agar.

5. The antibiotic discs-

- Individual antibiotic dispensers were used.

- A pair of forceps were used to remove an antibiotic disc from the dispenser: the forceps have to be sterile. Place the forceps in alcohol, flame the forceps until they catch on fire, and let the flame go out sterile forceps.

- Lightly touched each disc with sterile inoculating loop to make sure that it is in good contact with the agar surface. Incubated upside down and incubate at 37° C.

**Table 2.10. List of 14 different antibiotics used for Antibiogram and their potency**

| Name of the Antibiotics | Disc Code | Potency |
|---|---|---|
| Erythromycin | E | 15 µg |
| Nalidixic acid | NA | 30 µg |
| Tetracycline | TE | 30 µg |
| Polymyxin B | PB | 300 µg |
| Nitrofurantion | F | 300 µg |
| Streptomycin | S | 10 µg |
| Ciprofloxacin | CIP | 5 µg |
| Amoxicillin | AML | 10 µg |
| Amikacin | AK | 30 µg |
| Gentamycin | CN | 10 µg |
| Sulphamethoxazole | RL | 25 µg |
| Chloramphenicol | C | 30 µg |
| Ampicillin | AMP | 10 µg |
| Kanamycin | K | 30 µg |

# Chapter 3

# Results

In the present study, the infected shrimp were examined using both classical microbiology and molecular approach to identify the causative agent of AHPND. To conduct this study, samples were collected from 3 different location of south-west shrimp farming region of Bangladesh. Molecular characterization and antibiotic susceptibility test were performed on the *Vibrio parahaemolyticus* strains that causes AHPND.

## 3.1 Gross signs in farmed shrimp infected with AHPND



**Fig. 3.1. The picture represented the AHPND affected shrimp and its hepatopancreas that was collected from gher of Satkhira sadar Upazilla**

Moribund shrimp collected from farms affected with AHPND presented slightly expanded chromatophores, lethargy, erratic swimming, and empty guts. Diseased shrimp typically presented HP discoloration and an organ with an aqueous consistency and as the infection developed, the organ consistency became rubbery. After the dark membrane that overlays the HP was removed, a pale-colored organ was observed. Hepatopancreas (HP) often pale to white due to pigment loss in the connective tissue capsule was observed.Often soft shells and guts with discontinuous contents or no content were found in infected shrimp.Black spots or streaks sometimes were visible within the HP of infected shrimp.

## 3.2 Enumeration of Total bacterial density (cfu/g), Total *Vibrio* count from hepatopancreas and whole sample of shrimp

For total bacterial count and total *Vibrio* count, bacteria grown on Nutrient Agar (NA) plate, TCBS agar plate and HiChrome Agar plate were counted. The results are shown in the following table 3.1, 3.2 and 3.3.

**Table 3.1.Bacterial load (cfu/g) in shrimp collected from Satkhira Sadar Upazilla**

| Sample Name | | Satkhira | | |
|---|---|---|---|---|
| | | TBC | TVC | THC |
| Sampling 1 | SH1 | $3 \times 10^5$ | $6 \times 10^5$ | $3.8 \times 10^6$ |
| | AH2 | UC | UC | $1.2 \times 10^7$ |
| | M1H3 | UC | UC | UC |
| | OH4 | $2.9 \times 10^6$ | $1.6 \times 10^6$ | UC |
| | BH5 | UC | UC | UC |
| | M2H6 | UC | UC | $8 \times 10^6$ |
| | JH7 | UC | UC | $1.8 \times 10^7$ |
| Sampling 2 | ShW1 | $6.6 \times 10^7$ | $2.9 \times 10^6$ | $1.2 \times 10^7$ |
| | ShW2 | $3.61 \times 10^7$ | $2.16 \times 10^5$ | UC |
| | AnW1 | $3.87 \times 10^8$ | $2.40 \times 10^7$ | UC |
| | AbW1 | $5.69 \times 10^8$ | $2 \times 10^3$ | UC |
| | AbW2 | $6.37 \times 10^8$ | UC | UC |
| | BaW1 | $4.0 \times 10^6$ | $1.60 \times 10^8$ | UC |

In sampling 1, the bacterial load of affected shrimp samples on nutrient agar was from $3 \times 10^5$ cfu/g to infinity at Satkhira region. On the other hand, in sampling 2, the bacterial load of affected shrimp samples on nutrient agar was from $2 \times 10^3$ cfu/g to infinity. Total *Vibrio* count on TCBS agar from affected shrimp was ranged between $6 \times 10^5$ cfu/gto infinity in sampling 1, while total *Vibrio* count on TCBS was from $2 \times 10^3$ cfu/g to infinity in sampling 2. Besides, total *Vibrio* count on HiChrome agar from Satkhira region was from $3.8 \times 10^6$ cfu/g to infinity in sampling 1. In sampling 2, total *Vibrio* count on HiCrome agar was found between $1.2 \times 10^7$ cfu/g to infinity.

**Table3.2. Bacterial load (cfu/g) in shrimp collected from Mongla Upazilla.**

| Sample Name | | Mongla | | |
|---|---|---|---|---|
| | | TBC | TVC | THC |
| **Sampling 3** | AfHM | $1.12\times10^5$ | UC | UC |
| | BaHM | $1.6\times10^4$ | $2.40\times10^7$ | $1.88\times10^7$ |
| | JoHM | $2.16\times10^5$ | UC | UC |
| | IsWM | $1.60\times10^8$ | UC | UC |
| | BaWM | UC | UC | UC |
| **Sampling 4** | KBW1 | $1.5\times10^5$ | $2\times10^3$ | No Growth |
| | KHBW2 | $1.8\times10^5$ | $1\times10^3$ | No Growth |
| | SBW1 | $2.8\times10^5$ | $9\times10^3$ | No Growth |
| | AMW1 | $9\times10^3$ | No Growth | No Growth |
| | KAMW1 | $1.1\times10^4$ | No Growth | No Growth |
| | RAMW1 | $1.68\times10^5$ | No Growth | No Growth |
| | NMW1 | $5\times10^3$ | No Growth | No Growth |
| | AMW1 | $3.5\times10^4$ | No Growth | No Growth |
| | HBW1 | $9\times10^3$ | No Growth | No Growth |

In sampling 3, the bacterial load of affected shrimp samples on nutrient agar was from $1.6\times10^4$ cfu/g to infinity at Mongla Upazilla. On the other hand, in sampling 4, the bacterial load of affected shrimp samples on nutrient agar was from $5\times10^3$ cfu/g to $2.8\times10^5$ cfu/g. Total *Vibrio* count on TCBS agar from affected shrimp was ranged between $2.4\times10^7$ cfu/gto infinity in sampling 3, while total *Vibrio* count on TCBS was from $1\times10^3$ cfu/g to no growth in sampling 4. Besides, total *Vibrio* count on HiChrome agar from Mongla Upazilla was from $1.8\times10^7$ cfu/g to infinity in sampling 1. In sampling 4, no growth of Bacteria on HiChrome agar was found.

**Table3.3. Bacterial load (cfu/g) in shrimp collected from affected farms with AHPND in Morrelganj Upazilla under Bagerhat region.**

| Sample Name | | Bagerhat | | |
|---|---|---|---|---|
| | | TBC | TVC | THC |
| | MeHB | $1.68\times10^5$ | $1.84\times10^7$ | UC |
| | MiHB | $1.68\times10^5$ | $6.8\times10^6$ | $1.88\times10^7$ |
| | SeHB | $3.36\times10^5$ | UC | UC |
| | MaHB | $8.0\times10^4$ | UC | UC |
| **Sampling3** | AfHB | $3.52\times10^5$ | UC | UC |
| | JoHB | $2.16\times10^5$ | UC | UC |
| | MeWB | $3.0\times10^6$ | $1.8\times10^4$ | $3.2\times10^5$ |
| | MiWB | $2.4\times10^4$ | $1.60\times10^4$ | UC |

| | | | | |
|---|---|---|---|---|
| | SeWB | UC | UC | UC |
| | MaWB | $3.0\times10^7$ | $3.0\times10^4$ | $1.40\times10^4$ |
| | AfWB | $4.3\times10^8$ | $7.2\times10^4$ | UC |
| | JoWB | $1.76\times10^8$ | UC | UC |
| **Sampling4** | KBW1 | $1.5\times10^5$ | $2\times10^3$ | No Growth |
| | KHBW2 | $1.8\times10^5$ | $1\times10^3$ | No Growth |
| | SBW1 | $2.8\times10^5$ | $9\times10^3$ | No Growth |

In sampling 3, the bacterial load of affected shrimp samples on nutrient agar was from $2.4\times10^4$ cfu/g to infinity at Bagerhat region. On the other hand, in sampling 4, the bacterial load of affected shrimp samples on nutrient agar was from $1.5\times10^5$ cfu/g to $2.8\times10^5$ cfu/g. Total *Vibrio* count on TCBS agar from affected shrimp was ranged between $1.6\times10^4$ cfu/gto infinity in sampling 3, while total *Vibrio* count on TCBS was from $1\times10^3$ cfu/g to $9\times10^3$ cfu/g in sampling 4. Besides, total *Vibrio* count on HiCrome agar from Morrelganj Upazilla under Bagerhat District was from $1.4\times10^4$ cfu/g to infinity in sampling 3. In sampling 4, no growth of Bacteria on HiCrome agar was found.

**3.3 Isolation of the suspected *V. parahaemolyticus* isolates**

In this work, a total 46 isolates were isolated from different selective medium, among them 18 isolates were selected and purified for detail study towards identification. These 18 colonies were selected on the basis of colony morphology.

**Colony morphology of the selected isolates**

Colony of the selected isolates were found to be different in their form, elevation, margin, surface and color. The dissimilarities of colonial morphology of the selected 18 isolates were presented in table 3.4. Most of the colonies of the isolates were entire, round and smooth in nature.

**Table 3.4. Colony morphology of the 18 selected isolates suspected as *Vibrio parahaemolyticus***

| Isolate number | size | Shape | Pigmentation | Elevation | Margin | Surface |
|---|---|---|---|---|---|---|
| Vp2 | Small | Round | Green | Convex | Entire | Smooth |
| Vp4 | Large | Round | Green | Convex | Entire | Smooth |
| Vp6 | Large | Round | Green | Convex | Entire | Smooth |

| Vp7 | Large | Round | Green | Convex | Entire | Smooth |
|---|---|---|---|---|---|---|
| Vp9 | Small | Round | Green | Convex | Entire | Smooth |
| Vp11 | Small | Round | Green | Flat | Entire | Smooth |
| Vp21 | Medium | Round | Green | Convex | Entire | Smooth |
| Vp23 | Medium | Round | Green | Flat | Entire | Smooth |
| Vp24 | Small | Round | Torquise | Convex | Irregular | Smooth |
| Vp25 | Small | Irregular | Green | Convex | Entire | Smooth |
| Vp26 | Small | Round | Green | Convex | Entire | Smooth |
| Vp27 | Small | Round | Torquise | Flat | Irregular | Smooth |
| Vp30 | Small | Round | Green | Convex | Entire | Smooth |
| Vp35 | Medium | Irregular | Torquise | Convex | Irregular | Smooth |
| Vp38 | Small | Round | Torquise | Convex | Entire | Smooth |
| Vp39 | Small | Round | Green | Convex | Entire | Smooth |
| Vp42 | Medium | Round | Torquise | Convex | Entire | Smooth |
| Vp43 | Small | Round | Torquise | Convex | Entire | Smooth |

A.  Nutrient agar plate with Bacterial colony for Total bacterial count (TBC)

B.TCBS agar plate with Suspected colonies of *Vibrio* spp.

C. HiChrome agar plate with suspected colonies of *Vibrio* spp.

D. HiChrome agar plate with suspected colonies of *Vibrio* spp.

E.Streaking on TSA agar plate with 2% NaCl to obtain single colony of Suspected *V. parahaemolyticus*

F. Streaking on TSA agar plate with 2% NaCl to obtain single colony of Suspected *V. parahaemolyticus*

**Plate 3.1. Photographs showing bacterial colonies in different media**

**A. TCBS agar plate**

**B. TCBS agar plate**

**C. ChromAgar *Vibrio* plate**

**D. ChromAgar *Vibrio* pate**

**D. Hi Chrome agar plate**

**E. HiChrome agar plate**
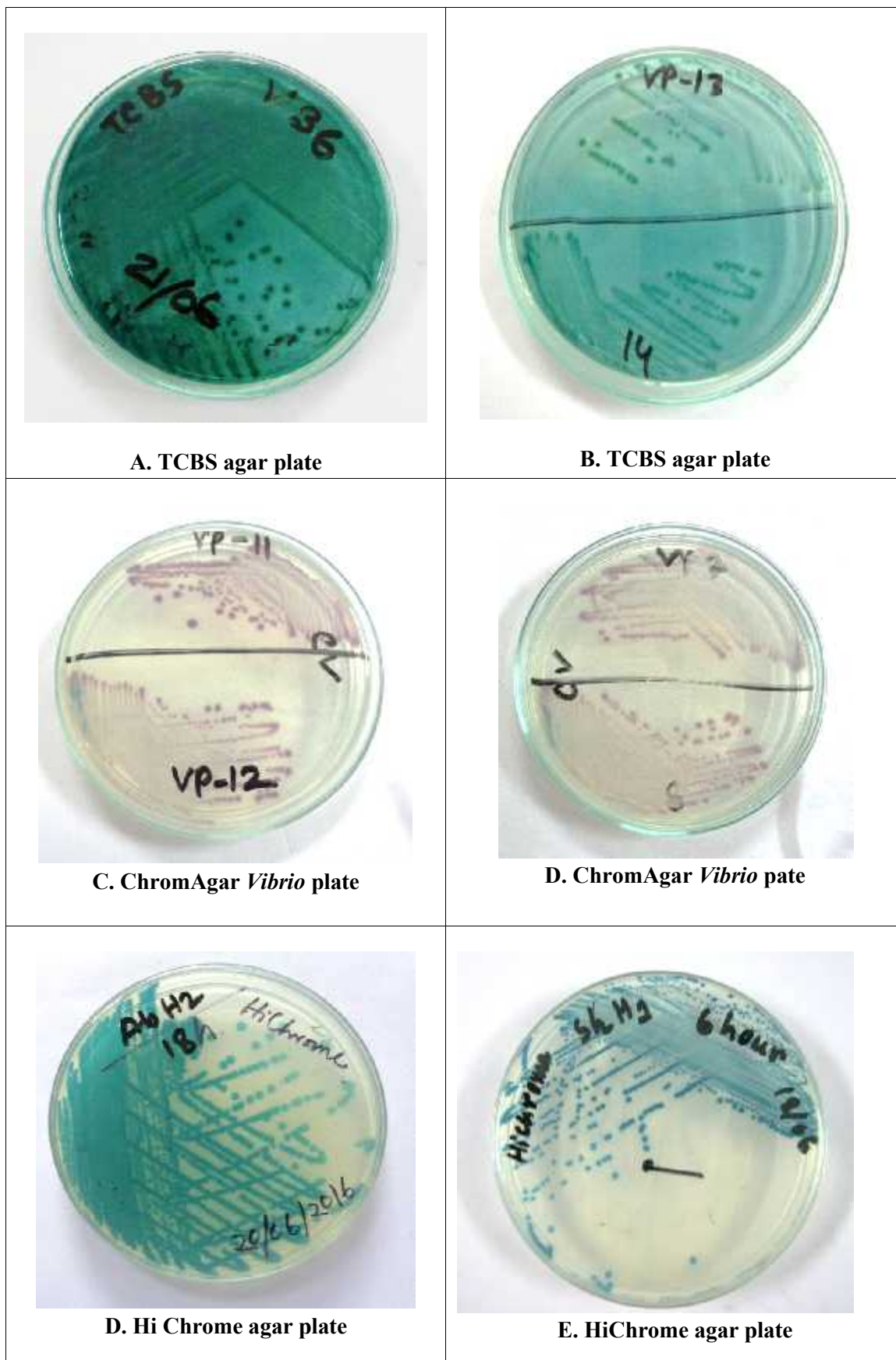
**Plate 3.2. Purification of suspected *V. parahaemolyticus* isolates by streak plate technique on three selective agar plates.**

**3.4 Molecular Identification and Characterization of suspected *V. parahaemolyticus* isolates**

The final concentration and purity of the 18 isolates measured before 16S rRNA sequencing with their corresponding colony ID and sample ID are summarized in table 3.6. The gel autoradiograph shows the size of the PCR product was 1.5 kb.

**3.4.1 Bacterial isolates of *ldh* positive/negative from shrimp ghers affected by AHPND**
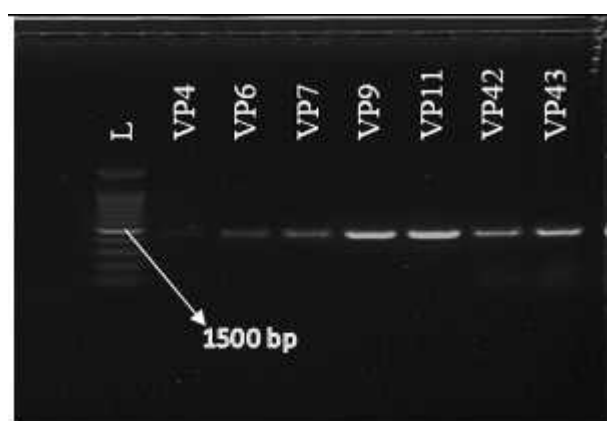


**Fig. 3.2. PCR Product profiles of *ldh* primer generated from 7 representative isolates: Vp2, Vp4, Vp6, Vp7, Vp9, Vp11, Vp42, Vp43 and L denotes DNA ladder of 1Kb**

In this study 18 representative isolates of Vpwere used for the detection of species specific pathogenicity of *V. parahaemolyticus* by using *ldh* primer. Detection for *ldh* gene fragment in the isolates showed positive result for *ldh*.

**Table 3.5. PCR detection results for lecithin-dependent hemolysin (*ldh*) in identified bacterial isolates**

| Isolates ID of Bacteria | *ldh* |
|---|---|
| Vp4 | + |
| Vp6 | + |
| Vp7 | + |
| Vp9 | + |
| Vp11 | + |
| Vp42 | + |
| Vp43 | + |

### 3.4.2 16S rRNA sequence based identification
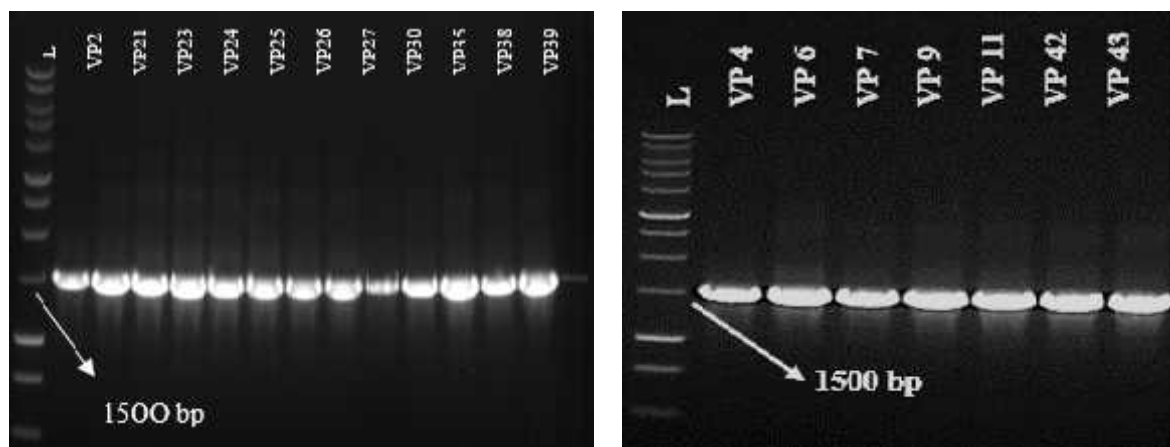


**Fig. 3.3.PCR Product profiles of 16S rRNA primer produced from 18 representative isolates: Vp2, Vp21, Vp23, Vp24, Vp25, Vp27, Vp30, Vp35, Vp38, Vp39, Vp43 and L denotes DNA ladder of 1kb Marker.**

The identification of the 16SrRNA gene sequences of 18 representative isolates (Vp2,Vp4,Vp6,Vp7,Vp9,Vp9,Vp11,Vp21,Vp23,Vp24,Vp25,Vp26,Vp27,Vp35,Vp38,Vp 39,Vp42,Vp43) of 46 isolates through nucleotide BLAST of NCBI is summarized on table 3.6 and 3.7.

It is clear from table that the sequences of all groups of isolates were matched with the strains presented in their respective following column. The table also provides information about scoring *viz*. maximum score, total score, percentage of query coverage, E value and percentage of identification of the identified sequences with NCBI Gene Bank Accession number of the matched sequences.

**Table 3.6. Similarity of sequences of 16S ribosomal RNA gene, partial sequence with BLAST search**

| Sl No. | Isolates ID | Description | Total score | Query coverage | E value | Identity | Accession no of our strains |
|---|---|---|---|---|---|---|---|
| 1 | Vp2 | *Vibrio parahaemolyticus* strain ECSMC9 (KU845385.1) | 2676 | 100% | 0.0 | 99% | KY565404 |
| 2 | Vp4 | *Vibrio parahaemolyticus* strain CHB-35 (KR347292.1) | 2571 | 99% | 0.0 | 99% | KY565405 |
| 3 | Vp6 | *Vibrio parahaemolyticus* strain Xmb045 (KT986171.1) | 2560 | 100% | 0.0 | 99% | KY565406 |
| 4 | Vp7 | *Vibrio parahaemolyticus* strain  NSP1 (JN188415.1) | 2575 | 100% | 0.0 | 99% | KY565407 |
| 5 | Vp9 | *Vibrio parahaemolyticus* strain XG409 (JQ948037.1) | 2575 | 99% | 0.0 | 99% | KY565408 |
| 6 | Vp11 | *Vibrio parahaemolyticus* strain M2-31 (KC210810.1) | 2538 | 99% | 0.0 | 99% | KY565409 |
| 7 | Vp21 | *Vibrio parahaemolyticus* strain M2-11 (KC210810.1) | 2571 | 99% | 0.0 | 99% | KY565410 |
| 8 | Vp23 | *Vibrio parahaemolyticus* strain  CHB-5 (KR347274.1) | 2573 | 99% | 0.0 | 99% | KY565411 |
| 9 | Vp24 | *Vibrio parahaemolyticus* strain | 2571 | 99% | 0.0 | 99% | KY565412 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | Aj2010072802A90(JF432066.1) | | | | | |
| 10 | Vp25 | *Vibrio parahaemolyticus* strain FORC_018 (CP013826.1) | 27998 | 100% | 0.0 | 99% | KY565413 |
| 11 | Vp26 | *Vibrio parahaemolyticus* strain L41 (KC884619.1) | 2573 | 99% | 0.0 | 99% | KY565414 |
| 12 | Vp27 | *Vibrio parahaemolyticus* strain CZN-9 (KR347248.1) | 2573 | 99% | 0.0 | 100% | KY565415 |
| 13 | Vp30 | *Vibrio parahaemolyticus* strain CHB-40 (KR347297.1) | 2573 | 99% | 0.0 | 99% | KY565416 |
| 14 | Vp35 | *Vibrio parahaemolyticus* strain CZN-34 (KR347270.1) | 2560 | 99% | 0.0 | 99% | KY565417 |
| 15 | Vp38 | *Vibrio parahaemolyticus* strain CZN-9 (KR347248.1) | 2567 | 99% | 0.0 | 99% | KY565418 |
| 16 | Vp39 | *Vibrio parahaemolyticus* strain CHB-33 (KR347290.1) | 2564 | 99% | 0.0 | 99% | KY565419 |
| 17 | Vp42 | *Vibrio parahaemolyticus* strain NSTH21 (KF886632.1) | 2555 | 99% | 0.0 | 99% | KY565420 |
| 18 | Vp43 | *Vibrio parahaemolyticus* strain CZN-7 (KR347246.1) | 2566 | 99% | 0.0 | 99% | KY565421 |

The amplified PCR products were sequenced and aligned with the 16S rRNA gene sequences in GenBank and the sequences showed (>99%) homology with the 16S rRNA gene of *V. parahaemolyticus* strain ECSMC9, *V. parahaemolyticus* strain Xmb045, *V. parahaemolyticus* strain NSP1,*V. parahaemolyticus* strainFORC_018.

The studied Vp2, Vp6, Vp7 and Vp15 strains were showed 100% query coverage and 99% homology with *V. parahaemolyticus* strain ECSMC9, Xm045 and FORC_018. All other isolates showed 99% query coverage and 99% homology with the isolates that are giving table 3.6. All groups were identified as *V. parahaemolyticus* which are gram negative bacteria that cause AHPND of shrimp.

In table, a total summary of identification of all isolates have shown with their corresponding isolates name that were collected from three different Upazilla of Satkhira and Bagerhat District.

**Table 3.7. Summary of molecular identification of 18 representative suspected *Vibrioparahaemolyticus*isolates for 16S rRNA sequencing from Black Tiger Shrimp**

| Sample ID | Isolate ID | Sample details | Identified species |
|---|---|---|---|
| Vp2 | SH1 | Shrimp, Satkhira | *V. parahaemolyticus* |
| Vp4 | AH2 | Shrimp, Satkhira | *V. parahaemolyticus* |
| Vp6 | MiH3 | Shrimp, Satkhira | *V. parahaemolyticus* |
| Vp7 | OH4 | Shrimp, Satkhira | *V. parahaemolyticus* |
| Vp9 | BH5 | Shrimp, Satkhira | *V. parahaemolyticus* |
| Vp11 | M2H6 | Shrimp, Satkhira | *V. parahaemolyticus* |
| Vp21 | AnH1 | Shrimp, Satkhira | *V. parahaemolyticus* |
| Vp23 | AbH2 | Shrimp, Satkhira | *V. parahaemolyticus* |
| Vp24 | AbH2 | Shrimp, Satkhira | *V. parahaemolyticus* |
| Vp25 | AbH2 | Shrimp, Satkhira | *V. parahaemolyticus* |
| Vp26 | BaH1 | Shrimp, Satkhira | *V. parahaemolyticus* |
| Vp27 | ShW1 | Shrimp, Satkhira | *V. parahaemolyticus* |
| Vp30 | AbW1 | Shrimp, Satkhira | *V. parahaemolyticus* |
| Vp35 | BaHM | Shrimp, Mongla | *V. parahaemolyticus* |
| Vp38 | MiHB | Shrimp, Bagerhat | *V. parahaemolyticus* |

| Vp39 | MeHB | Shrimp, Bagerhat | *V. parahaemolyticus* |
| Vp42 | AfHB | Shrimp, Bagerhat | *V. parahaemolyticus* |
| Vp43 | SeHB | Shrimp, Bagerhat | *V. parahaemolyticus* |

### 3.4.3 Multiple Sequence Alignment

A multiple sequence alignment is shown by view CLUSTALW to compare the sequences. After comparing the obtained sequence of 18 *V. parahaemolyticus* sp. Group (Vp2, Vp4, Vp6, Vp7, Vp9, Vp11, Vp21, Vp23, Vp24, Vp25, Vp30, Vp35, Vp38, Vp39), total 16 sites were found polymorphic. Therefore, the dissimilarity was 1.15% (16/1386).

Among 18 *V. parahaemolyticus* sp. sequence, Vp6 and Vp9 were exhibited the highest number of polymorphic sites with each other. A total 10 regions (405, 406, 407, 408, 409, 420, 421, 422, 423, 425, and 426) were found dissimilarity on these strains.

Six strains (Vp2, Vp4, Vp21, Vp27, Vp35 and Vp39) were highly similar each other on their polymorphic region but dissimilar to only two site (34, 35) of them. Vp2 were collected from Satkhira (Safiqul, 22Bigha), Vp4 were collected from Satkhira (Abidullah, 30 Bigha), Vp21 were collected from Satkhira (Anwarul, 10 Bigha), Vp27 were sampled from Satkhira (Shahbuddin 1, 15 Bigha), Vp 35 were collected from Mongla (Bappi, 2.5 Bigha) and Vp39 from Bagerhat (Methun, 6 Bigha) region.

On the other hand Vp11, Vp35, Vp39, Vp43 show their dissimilarity in common sites (1207). Among the aligned sequences, Vp11 were sampled from Satkhira (Mossaraf, 20 Bigha), Vp43 were sampled from Bagerhat (Selim, 8 Bigha). Only one site (985) of Vp42 was found dissimilar that was collected from Bagerhat regions.
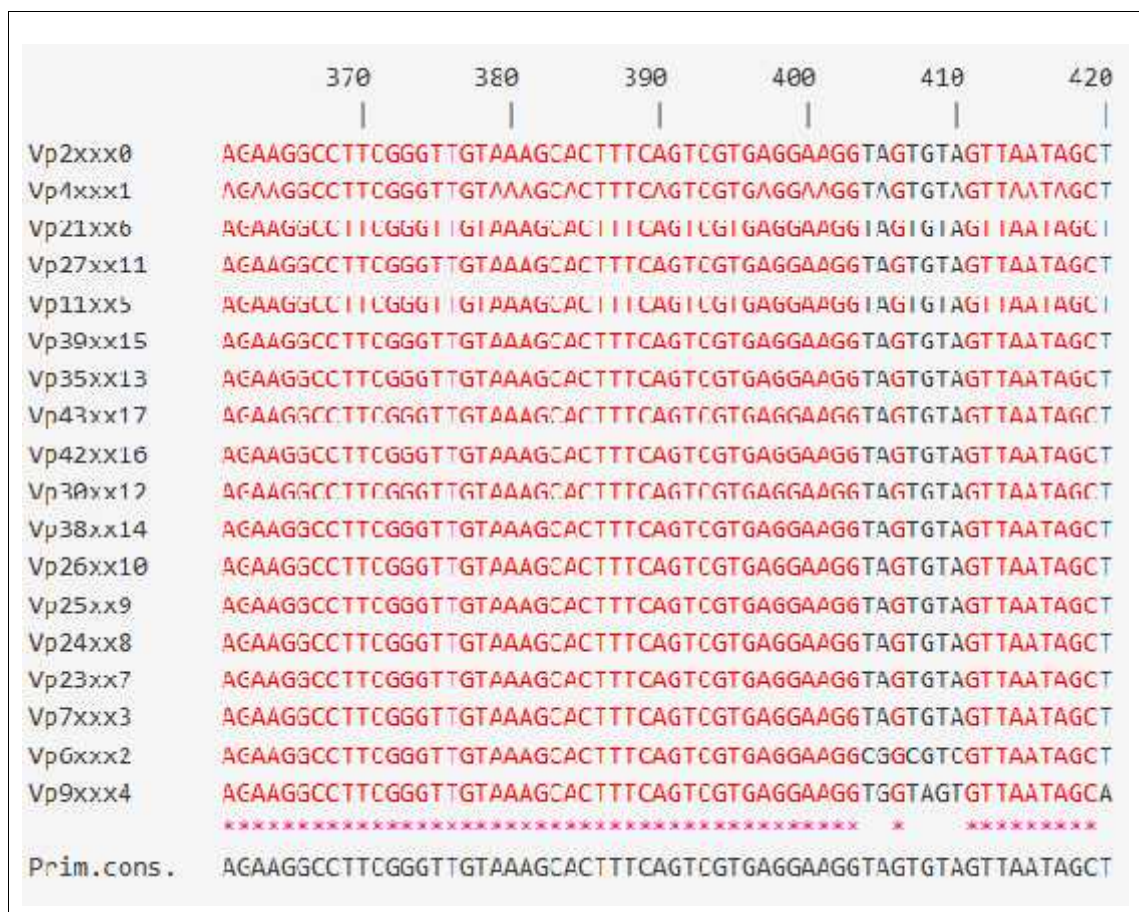
**Fig. 3.4.A Multiple sequence alignment of 16S rRNA gene fragments of the closely related group of 18 Vp strains where black among the red indicates polymorphic sites. (CLUSTLAW view, alignment width 60). Alignment length of *Vibrio parahaemolyticus* isolates 1386 bp.**

**Alignment Data for VP isolates**

Alignment length: 1386

Identity: 1370 is 98.85%

Strongly similar: 0 is 0.00%

Weakly similar: 0 is 0.00 %

Different: 16 is 1.15 %
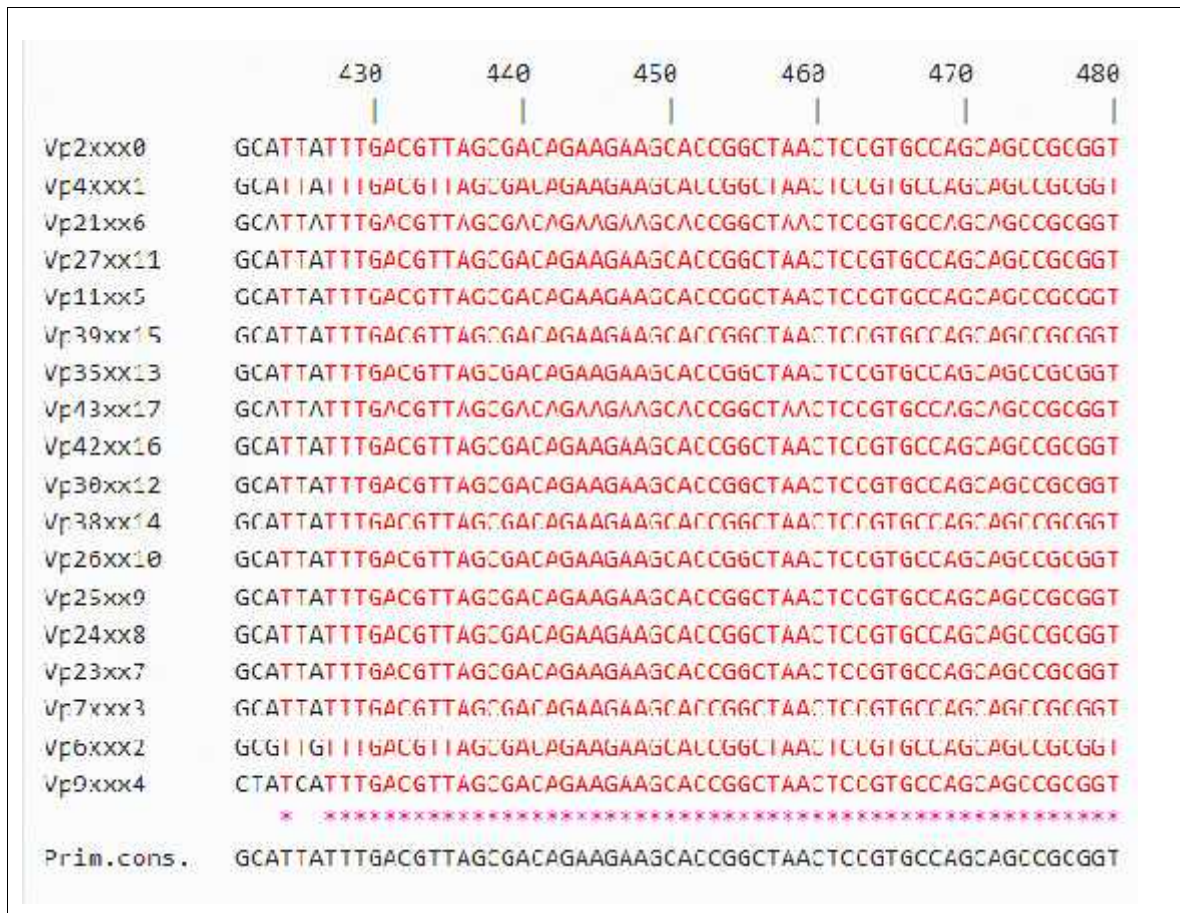
**Fig. 3.4.BMultiple sequence alignment of 16S rRNA gene fragments of the closely related group of 18 Vp strains where black among the red indicates polymorphic sites. (CLUSTLAW view, alignment width 60). Alignment length of *Vibrio parahaemolyticus* isolates 1386 bp.**

### 3.4.4 Phylogenetic Analysis

Phylogenetic analysis by constructing the phylogenetic tree based on the partial 16S rRNA gene sequences of the representative 18 Vp isolates using neighbor-joining and BioNJ algorithms confirmed the taxonomic position of the isolates (Vp2, Vp4, Vp6, Vp7, Vp9, Vp11, Vp21, Vp23 Vp24, Vp25, Vp26, Vp27, Vp30, Vp35, Vp38, Vp39, Vp42, Vp43) and the comparison of these bacterial strain sequences with other homologous bacterial sequences.

The phylogenetic tree indicates that Vp21, Vp4 and Vp2 found closely related to Vp27 which supporting their similarity with the allocated strains Vp strains CHB-35 and Vp strains ECSMC9.It is clear that Vp11 and Vp39 are closely related to Vp35 and Vp43 which supporting their similarity with the allocated strains Vp strains Xm045. Phylogenetic tree also confirmed that the taxonomic position of Vp42, Vp7, Vp23, Vp24, Vp25, Vp26, Vp30, Vp38 supporting their similarity with the Vp6 and Vp9 which is closely related with the allocated strains Vp strains Xmo45.

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (*Tamura* et.al,1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 27 V. parahaemolyticus nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There were a total of 1384 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

**Phylogeny Test**

Test of Phylogeny: Bootstrap method

No. of Bootstrap Replications: 1000

Substitutions Type: Nucleotide

Model/ Method: Tamura-Nei model

Rates among sites: Uniform rates

ML Heuristic Method: Nearest-Neighbor-Interchange (NNI)

No of Sites: 1386
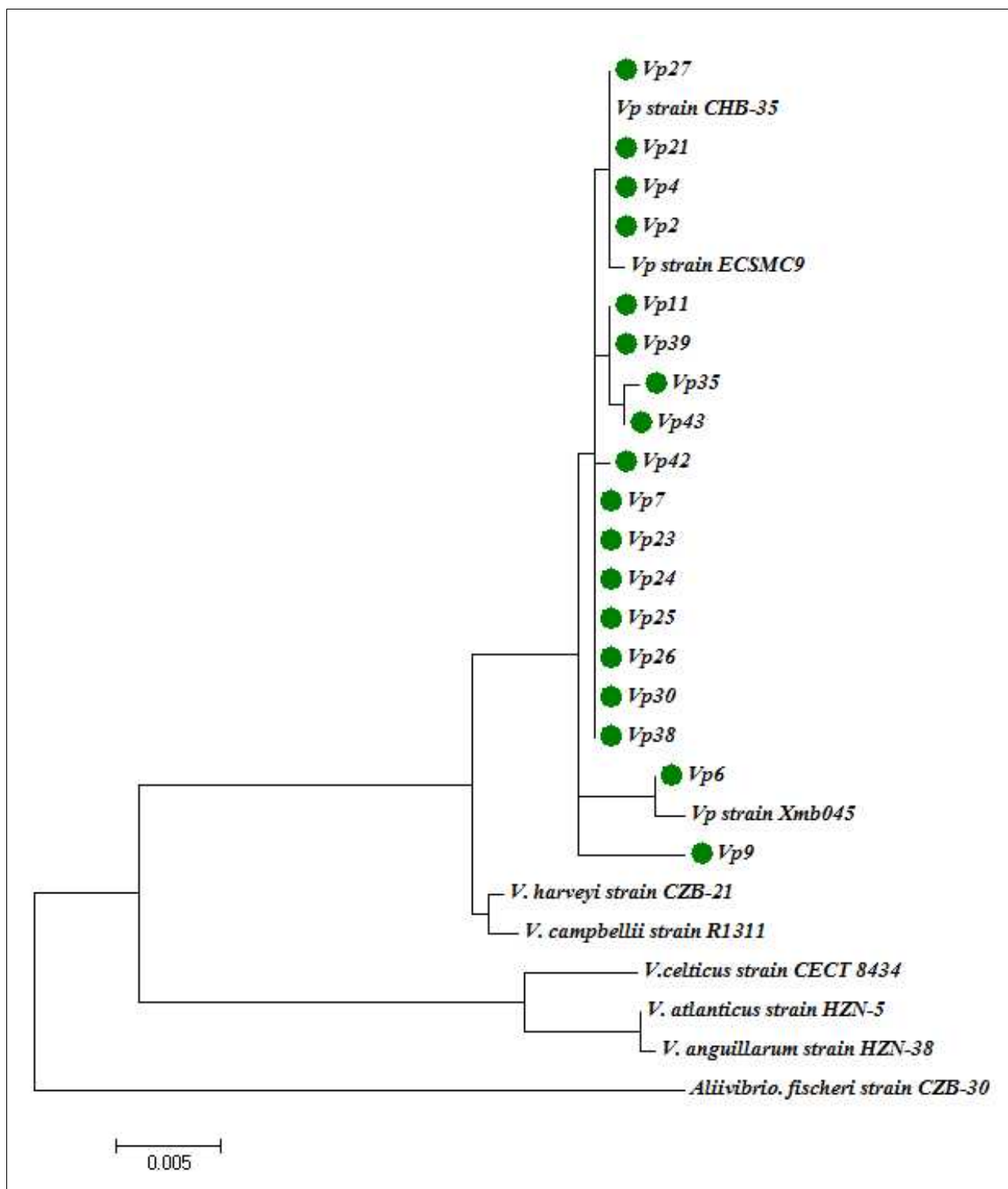
**Fig. 3.5.Molecular Phylogenetic analysis by Maximum Likelihood method**

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura et al.,1993). The tree with the highest log likelihood (-2805.5279) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances

estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 27 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1384 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Tamura et al., 2006).

**3.4.5 Detection of Human pathogenic *V. parahaemolyticus* isolatesusing the tdh gene primers**
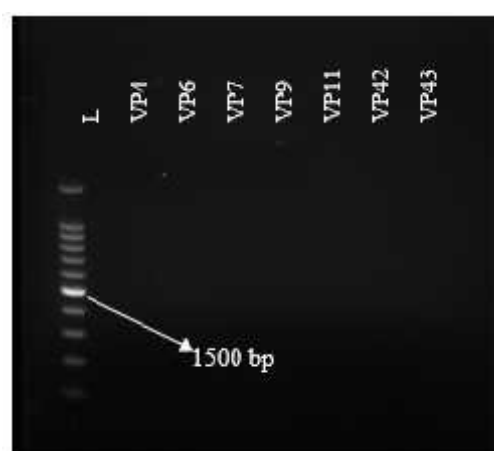


**Fig. 3.6.*tdh* PCR amplicons obtained from representative *V. parahaemolyticus* isolates.**

18*Vibrio parahaemolyticus*isolates were used for detection of human pathogenicity by using *tdh* primer. Detection for *tdh* gene fragment in the isolates showed negative results for *tdh.*

**3.4.6 Successful PCR detection of VP<sub>AHPND</sub> isolates using AP3 primer**
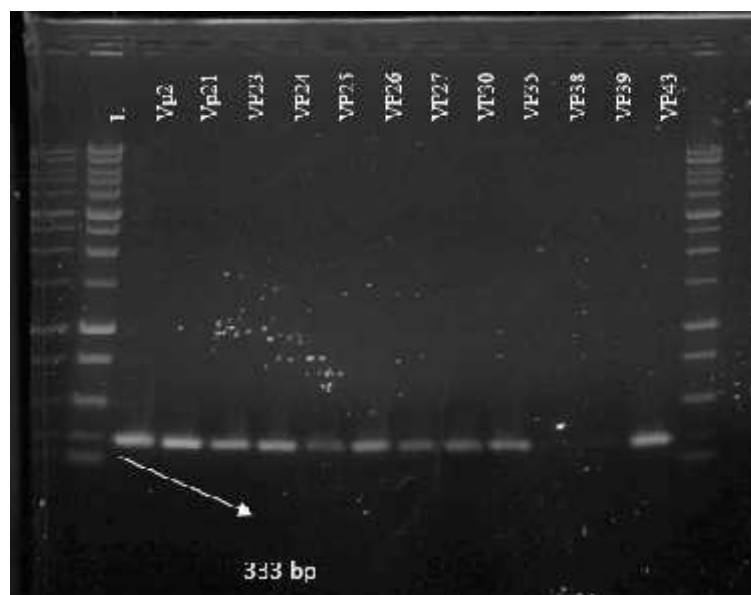


**Fig. 3.7. PCR Product profiles of AP3 primer produced from 12 representative isolates: VP2, VP21, VP23, VP24, VP25, VP27, VP30, VP35, VP38, VP39, VP43 and L denotes DNA ladder of 1kb Marker.**
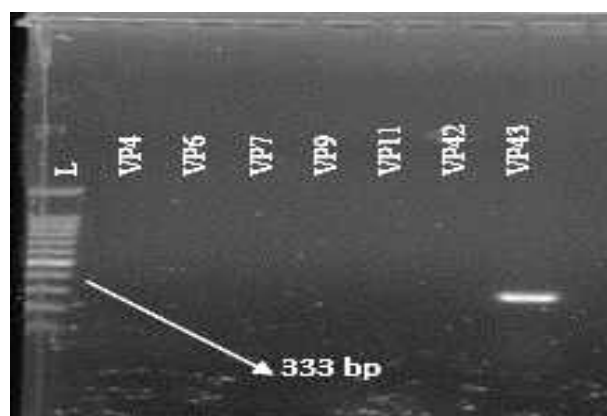


**Fig. 3.8. Agarose gel of PCR amplicons from VP<sub>AHPND</sub> using the AP3 method. Here L denotes DNA ladder of 1kb Marker.**

The materials and methods section described a PCR protocol called the AP3 method for amplification of the complete ToxA gene to detect VPAHPND isolates.The primers are specific for *V. parahaemolyticus* strains causing AHPND. In present study, out of 18 isolates 12 isolates were positive for AP3 specific primer that indicated those shrimp were infected with *V. parahaemolyticus* AHPND (Table 3.9). Six isolates were negative for AP3 specific primer. To validate the test, a total of 18 representative bacterial isolates

were tested in this way which were commonly found in shrimp hepatopancreas. Results for all 12 AHPND isolates were positive with the test, while results for all the remaining isolates were negative.

**Table3.8. Detection of VP$_{AHPND}$ isolates using AP3 primer based PCR assay.**

| Isolates name | Region | Gher name | AP3 |
|---|---|---|---|
| Vp4 | Satkhira | Abidullah | - |
| Vp6 | Satkhira | Mossarraf | - |
| Vp7 | Satkhira | Obydullah | - |
| Vp9 | Satkhira | Babul | - |
| Vp11 | Satkhira | Mossarraf | - |
| Vp2 | Satkhira | Safiqul | + |
| Vp21 | Satkhira | Anwarul | + |
| Vp23 | Satkhira | Abdullah 1 | + |
| Vp24 | Satkhira | Abdullah 2 | + |
| Vp25 | Satkhira | Abdullah 2 | + |
| Vp26 | Satkhira | Bablu | + |
| Vp27 | Satkhira | Shahbuddin 1 | + |
| Vp30 | Mongla | Abdullah 2 | + |
| Vp35 | Bagerhat | Bappi | + |
| Vp38 | Bagerhat | Mizan Mia | + |
| Vp39 | Bagerhat | Methun | + |
| Vp42 | Bagerhat | Afsar | - |
| Vp43 | Bagerhat | Selim | + |

**3.4.7 Successful PCR detection of VP<sub>AHPND</sub> isolates using AP4 primer**
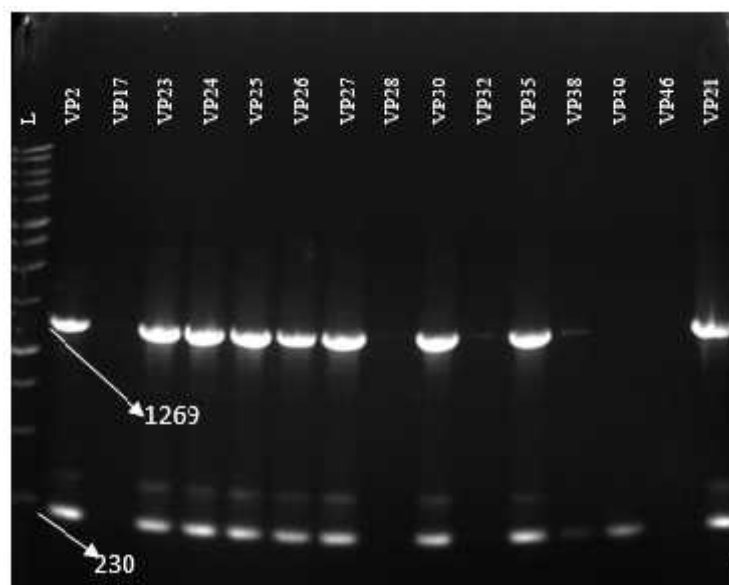


**Fig. 3.9.Agarose gel electrophoresis of PCR amplified DNA fragments from representative isolates using AP4 nested PCR: Vp2, Vp17, Vp21, Vp23, Vp24, Vp25, Vp26, Vp27, Vp28, Vp30, Vp32, Vp35, Vp38, Vp39, Vp46 and L denotes DNA ladder of 1kb Marker**

From 46 bacterial isolates obtained from the hepatopancreas and whole sample of individual shrimp specimens from the AHPND outbreaks shrimp gher by streaking on TSA plates, 18 isolates were arbitrarily selected and named as Vp4, Vp6, Vp7, Vp9, Vp11, Vp42, Vp43, Vp2, Vp21, Vp23, Vp24, Vp25, Vp26, Vp26, Vp27, Vp30, Vp35, Vp38 and Vp39. They were examined for growth on TCBS agar, HiCrome agar, ChromAgar*Vibrio*and all were able to grow and to produce green, Torquise and purple colonies were subjected to PCR using the specific primer pairs AP4.A total 14 of the isolates were PCR positive for the *V. parahaemolyticus* AHPND by AP4 method (Fig.3.9).
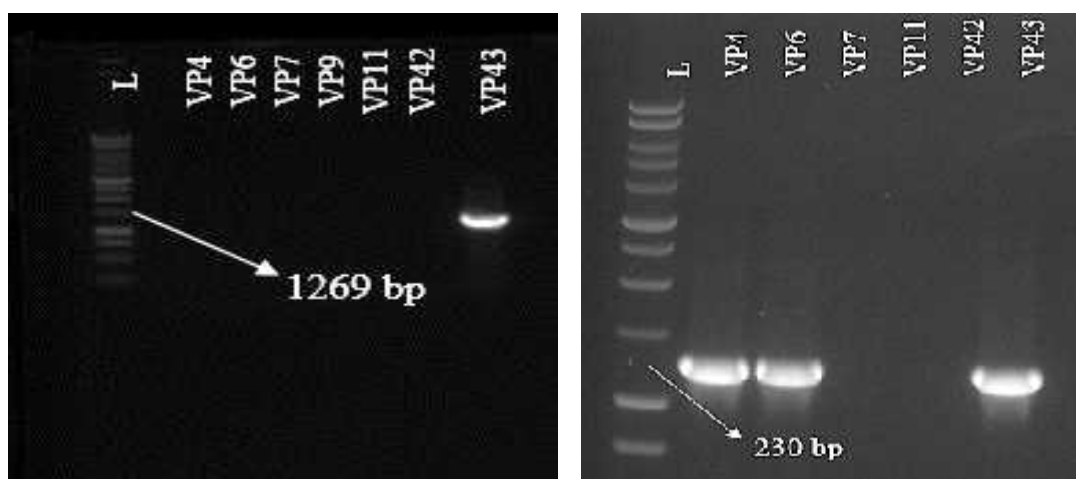
**Fig. 3.10. AP4 (1ˢᵗ and 2ⁿᵈ step) nested PCR amplicons obtained from representative**
***V. parahaemolyticus* isolates.**

In this work *V. parahaemolyticus* AHPND was isolated from 15 different ghers.For AP4
primer, Vp4, Vp9 andVp11, these three colonies selected from shrimp samples of the
three ghers (Abidullah 30 Bigha, Babul 20 Bigha and Mossaraf 20 Bigha) of Alipur,
Satkhira were negative for *V. parahaemolyticus* AHPND and indicated that those shrimp
might not be infected with *V. parahaemolyticus* AHPND or a mild infection might have
occurred; therefore, more bacterial isolates would be needed for confirmation. It was of
interest that isolates 18 *V. parahaemolyticus* mostly obtained from Satkhira region
shrimp sample. Only one isolate (Vp35) and three isolates (Vp38, Vp39 and Vp43) were
positive for AP4 isolated from Mongla and Bagerhat area.

**Table 3.9:Detection of VP$_{AHPND}$ isolates using AP4 primer based PCR assay.**

| Isolate Name | Region | AP4 primer based PCR Assay |
|:---:|:---:|:---:|
| Vp2 | Satkhira | + |
| Vp17 | Satkhira | - |
| Vp21 | Satkhira | + |
| Vp23 | Satkhira | + |
| Vp24 | Satkhira | + |
| Vp25 | Satkhira | + |
| Vp26 | Satkhira | + |
| Vp27 | Satkhira | + |

| | | |
|---|---|---|
| Vp28 | Satkhira | - |
| Vp30 | Mongla | + |
| Vp32 | Mongla | - |
| Vp35 | Bagerhat | + |
| Vp38 | Bagerhat | - |
| Vp39 | Bagerhat | + |

## 3.5 Antibiotic Susceptibility Test of AHPND positive *V. parahaemolyticus* isolates

During present study 18 bacterial isolates of AHPND affected shrimp were tested against 14 common antibiotics viz. Amikacin (30µg), Ampicillin (10µg), Amoxicillin (10µg), Chloramphenicol (30µg), Ciprofloxacin (5µg), Erythromycin (15µg), Gentamycin (10µg), Kanamycin (30µg), Nalidixic Acid (30µg), Nitrofurantion (300µg), Polymyxin B (300unit), Streptomycin (10µg), Sulphamethoxazole (25µg) and tetracycline (30µ). Result of antibiotic susceptibility test are shown in table 3.11.

All isolates of *V. parahaemolyticus* were resistant to Gentamycin where all isolates of *V. parahaemolyticus* showed sensitive to Chloramphenicol, Nitrofurantion, Tetracycline and Nalidixic Acid. Among 18 isolates of *V. parahaemolyticus*, 16 isolates showed intermediate and 3 isolates showed sensitivity to Ciprofloxacin. On the other hand, 17 isolates showed Amikacin sensitivity and 2 isolates showed intermediate but no resistant isolate was found.

Ampicillin, Erythromycin, Streptomycin and Sulphamethoxazole were sensitive, intermediate and resistance for all isolates of *V. parahaemolyticus*. Amoxycillin and Polymyxin were sensitive and resistant for all isolates of *V. parahaemolyticus* but no isolates were intermediate for Amoxycillin and Polymyxin.

**Table 3.10. Result of Antibiotic susceptibility test against 14 Antibiotics for 18 *V. parahaemolyticus* isolates**

| Isolate name | Amikacin (30µg) | Amoxycillin(10µg) | Ampicillin (10µg) | Chloramphinocol (30µg) | Ciprofloxacin (5µg) | Erythromycin (15µg) | Gentamycin (10µg) | Kanamycin (30µg) | Nalidixic acid (30µg) | Nitrofurantion (300unit) | Polymyxin (300unit) | Streptomycin (10µg) | Sulphamethoxazole (25µg) | Tetracycline (30µg) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Vp2 | S | R | R | S | I | S | R | S | S | S | S | I | R | S |
| Vp4 | S | R | R | S | I | I | R | S | S | S | R | S | I | S |
| Vp6 | I | R | R | S | I | S | R | I | S | S | S | S | I | S |
| Vp7 | S | R | R | S | I | S | R | I | S | S | S | S | S | S |
| Vp9 | S | R | R | S | I | S | R | S | S | S | S | S | I | S |
| Vp11 | S | R | R | S | I | S | R | S | S | S | S | S | R | S |
| Vp17 | S | R | R | S | I | I | R | I | S | S | S | I | I | S |
| Vp19 | S | R | S | S | I | I | R | I | S | S | R | I | I | S |
| Vp21 | S | R | S | S | I | I | R | I | S | S | S | I | S | S |
| Vp24 | S | R | S | S | I | S | R | I | S | S | S | R | I | S |
| Vp26 | S | R | S | S | I | S | R | S | S | S | S | R | R | S |
| Vp27 | S | R | R | S | I | S | R | S | S | S | S | S | S | S |
| Vp35 | S | I | S | S | S | I | R | S | S | S | S | S | S | S |
| Vp38 | S | R | S | S | S | S | R | S | S | S | S | S | I | S |
| Vp39 | S | S | S | S | I | S | R | S | S | S | S | I | S | S |
| Vp41 | S | R | S | S | I | R | R | I | S | S | S | R | R | S |
| Vp42 | I | R | S | S | I | I | R | I | S | S | S | I | S | S |
| Vp43 | S | R | R | S | S | S | R | S | S | S | S | I | I | S |

*S= Sensitivity, I= Intermediate, R= resistant

**Table 3.11. Percentage of antibiotic sensitivity, intermediate and resistance for 18 *V. parahaemolyticus*isolates against 14 antibiotics**

| Name of Antibiotics | Isolates of Bacteria (N=18) | | |
|---|---|---|---|
| | R% | I% | S% |
| Amikacin (30µg) | 0% | 11.11% | 88.89% |
| Amoxycillin(10µg) | 88.89% | 5.56% | 5.56% |
| Ampicillin (10µg) | 44.45% | 0% | 55.55% |
| Chloramphinocol (30µg) | 0% | 0% | 100% |
| Ciprofloxacin (5µg) | 0% | 16.67% | 83.33% |
| Erythromycin (15µg) | 38.89% | 5.55% | 55.55% |
| Gentamycin (10µg) | 100% | 0% | 0% |
| Kanamycin (30µg) | 0% | 44.45% | 55.55% |
| Nalidixic acid (30µg) | 0% | 0% | 100% |
| Nitrofurantion (300unit) | 0% | 0% | 100% |
| Polymyxin (300unit) | 11.11% | 0% | 88.89% |
| Streptomycin (10µg) | 11.11% | 44.45% | 44.45% |
| Sulphamethoxazole (25µg) | 22.22% | 44.45% | 33.33% |
| Tetracycline (30µg) | 0% | 0% | 100% |

*R= Resistant, S=Sensitive, I= Intermediate

Culture and sensitivity showed all the strain were resistant to Gentamycin whereas all the strain showed sensitivity to Chloramphenicol, Nalidixic Acid,Nitrofurantion and Tetracycline (Table 3.13).

**Table 3.12. Susceptibility of 18 representative AHPND positive isolates of *V. parahaemolyticus* to tested antibiotics**

| Isolates name | Antibiotics | | |
|---|---|---|---|
| | Sensitive | Intermediate | Resistant |
| Vp2 | AK, C, E, K, NA,  F, PB, TE | CIP, S | AML, AMP, CN, RL |
| Vp4 | AK, C, K, NA, F, S, TE | CIP, E, RL | AML, AMP,CN |
| Vp6 | C, E, NA, F, PB, S, TE | AK, CIP, K, RL | AML, AMP,CN, |
| Vp7 | AK, C, E, NA, F, PB, S, TE, RL | CIP, K | AML, AMP, CN |

| | | | |
|---|---|---|---|
| Vp9 | AK, C, E, K, NA, F, PB, S, TE | CIP, RL | AML, AMP, CN |
| Vp11 | AK, C, E, K, NA, F, PB, S, TE | CIP | AML, AMP, CN, RL |
| Vp17 | AK, C, NA, F, PB, TE | CIP, E, K, S, RL | AML, AMP, CN |
| Vp19 | AK, AMP, AML, NA, F, TE | CIP, E, K, S, RL | AML, AMP, CN |
| Vp21 | AK, AMP, C, NA, F, PB, RL, TE | CIP, E, CN, S | AML, CN |
| Vp24 | AK, AMP, C, E, NA, F, PB, TE | CIP, K, RL | AML, CN, S |
| Vp26 | AK, AMP, C, E, K, NA, F, PB, TE | CIP | AML, AMP, CN |
| Vp27 | AK, C, E, K, NA, F, PB, RL, TE | CIP | AML, AMP, CN |
| Vp35 | AK, AMP, C, CIP, K, NA, F, PB, S, RL, TE | AML, E | CN |
| Vp38 | AK, AMP, C, CIP, E, K, NA, F, PB, S, TE | RL | AML, CN |
| Vp39 | AK, AML, AMP, C, E, K,NA, F, PB, RL, TE | CIP, S | CN |
| Vp41 | AK, AMP, C, NA, F, PB,TE | CIP, K | AMP, E, CN, S, RL |
| Vp42 | C, CIP, NA, F, PB, RL, TE | AK, C, E, K,S | AML, CN |
| Vp43 | AK, C, CIP, K, E, NA, F, PB ,TE | S, RL | AML, AMP, CN |

**A.Antibiogram of Vp 3 against Nitrofurantion, Nalidixic Acid, Polymyxin B, Kanamycin**

**B. Antibiotic susceptibility of Vp39 against Ampicillin, Amoxycillin, Amikacin, Ciprofloxacin**

**C.Antibiotic susceptibility of Vp21 against Nitrofurantion, Nalidixic Acid, Polymyxin B, Kanamycin**

**D.Antibiotic susceptibility of Vp 17 against Ampicillin, Amoxycillin, Amikacin**

**Plate 3.3. Antibiotic susceptibility of AHPND positive *V. parahaemolyticus*where clear zone indicates the degree of antibiotic sensitivity.**

# Chapter 4

# Discussion

*Vibrio parahaemolyticus* is ubiquitous in the marine environment. Both pathogenic and nonpathogenic strains exist. The isolates that are responsible for AHPND are extremely virulent. With the intensification of shrimp farming worldwide, new pathogens are seen to be emerged frequently. The first step in preventing the step of a newly recognized disease is to identify or detect the causative agent. The present study was conducted to detect the presence of *V. parahaemolyticus* and to confirm their virulence properties by the presence of *tdh*. Testing by PCR has become a standard for screening of major disease causing agents in cultured shrimp including viruses, bacteria and parasites that may cause diseases in ghers.

## 4.1 Disease signs in shrimp samples suspected with AHPND

Moribund shrimp collected from farms affected with AHPND presented slightly expanded chromatophores, lethargy, erratic swimming, and empty guts. Diseased shrimp typically presented HP discoloration and an organ with an aqueous consistency and as the infection developed, the organ consistency became rubbery. After the dark membrane that overlays the HP was removed, a pale-colored organ was observed. Hepatopancreas (HP) often pale to white HP due to pigment loss in the connective tissue capsule was observed. Tran et al. (2013) reported that Shrimp with early AHPND will show a pale to white HP due to pigment loss in the HP R-cells, as well as atrophy of the HP that may reduce the expected size of theorgan by 50% or more. In the terminal phase of the disease, black streaks or spots (due to melanin deposition from hemocyte activity) appear in the HP. Lai et al. (2015) reported that an atrophied pale hepatopancreas is the most commonly observed sign of AHPND in diseased shrimp.

## 4.2 Quantitative enumeration of bacterial density in shrimp samples

In this study, comparative analysis of TBC, TVC on TCBS and on HiChrome showed little variation of AHPND affected shrimp from region to region. In Satkhira Sadar Upazilla TBC was ranged from $3 \times 10^5$ to $6.37 \times 10^8$ cfu/g. Infinity number of TBC was found in some gher in Satkhira Sadar Upazilla. On the other hand, TBC in Mongla Upazilla was ranged from $1.12 \times 10^5$ to $1.60 \times 10^8$ cfu/g. Like Satkhira Sadar Upazilla, infinity TBC was found in only one gher of Mongla from which AHPND affected

shrimp was collected. In Morrelganj Upazilla under Bagerhat district TBC was found $8.0\times10^4$ to $4.3\times10^8$ cfu/g. Infinity number of TBC was found in only one gher of Bagerhat Upazilla.

The bacterial abundance of $10^6$–$10^8$ cfu/g indicates the importance of bacteria in maintaining sediment and water quality, influencing the health status of cultured penaeids (Gopal et al., 2015). Solomon et al. (2012) reported that Total aerobic count ranged from 1.84 x $10^7$ to 3.1 x $10^7$ cfu/g with an average of 2.6 x $10^7$ cfu/g or log count/g of 7.39 $\pm$ 0.14. Yousuf etal. (2008) found that the total counts ranged from $2.04\times10^2$ to $4.5\times10^5$ and $1.08\times10^2$ to$1.2\times10^5$ cells for the shrimp and prawn samples respectively. According to the International Commission on the Microbiological Speciation of Foods (ICMSF, 1986) guideline, acceptable limit of total bacterial counts for giant prawns and white fish are $10^6$ and $5\times10^5$ cfu/g. In this study, total bacterial count was maximum $6.37\times10^8$ cfu/g which exceed the acceptable limit. So this study clarified that the collected shrimp samples from three different region were unacceptable for the consumption.

On total *Vibrio* like colony count, enumerated bacterial count was ranged from $2\times10^3$ to $1.6\times10^6$ cfu/g for representative gher of Satkhira Sadar Upazilla from which sample was collected. But uncountable number of total *Vibrio* like colony count was found in some gher of Satkhira Sadar Upazilla. On the other hand in Mongla Upazilla total *Vibrio* like colony count was found from $1\times10^3$ to $2.40\times10^7$ cfu/g. Interestingly both uncountable total *Vibrio* like colony and no growth of bacteria were found in representative gher of Mongla Upazilla from which samples were collected. While total *Vibrio* like colony count was found from $1\times10^3$ to $1.84\times10^7$ cfu/g in Morrelganj Upazilla under Bagerhat District. Infinity number of total *Vibrio* like colony count was also found in Morrelganj Upazilla under Bagerhat District.

Vibriosconstitute a major portion of the microbiota in brackish water pond ecosystem. In shrimp farms from India, Otta et al. (1999) noted that *Vibrio* species accounted for 38–81% of the bacterial biota. Gopal et al. (2015) reported that the water samples analyzed showed a higher density of culturable vibrios in west coast (~$10^4$ cfu/ ml) compared to east coast (~$10^2$ cfu/ml) samples, confirming an earlier report by Otta et al. (1999).Hossain et al. (2012) found that Total *Vibrio* counts were ranged up to 2.5 × $10^3$ cfu/gand 60 cfu/g inshrimp and Gher water samples, respectively. Results revealed that

the total *Vibrio* count were found to be higher in samples taking from the market shops in Dhaka city than Gher shrimp samples and the live shrimps were not contaminated with *Vibrio* species but dead shrimps werefound to be contaminated, which were collected from shrimp Gher. The mean density of *Vibrio* spp. found in the hepatopancreas was $4.30×10^4$ cfu/g, in the intestine $2.10×10^6$cfu/g and for the stomach $1.29×10^6$cfu/g that was found by Gomez-Gil et al. (1998) in their study.

According to the recommendation of International Association of Microbiological Societies (IAMS), fresh and frozen fish and shrimp should be free of *Vibrio* (0 cfu/g). The present study revealed that microbial quality of AHPND affected shrimp of selected regions were not acceptable due to presence of *Vibrio*. According to the guideline of ICMSF (1986), acceptable limit of *Vibrio cholerae* counts for giant prawns and white fish are 0 cfu/g. From the result of total *Vibrio* count of present study we can conclude that total *Vibrio* count were beyond the standard value suggested by IAMS and ICMSF, which indicate the unacceptability as food for human consumption.

Total *Vibrio*count on HiChrome, enumerated bacterial count was ranged from $3.8x10^6$to $1.8x10^7$cfu/g for representative gher of Satkhira Sadar Upazilla from which sample was collected.But Uncountable total *Vibrio* like colony count was found in some gher of Satkhira Sadar Upazilla. On the other hand in Mongla Upazilla total *Vibrio* like colony count was found from $1.88×10^7$ cfu/gto infinity. Most of the representative gher of Mongla Upazilla from which samples were collected showed no growth of bacteria on HiChrome agar plate. While *Vibrio* like colony on HiChrome agar was found from $1.40×10^4$to $1.88×10^7$cfu/g in Morrelganj Upazilla of Bagerhat Upazilla. No growth of bacteria on HiChromeagaralso found in some gher of Morrelganj Upazilla under Bagerhat district.

## 4.3 Molecular identification of bacterial isolates by using *ldh* primer

 Positive PCR results were obtained with all the suspected isolates for the lecithin-dependent hemolysin (*ldh*) gene that is considered to be a species-specific marker for *V. parahaemolyticus*. From the result we have concluded that the representative isolates are *V. parahaemolyticus* which create early mortality syndrome EMS). Joshiet al.(2014) reported that *ldh* gene was not detected by PCR in the six isolates that causing AHPND.Soto-Rodriguezet al. (2014) showed that in total, 294 bacterial isolates were

recovered from the diseased shrimp and pond water. Of these, 37were negative for the human-toxigenic genes *tdh* and *trh*; therefore, *V. parahaemolyticus* was presumably non toxigenic to human. Bacterial identification was confirmed with the gene coding for the thermolabile hemolysin (*tlh*) that is specific for *V. parahaemolyticus* (Bej et al., 1999). The 35 isolates were positive for *tlh* and negative for human toxigenic genes *tdh* and *trh*. The *tlh* hemolysin of *V. parahaemolyticus* does not cause lysis in vertebrate erythrocytes (Bej et al., 1999) as occurs with the *tdh* and *trh* hemolysin of the same species that are considered virulence factors in human and animal pathogens (Zhang et al., 2005). We concluded that all the isolates belong to *V. parahaemolyticus.*

**4.4 Molecular identification of bacterial isolates by 16S rRNA sequencing**

The use of 16S rRNA gene sequences in the present study to investigate bacterial phylogeny and taxonomy has been by far the most housekeeping genetic marker used for a number of reasons (Ferris et al., 1996; Weller et al., 1991; Nubel et al., 1997). The 16S rRNA gene (1500 bp) is large enough for informatics purposes (Patel, 2001). In this study, the 16S rRNA gene sequencing confirmed the identity of the 18 representative bacterial isolates from suspected AHPND affected shrimp by using 1386 bp of 16S rRNA gene. Before the sequencing genomic DNA were amplified by using 2 universal primers, 27F and 1492R, which allowed for the identification of bacteria up to the species level (Jiang et al., 2006).

Current study strongly suggest that all of the isolates from representative sample identified as *V. parahaemolyticus* that were responsible for causing AHPND in cultured shrimp.Joshi et al. (2014) suggested , bacterial isolates were subjected to analysis of the small subunit (SSU) ribosomal RNA (16S rRNA) sequences and PCR analysis for standard markers used in identification of *V. parahaemolyticus*. Current investigation strongly suggests that the isolates confirmed through the media test were the strains of *V. parahaemolyticus*.

A multiple sequence alignment is shown by view CLUSTALW to compare the sequences. After comparing the obtained sequence of 18 *V. parahaemolyticus*, total 16 sites were found polymorphic. Therefore, the dissimilarity was 1.15% (16/1386). It is confirmed that Vp2, Vp4 and Vp21 found closely related to Vp27 which supporting their similarity with the allocated VP strains CHB-35 and VP strains ECSMC9.It is clear that Vp11 and Vp39 are closely related to Vp35 and Vp43 which supporting their similarity

with the allocated strains VP Xm045. The taxonomic position of Vp42, Vp7, Vp23, Vp24, Vp25, Vp26, Vp30, Vp38 supporting their similarity with the Vp6 and Vp9 which is closely related with the allocated strains VpXmo45.

Construction of phylogenetic tree, involved by a total 27 (18 of experimental sequences with 9 downloaded sequences from NCBI Gene Bank) nucleotide sequence that supports the findings of multiple sequence alignment. By using neighbor-join and BioNJ algorithms confirmed the taxonomic position of the 18 representative isolates and the comparison of these bacterial strain sequences with other homologous bacterial sequences. The percentages of replicate trees in which the associated taxa clustered together in bootstrap test where 1000 replications were used. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura et al., 1993). Joshi et al. (2014) identified of the Bacterial species in diseased HP by Sequences Analysis of 16S rRNA Amplified PCR Products. They reported that among 50 clones, 37 clones were identified as *V. parahaemolyticus*, while 3 clones were as *V. vulnificus, V. harveyi and V. chaquassi.* The remaining 10 clones were excluded from BLAST analysis due to non-specific sequences and short length.Based on 16S rRNA sequences, the isolate from East Malaysia shrimps was closely associated with *Vibrio sinaloensis* (85% homology) and could also produce AHPND histopathology (Linthong et al.,2014).

## 4.5 Identification of human pathogenic strains of *V. parahaemolyticus* using *tdh* primer

The presence of virulence factor on *V. parahaemolyticus*genome is *tdh* gene which code for haemolysin product namely thermostable direct hemolysin (*tdh*) (Honda et al., 1993). Shirai et al. (1990) suggested that, studies using *tdh* or *trh* related hemolysin or both gene probes on *V.parahaemolyticus*strains showed a strong correlation between clinically significant strains and presence of either of these genes, implying that *tdh* and *trh* genes are virulence factors in *V. parahaemolyticus*. The present study revealed that the representative isolates were negative for human toxigenic gene *tdh*. The negative detection of *tdh*, which is human pathogen markers (Taniguchi et al., 1985), indicated that those strains of *V. parahaemolyticus*are not infectious to human. This gene with 230bp size did not appear on the gel. These results were in agreement with previous studies that reported *tdh* genes were found in very low number (1-7%) among

environmental and seafood samples (Nordstrom et al., 2003). Based on this result, all the isolates under this study do not contain virulence properties that have strong correlation to the ability of causing diseases, which means that they are non-pathogenic for human. Detection *tdh* genes is important to study the distribution of pathogenic strains especially in seafood. Most of the seafood from tropical region especially Southeast Asia is known to have high risk of the *V. parahaemolyticus* presence with percentage between 20 - 70% (Wong et al., 1999). This is because of the high marine temperature (25–35°C) and thus the occurrence and distribution of *V. parahaemolyticus* is all year round. The hotmarine water is a major contribution factorto the occurrence of high percentage *V.parahaemolyticus* in the cockle samples. In addition, Joshi et al. (2014) summarized that *tdh* gene was not detected by PCR in the six isolates that causing AHPND.

The AHPND is thought to be caused by *V. parahaemolyticus*, which is typically known to be a human pathogenic bacterium. Our PCR assays showed that none of the AHPND associated strains possess *tdh* genes of human pathogenic strains. Markegardet al. (2009) conducted Real-time PCR on *tlh* and *tdh* on 12 *V. parahaemolyticus* strains, one*V. alginolyticus* and one *V. vulnificus* strains. As expected, *V. alginolyticus* and *V. vulnificus* were negative for *tlh*-and *tdh*-PCR. All *V. parahaemolyticus* strains were positive for *tlh*. These results confirmed the primers and probes were specific for *V. parahaemolyticus*. Xu et al. (2016) worked on 145 *V. parahaemolyticus* isolates to confirm and test for the presence of *trh* and *tdh*. But they found that none of the isolates possessed the genes. Soto-Rodriguez et al. (2015) recovered 294 bacterial isolates from the diseased shrimp and pond water. Of these, 37 were positive for *tlh* and negative for the human-toxigenic genes *tdh* and *trh*; therefore, *V. parahaemolyticus* was presumably no toxigenic to humans. *V. parahaemolyticus* isolates were obtained from the HL (one isolate), HP (fourteen isolates), ST (twenty isolates), and pond water (one isolate) from May (twenty one isolates), June (two isolates), July (five isolates), August (eight isolates), and one undetermined date. The 37 *tlh*-positive, *tdh*-negative, and *trh*-negative isolates were fingerprinted using ERIC-PCR, and several clones were detected. Based on this result, all of the isolates under this study do not contain virulence properties that have strong correlation to the ability of causing diseases, which means that they are non-pathogenic or different from human pathogenic strains.

**4.6 Molecular identification of AHPND positive isolates using AP3 primer**

In present study, out of18 isolates 12 isolates were positive for AP3 specific primer that indicated those shrimp were infected with *V. parahaemolyticus* AHPND. The AP3 method for detection of the small AHPND toxin gene (AHPND ToxA) has proven effective for detection of bacteria that cause acute hepatopancreatic necrosis disease (AHPND).The AP3 primer set produced a 333 bp amplicon in an intergenic region that was present in all pathogenic strains tested.The evaluation of these tests clearly showed that the diagnostic tests based on the first AP3 had the highest degree of differentiation between the pathogenic and nonpathogenicstrains of *V. parahaemolyticus*.These results differ from Sirikharin et al. (2014), who showed predictive values of 100% using Asian strains.All the selected isolates formed green colonies in TCBS. Thirty-five isolates were positive with AP3 primers, showing a fragment of 336 bp. Kongrueng et al.(2014) used a PCR method targeted to the unique DNA sequences derived from the plasmid (AP2 primers) and the toxin gene (AP3 primers) of *V. parahaemolyticus* that caused acute hepatopancreatic necrosis disease (AHPND), a total of 33 of 108 isolates were positive. In contrast, all 63 and 66 isolates of clinical and environmental *V. parahaemolyticus*, respectively, obtained previously from 2008 to 2014 in the same area were negative. Soto-Rodriguez et al. (2015) used AP3 primer for the detection of pathogenic and nonpathogenic *V. parahaemolyticus* strains.However, AP3 showed a false-positive result in this study (strain M06-04) and produced a predictive positive value of 90%. These results differ from Sirikharin et al. (2014) who showed predictive values of100% using Asian strains. Lai et al. (2015) performed PCR assay for seven bacterial strains to confirm the presence of the AP1, AP2 and *Pir* sequences that are associated with the AHPND causing strains of *V. parahaemolyticus*. They found that three virulent Thailand strains, ThV-1, ThV-16 and 5HP and one of the Vietnam strain, M1-1, were positive for all three sequences.

**4.7 Molecular identification of AHPND positive isolates using AP4primer**

The result from this research revealed that 18isolates of *V. parahaemolyticus* that cause AHPND of shrimp that was collected from three different southern regions of Bangladesh. These 14 isolates were confirmed by AP4 nested PCR method.The specimens with severe $VP_{AHPND}$ infections show two additional PCR product bands of 1269 and 230 bp resulting from cross reaction between the nested primers and residual

first-step AP4 PCR primers plus DNA template included with the 1st-step AP4 PCR product in the template solution used for the 2<sup>nd</sup> step AP4 nested PCR step. Using the bacterial isolates, the AP4 method was gave positive and negative predictive values for detection of VP$_{AHPND}$.

AP4 nested PCR method that has high sensitivity and specificity for detection of VP$_{AHPND}$ at lower levels than possible using previously published methods and without cross reactions with non-AHPND bacterial DNA or potential host DNA. AP4 method is 100 times more sensitive than AP3 method for the detection of AHPND causing bacteria (Dangtip et al., 2014).

Han et al. (2015) reported that the 69 kb plasmid pVPA3-1 was identified in *V. parahaemolyticus* strain 13-028/A3 that can cause Acute Hepatopancreatic Necrosis Disease (AHPND). To determine the copy number of the pVPA3-1 plasmid, they applied a comparative qPCR to determine the relative quantity of the *Pir*B-like gene (which encodes a *Pir*B toxin-like protein harbored by this plasmid, Insecticidal toxin genes within the plasmid') versus the *toxR* gene. The *toxR* gene is used as an endogenous control; ToxR protein is a transcriptional regulator for outermembrane proteins and has been used as a species-specific marker (Kim et al., 1999). Only one copy of the *toxR* gene is found in chromosome I in the *V. parahaemolyticus* strains RIMD 2210633 and BB22OP.

**4.8 Antibiotic susceptibility of AHPND positive *V. parahaemolyticus* isolates**

Antibiotics are frequently used in aquaculture, and antibiogram patterns can be used to evaluate different populations of organisms. Inpresent study, all 18 isolates showed multiple antibiotic resistance against 14 different antibiotics where all 18 isolates were sensitive to Chloramphinocol (30μg), Nalidixic acid (30μg), Nitrofurantion (300unit).De Meloet al.(2011) reported that in antibiotic susceptibility test five strains (50%) presented multiple antibiotic resistance to ampicillin (90%) and Amikacin (60%), while two strains (20%) displayed intermediate-level of resistance to Amikacin. All strains were sensitive to chloramphenicol. Intermediate-level of susceptibility and/or resistance to other antibiotics ranged from 10 to 90%, with emphasis on the observedgrowing intermediate-level of resistance to ciprofloxacin. In contrast, a study ofMolitoris et al. (1985)isolating *V. parahaemolyticus* from seawater and various seafoods (fish, crabs and shrimps), a total of 92 different antibiotic resistance patterns were observed, 3.5% of

which included ampicillin. Another study, however, reported over 50% of *V. parahaemolyticus* strains isolated from fresh and frozen seafood to be resistant to ampicillin.Xu et al. (2016) reportedthe antimicrobialresistance patterns of these 145 isolates to 12 antimicrobial agents revealed that most of the isolates resistant to streptomycin, with resistance and intermediate rates of 86.2 % and 11.7 %, respectively. In addition, the isolates exhibited relatively high resistance rates, of 49.6 %, 43.5 %, 35.9 %, and 22.1 %, for ampicillin, cefazolin, cephalothin, and kanamycin, respectively. However, it was surprising to note that strain 58, isolated from a fish sample, was a multi-drug-resistant strain, which showed resistance to 7 antibiotics: streptomycin, cephalothin, ampicillin, tetracycline, kanamycin, trimethoprim Sulphamethoxazole and cefazolin. All of the examined isolates were susceptible to azithromycin and chloramphenicol. Among the remaining tested antibiotics, the next-highest susceptibility rates were observed for Nalidixic acid (97.2 %), ciprofloxacin (91.7 %), tetracycline (83.4 %), trimethoprim-sulfamethoxazole (75.2 %), and gentamicin (62.8 %). Kongrueng *et al*. (2014) evaluated 33 *V. parahaemolyticus* AHPND isolates and 16 environmental *V. parahaemolyticus* isolates to test the antibiotic susceptibility. All *V. parahaemolyticus* AHPND isolates were resistant to ampicillin (Amp) and erythromycin (E), whereas they were susceptible to tetracycline (T), chloramphenicol (C), sulphamethoxazole/trimethoprim (SXT), gentamycin (CN) and norfloxacin (NOR) .

Hossain et al. (2012) demonstrated that, around 18% isolated strains of *V. parahaemolyticus* were resistant to ampicillin and all the strains were sensitive to the chloramphenicol (30µg), Nitrofurantion (300µg), ciprofloxacin (5µg) and Polymyxin B (300µg). The study support oxytetracycline and erythromycin are commonly used antibiotics in Bangladesh and these drugs mainly defer plasmid-mediated resistance in aquatic bacteria (Graslund et al., 2001). In present study around all of the isolates of *V. parahaemolyticus* showed resistance against gentamycin. Since FDA legalized oxytetracycline in addition to four more drugs for use in US aquaculture but it is essential to control their use in prescribed doses for safe use in Bangladesh (FDA, 1997). The present study also recommends six drugs- Nalidixic Acid, Nitrofurantion, Azithromycin, Chloramphenicol, Ciprofloxacin and tetracycline that can be considered for controlling Vibriosis, provided the drugs are discharged in appropriate doses once the target pathogens are identified.

**Chapter 5**

**Conclusion and Recommendations**

**5.1 Conclusion**

The study was conducted to check the presence of AHPND in southern shrimp farming regions of Bangaldesh. A total of forty six isolates of *Vibrioparahaemolyticus* were obtained from the samples collected from three different sampling area. *V. parahaemolyticus*,the causative agent of AHPND was primarily identified using species specific PCR of *ldh* and16S rRNA gene sequencing.Eighteen representative isolates were confirmedas AHPND positive using AP3 and AP4 nested PCR assay. The thermostable direct hemolysin (*tdh*) gene also used to check human pathogenicity. But none of the isolates showed positive result for *tdh* primer based PCR assay. From this result it can be said that the AHPND affected shrimp were not human pathogenic. On the other hand, antibiotic susceptibility test was used for further characterization of the isolated *V. parahaemolyticus* strains. This report also agreed with the mortalities of shrimp that occurred within 30 days after stocking in gher of three different south-west regions of Bangladesh which was affected by AHPND.

**5.2 Recommendations**

Based on the findings of the current study, several recommendations could be mentioned.

1. More research must be performed on the aspect of control or remediation of this disease to avoid the high economic loss and human health hazards.

2. Local government should take necessary action or steps, including quarantine to confirm biosecurity.

3. The study was limited for the identification of *V. parahaemolyticus* bacteria by using 16S rRNA sequencing with culture dependent method. More accurate result might be determined by histopathological study, reverse gavage test, multi locus gene, and next generation sequencing to characterize the AHPND positive*V. parahaemolyticus*.

4. Current study was conducted only in very short periods, so further studies are to be needed for better understanding mechanisms of AHPND disease.

5. This study was conducted on three different location of south-west regions of Bangladesh, research should perform in other regions of Bangladesh where shrimp cultured.

6. The transmission route of *V. parahaemolyticus* in shrimp farms could be checked that are responsible for AHPND in cultured shrimp.

7. Farm management technique to prevent AHPND in cultured shrimp should be taken in consideration.

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# Appendices

**Appendix A**

**Media Composition**

### 1.1 Luria-Bertani Media
A) NaCl-1g
B) Pepsin Digest of casein-1g
C) Yeast of extract- 0.5g
D) Agar powder- 1.5 g
E) PH- 7.13
F) Water- 1L

### 1.2 TCBS Media
A) TCBS agar- 88 g
B) Water- 1L

### 1.3 Nutrient Agar Media
A) Agar- 15g
B) Peptone- 5g
C) Beef Extract- 3g
D) NaCl- 5g
E) PH- 6.8-7.2
F) Water- 1L

### 1.4 Alkaline Peptone Water
A) NaCl- 10 g
B) Peptone- 10 g
C) PH- 8.5±0.2
D) Water- 1L

### 1.5 CHROME Agar Media
A) CHROME Agar- 74.8 g
B) Water-1L

### 1.6 Tryptic Soya Agar Media
A) Tryptic Soya Agar- 30 g
B) Bacteriological Agar- 15g
C) Salt- 20 g
D) Water- 1L

## Appendix 2

## Information about samples and their work

| Sample no. | Isolate symbol | Region | Gher name | Volume of gher | Media | Condtion of culture | 16S rRNA | LDH | TDH | AP3 | AP4 (1st PCR) | AP4 (2nd PCR) | AP4 final | DNA extraction | Antibiogram |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| VP2 | SH1 | Satkhira(alipur) | Safiqul | same | HiChrome | same | Yes | | | Yes | Yes | Yes | Yes | Yes | Yes |
| VP3 | SH1 | Satkhira(alipur) | Safiqul | same | TCBS | APW6 | | | | | Yes | Yes | | Yes | |
| VP4 | AH2 | Satkhira(alipur) | Abidullah | 30 bigha | HiChrome | Direct plate | Yes | Yes | Yes | Yes | Yes | Yes | | Yes | Yes |
| VP5 | AH2 | Satkhira(alipur) | Abidullah | same | HiChrome | APW6 | | | | | Yes | Yes | | Yes | |
| VP6 | M1H3 | Satkhira(alipur) | Mossarraf | 1 bigha | Chrome*Vibrio* agar | APW6 | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| VP7 | OH4 | Satkhira(alipur) | Obydullah | 1.5 bigha | TCBS | Direct plate | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| VP8 | OH4 | Satkhira(alipur) | Obydullah | same | HiChrome | APW6 | | | | | Yes | Yes | | Yes | |
| VP9 | BH5 | Satkhira(alipur) | Babul | 20 bigha | HiChrome | APW6 | Yes | Yes | Yes | Yes | Yes | Yes | | Yes | Yes |
| VP10 | M2H6 | Satkhira(alipur) | Mossarraf | 20 bigha | HiChrome | Direct plate | | | | | Yes | Yes | | Yes | |
| VP11 | M2H6 | Satkhira(alipur) | Mossarraf | same | Chrome*Vibrio* agar | APW6 | Yes | Yes | Yes | Yes | Yes | Yes | | Yes | Yes |
| VP12 | JH7 | Satkhira(alipur) | Jahangir | 7 bigha | HiChrome | APW6 | | | | | Yes | Yes | | Yes | |
| VP13 | SH1 | Satkhira(alipur) | Safiqul | 20 | HiChrome | APW6 | | | | | | | | | |

| | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | bigha | | | | | | | | | | | |
| VP14 | AH2 | Satkhira(alipur) | Abidullah | 30 bigha | HiChrome | 18h | | | | | | | | | |
| VP15 | OH4 | Satkhira(alipur) | Obydullah | 1.5 bigha | Chrome*Vibrio* agar | 18h | | | | | | | | | |
| VP16 | JH7 | Satkhira(alipur) | Jahangir | 7 bigha | Chrome*Vibrio* agar | 18h | | | | | | | | | |
| VP17 | ShH1 | Satkhira(alipur) | Shahbuddin1 | 15 bigha | HiChrome | 18h | | | | | Yes | Yes | Yes | Yes | Yes |
| VP18 | ShH1 | Satkhira(alipur) | Shahbuddin1 | same | HiChrome | 6h | | | | | Yes | Yes | | Yes | |
| VP19 | ShH2 | Satkhira(alipur) | Shahbuddin2 | 20 bigha | TCBS | 6h | | | | | Yes | Yes | | Yes | Yes |
| VP20 | ShH2 | Satkhira(alipur) | Shahbuddin2 | same | HiChrome | 6h | | | | | Yes | Yes | | Yes | |
| VP21 | AnH1 | Satkhira(alipur) | Anwarul | 10 bigha | TCBS | 6h | Yes | | | Yes | Yes | Yes | Yes | Yes | Yes |
| VP22 | AbH1 | Satkhira(alipur) | Abdullah1 | 1.5 bigha | TCBS | 6h | | | | | Yes | Yes | | Yes | |
| VP23 | AbH1 | Satkhira(alipur) | Abdullah1 | same | HiChrome | 6h | Yes | | | Yes | Yes | Yes | Yes | Yes | Yes |
| VP24 | AbH2 | Satkhira(alipur) | Abdullah2 | 2 bigha | TCBS | 6h | Yes | | | Yes | Yes | Yes | Yes | Yes | Yes |
| VP25 | AbH2 | Satkhira(alipur) | Abdullah2 | same | HiChrome | 6h | Yes | | | Yes | Yes | Yes | Yes | Yes | |
| VP26 | BaH1 | Satkhira(alipur) | Bablu | 20 bigha | HiChrome | 6h | Yes | | | Yes | Yes | Yes | Yes | Yes | Yes |
| VP27 | ShW1 | Satkhira(alipur) | Shahbuddin1 | 15 bigha | HiChrome | 6h | Yes | | | Yes | Yes | Yes | Yes | Yes | |

| VP28 | ShW2 | Satkhira(alipur) | Shahbuddin2 | 20 bigha | HiChrome | 6h | | | | | Yes | Yes | Yes | Yes | |
|------|------|------------------|-------------|----------|----------|----|--|--|--|--|-----|-----|-----|-----|--|
| VP29 | AnW1 | Satkhira(alipur) | Anwarul | 10 bigha | HiChrome | 6h | | | | | Yes | Yes | | Yes | |
| VP30 | AbW2 | Satkhira(alipur) | Abdullah2 | 2 bigha | HiChrome | 6h | Yes | | | Yes | Yes | Yes | Yes | Yes | |
| VP31 | Abw2 | Satkhira(alipur) | Adullah2 | 2 bigha | HiChrome | 6h | | | | | Yes | Yes | | Yes | |
| VP32 | BaW1 | Satkhira(alipur) | Bablu | 20 bigha | HiChrome | Direct plate | | | | | Yes | Yes | Yes | Yes | |
| VP33 | BaW1 | Satkhira(alipur) | Bablu | same | TCBS | Direct plate | | | | | Yes | Yes | | | |
| VP34 | IsHM | Mongla | Ismail | 10 bigha | HiChrome | 6h | | | | | | | | | |
| VP35 | BaHM | Mongla | Bappi | 2.5 bigha | HiChrome | 6H | Yes | | | Yes | Yes | Yes | Yes | Yes | Yes |
| VP36 | AfWB | Bagherhat | Afsar | 7 bigha | TCBS | 6h | | | | | Yes | yes | | Yes | |
| VP37 | MiHB | Bagherhat | Mizan mea | 5 bigha | TCBS | $(10^{-3})$ | | | | | Yes | yes | | Yes | |
| VP38 | MiHB | Bagherhat | Mizam mea | 5 bigha | TCBS | 6h | Yes | | | Yes | Yes | Yes | Yes | Yes | Yes |
| VP39 | MeHB | Bagherhat | Methun | 6 bigha | TCBS | Direct plate | Yes | | | Yes | Yes | Yes | Yes | Yes | Yes |
| VP40 | MeHB | Bagherhat | Methun | same | HiChrome | 6h | | | | | Yes | Yes | | Yes | |
| VP41 | JoHB | Bagherhat | Jolil | 10 bigha | HiChrome | 6h | | | | | Yes | Yes | | Yes | Yes |
| VP42 | AfHB | Bagherhat | Afsar | 7 bigha | HiChrome | Direct plate | Yes | Yes | Yes | Yes | Yes | Yes | | Yes | Yes |

| VP43 | SeHB | Bagherhat | Selim | 8 bigha | HiChrome | 6h | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes | Yes |
| VP44 | BaWM | Mongla | Bappi | 2.5 bigha | HiChrome | 6h | | | | | Yes | Yes | | Yes | |
| VP45 | MiWB | Bagherhat | Mizan mea | 5 bigha | TCBS | $(10^{-3})$ | | | | | Yes | Yes | | Yes | |
| VP46 | MeHB | Bagherhat | Methun | 6 bigha | TCBS | $(10^{-3})$ | | | | | Yes | Yes | Yes | Yes | |
| VP47 | JoWB | Bagherhat | Jolil | 10 bigha | HiChrome | 6h | Absent | | | | | | | | |
| VP48 | AfWB | Bagherhat | Afsar | 7 bigha | HiChrome | 6h | | | | | Yes | Yes | | Yes | |
| VP49 | SeWB | Bagherhat | Selim | 8 bigha | HiChrome | 6h | | | | | Yes | Yes | | Yes | |

## Appendix C

## 16S rRNA Sequences of 18 representative isolates of *Vibrio parahaemolyticus* in this thesis work (1386 bp)

>Vp2
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GTCGAGCGGAACGAGTTATCTGAACCTTCGGGGGACGATAACGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCCTGATGTGGGGGATAACC
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GGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTAGTGTAGTTA
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AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGGCCTTGAGCCGTGGCTTTCGGAGCTAACGCGTTAAGTAGACC
GCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTA
CCTACTCTTGACATCCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCGAGTAATGTCGGGAACTCCAGGGAGACTGCCGGTGATAAACCGGAGGAAGGTGGGGACGA
CGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACAGAGGGCGGCCAACTTGCGAAAGTGAGCGAATCCCAAAAAGTGCGT
CGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACC
GCCCGTCACACCATGGGAGTGGGCTGCAAAAGAAGT
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>Vp4
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GAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGGCCTTGAGCCGTGGCTTTCGGAGCTAACGCGTTAAGTAGACC
GCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTA
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CGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACC
GCCCGTCACACCATGGGAGTGGGCTGCAAAAGAAGT
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>Vp6
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CGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACC
GCCCGTCACACCATGGGAGTGGGCTGCAAAAGAAGT
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>Vp7
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ATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGGCCTCTCGCGTCAGGATATGCCTAGGTGGGATTAGCTAGTTGGTGA
GGTAAGGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGCACAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGTCGTGAGGAAGGTAGTGTAGTTA
ATAGCTGCATTATTTGACGTTAGCGACAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGC
GTAAAGCGCATGCAGGTGGTTTGTTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAATTGCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTA
GAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGG
AGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGGCCTTGAGCCGTGGCTTTCGGAGCTAACGCGTTAAGTAGACC
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CCTACTCTTGACATCCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGG
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