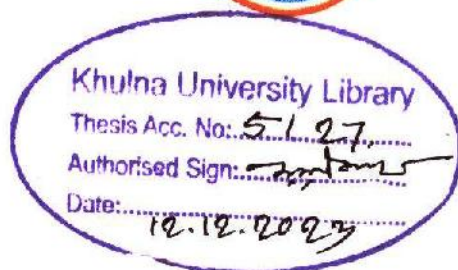


**Molecular Diagnosis of White Spot Syndrome Virus (WSSV) and Acute
Hepatopancreatic Necrosis Disease (AHPND) Causative Agent in Black
Tiger Shrimp Hatcheries**

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Student ID: MS-200604
Session: 2021-2022



Fisheries and Marine Resource Technology Discipline
Khulna University, Khulna
Khulna-9208, Bangladesh

November, 2022

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**A Thesis
by**

**Munna Khatun
Examination Roll No: MS-200604
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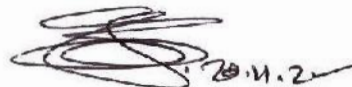
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**Molecular Diagnosis of White Spot Syndrome Virus (WSSV) and Acute
Hepatopancreatic Necrosis Disease (AHPND) Causative Agent in Black
Tiger Shrimp Hatcheries**

**Approved as to the style and content
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November, 2022

Declaration

The results presented in the thesis with the above-mentioned title are entirely of the candidate's own investigations and no part of the results has been accepted for any degree nor it is being concurrently submitted for any degree.



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November, 2022

DEDICATED
TO
MY BELOVED FAMILY



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All praises are to the Supreme Being “ALLAH”, creator and ruler of the universe, whose mercy keeps me alive and enables me to pursue my education in Fisheries and Marine Resource Technology Discipline and to complete my thesis for the degree of Master of Science (MSc.) in Fish Genetics and Biotechnology.

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Abstract

Shrimp aquaculture is a very promising and fast-developing industry in Bangladesh that contributes about 3.78% of the national GDP. However, this sector has been suffering from different diseases especially viral and bacterial diseases. White Spot Disease (WSD) and Acute Hepatopancreatic Necrosis Disease (AHPND) are considered serious intimidation to the commercial tiger shrimp (*Penaeus monodon*) farming industry. White spot syndrome virus (WSSV), a DNA virus, and *Vibrio parahaemolyticus*, a bacterium, are the causative agent of WSD and AHPND, respectively. The study aimed to investigate the scenario of viral and bacterial contamination in broods, nauplii, zoea, mysis, post larvae (PL) of shrimp, and other hatchery inputs like artemia, algae, squid, and mussel in commercial shrimp hatcheries in Cox's Bazar. The prevalence of these dreadful viral and bacterial infections was studied in 10 randomly chosen hatcheries in Cox's Bazar district of Bangladesh. WSSV and AHPND causative agents were detected using nested polymerase chain reaction (PCR). WSSV was detected using nested polymerase chain reaction (PCR) using two pairs of primers, 146F1/146R1 and 146F2/146R2, amplifying the 1447 bp and 941 bp fragments, respectively. AP4F1/AP4R1 and AP4F2/AP4R2 primers pairs were used to detect AHPND causative agent amplifying the 1269bp and 230bp, respectively. Out of 118 randomly selected samples including brood, PL, nauplii, zoea, mysis of shrimp, artemia, algae, squid, and mussel, 28.81% and 5.08% of the total samples were found positive for WSSV and AHPND, respectively. The study showed that the diseases affected all stages of the life cycle of shrimp as well as squid, mussel, and other hatchery inputs were also potential hosts for WSSV and AHPND. Hence, the present study suggests focusing on ensuring the production of specific-pathogen-free (SPF) shrimp PL in commercial shrimp hatcheries by maintaining proper biosecurity management. The findings of this study would aid farmers and decision-makers in developing plans to protect the shrimp sector from WSD and AHPND and thereby maintain sustainable shrimp farming in Bangladesh.

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List of Abbreviations and Non-English Words

AHPND	Acute Hepatopancreatic Necrosis Disease
Bp	basepair
BLAST	Basic Local Alignment Research Tool
DNA	Deoxyribonucleic acid
DoF	Department of Fisheries
F	Forward
FAO	Food and Agriculture Organization
GDP	Gross Domestic Product
<i>Gher</i>	Farms for production of crab
<i>In situ</i>	In the original place
Kb	kilo base pair
MT	Metric ton
OIE	World Organization for Animal Health
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PL	Post Larvae
R	Reverse
USA	United States of America
USD	United States Dollar
WSSV	White Spot Syndrome Virus
WSD	White Spot Disease

CHAPTER ONE

(INTRODUCTION)

Introduction

1.1. Introduction

Bangladesh is the 5th largest aquaculture producing country in the world next to China, India, Vietnam and Indonesia. In 2019–2020, Bangladesh produced 43.033 lakh metric tons of fish. (DoF, 2020). *Penaeus monodon*, usually known as black tiger shrimp, locally called *Bagda* is the most important fisheries product of Bangladesh. It is a marine crustacean and dispersed nearby the Indo-Pacific territory extending from northern Australia to the eastern coast of Africa, the Arabian Peninsula, Southeast Asia, and the Pacific Ocean (Waqairatu *et al.*, 2012). Among the fisheries product, shrimp aquaculture is a very promising and fast developing industry in Bangladesh. After ready-made garments, shrimp and prawn sector is second-largest export sector in Bangladesh. Shrimp culture contributes 71.4 % to the total national production (BBS, 2017) and 3.78% GDP in Bangladesh (DoF, 2020). In the southwest region of Bangladesh, around 75% of shrimp farms are situated. The coastal area of these region is mostly led by black tiger shrimp (*Penaeus monodon*) production. The shrimp production was 127601 MT in 2019-2020, among this 87% (111215 MT) was produced from Khulna division in the year 2019-2020 (DoF, 2020). In Bangladesh, shrimp farming has promptly prolonged since 1980s (Debnath *et al.*, 2014). In the previous two decades coastal shrimp culture in Bangladesh has extended vividly providing that substantial economic profits to the farmers and the country. Bangladesh contributes 2.5% in the world shrimp market (DoF, 2020). The prosperous shrimp farming industry produced a variety of job opportunities. There are approximately 5000–6000 families occupied in the shrimp processing sector and other businesses, and there are approximately 87,000 people participating in this field both directly and indirectly (BBS, 2017).

Different diseases are the main drawbacks for shrimp sector in worldwide. The most common shrimp diseases are caused by viruses, bacteria and parasites. White Spot Syndrome Virus (WSSV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Monodon Baculovirus (MBV), Hepatopancreatic Parvovirus (HPV), and Yellow Head Virus (YHV) are the main causative agents of shrimp viral diseases.

White Spot Syndrome Virus (WSSV) is one of the viruses that causes heavy mortalities and a serious production loss occurs to the shrimp farming. In Taiwan, this virus was first discovered in 1992 (Chou *et al.*, 1995). It caused a serious production loss to the shrimp farming sector in many countries of Asia and Latin America that time (Wongteerasupaya *et al.*, 1995). WSSV is

currently regarded as the most severe issue of shrimp culture sector in Asia. All *P. monodon* life phases are affected by this virus, which causes 100% mortality within 3- 10 days of the beginning of clinical symptoms. (Chou *et al.*, 1995; Wang *et al.*, 1995; Karunasagar *et al.*, 1997). White spot syndrome virus (WSSV) is a large enveloped double-stranded DNA virus that belongs to the family Nimaviridae, genus Whispovirus. The length of the total viral genome is about 292.976 kb and it is a circular shaped virus (van Hulten *et al.*, 2001).

Complete genome sequences of 12 isolates have been done including four from China (GenBank accession numbers AF332093, KT995472, KT995470) (Yang *et al.*, 2001; Li *et al.*, 2017), one from respectively of Thailand (AF369029) (van Hulten *et al.*, 2000), Taiwan (AF440570) (Tsai *et al.*, 2000), Korea (JX515788), India (MG702567) (Vinaya *et al.*, 2018), Brazil (MF784752) (Danta *et al.*, 2018), Mexico (KU216744) (Rodriguez *et al.*, 2016), Australia (MF768985) (Oakey *et al.*, 2018) and Ecuador (MH090824) (Restrepo *et al.*, 2018). The WSSV genome exhibits a consistent 58.9% A/T composition over the whole genome. WSSV has a wide range of host (Karunasagar *et al.*, 1997). It affects almost all species of cultured shrimp (Lo *et al.*, 1996). WSSV infects different species the penaeid shrimp including *P. japonicus*, *P. monodon*, *P. chinensis*, *P. pericillatus* etc. WSSV also infect other crustacean like lobster, crabs and other decapods (Lightner, 1996). WSSV affect shrimps of all age groups, sizes, all sexes and in all aquaculture systems (Chang *et al.*, 1996; Maeda *et al.*, 1998; Hossain *et al.*, 2001). WSSV infect larvae, post larvae (PL), brood stock (Kou *et al.*, 1998). The frequently expressed clinical sign of WSSV is the presence of white spot in the exoskeleton and epidermis of the diseased shrimp (Peinado-Guevara and Lopez- Meyer, 2006). Using *in situ* hybridization, vertical transmission of the virus in oocytes are ensured by observing the presence of virus in follicle cells and connective tissue cells in the ovary. It has been revealed that WSSV can be found in both wild and hatchery-raised PL by some studies (Lo *et al.*, 1997). PL which are collected from wild sources considered as a major route for WSSV into culture farm (Mushiake *et al.*, 1999; Flegel and Aldaysaz, 1998). White spot viral infection is classified into two types according to Wang *et al.*, 1995. Acute infectious type I is highly lethal in species including *P. monodon*, *P. indicus*, and *P. penicillatus*. Type II is dormant and uses wild crabs, wild lobsters and *Macrobrachium sp.* as harbor. In 1994, WSSV outbreak happened in culture penaeid shrimp of semi-intensive farms of Cox's Bazar region. The disease scattered to Khulna region, the southwest part of the country, affecting approximately 90% of traditional shrimp farms and causing a 20% drop of the shrimp production at that time. For this reason, in 1998, shrimp exports declined 25,742 tons to 18,630 tons (Alam *et al.*, 2007). The first study was

conducted by Hossain *et al.*, 2015, to investigate the prevalence and distribution of WSSV in cultured shrimps of the coastal regions in Bangladesh. They used conventional PCR utilizing VP28 and VP664 gene-specific primers. They found 65% samples were positive. According to Dipankar Chakroborty *et al.*, 2020, the lowest 30% and the highest 72% prevalence of WSSV are found from January to March. Increasing the temperature ($22\pm0.71^{\circ}\text{C}$) to March ($27\pm1^{\circ}\text{C}$) increased the prevalence the viral infection (Chakroborty *et al.*, 2020).

Another severe infection, a bacterial infection is Acute Hepatopancreatic Necrosis Disease (AHPND). It is an emerging disease that is causing mass mortality in shrimp sector of many Asian and American countries (Devadas *et al.*, 2019 and Hossain *et al.*, 2020). In 2009, this bacterial disease was first reported to cause mass mortality in China then it was found in Vietnam, Malaysia, Thailand, Mexico, Philippines and South America in 2011, 2011, 2012, 2013, 2015 and 2016 respectively (Leobert *et al.*, 2015 and Soto-Rodriguez *et al.*, 2015). *Vibrio parahaemolyticus* is the causative agent of AHPND (Wang *et al.*, 2015). This bacterium contains a 70-kbp plasmid and the Pir (Phototransducing insect-related) toxins encoded by the plasmid are mainly responsible for shrimp mortality in AHPND (Tran *et al.*, 2013). The first genome sequencing report of Bangladesh was conducted to MSR16 and MSR17, AHPND positive *V. parahaemolyticus* strains (Ahmed *et al.*, 2019). In the beginning of the disease outbreak, this disease was named as Early Mortality Syndrome (EMS) because of the mass mortality of shrimp within few days after PL stocking. *Vibrio parahaemolyticus* moves through oral route and then exists in the shrimp gastrointestinal tract and release poison that causes tissue damage and infirmity of the shrimp digestive system. Hence, the name of the disease was changed to acute hepatopancreatic necrosis disease (AHPND) (Tran *et al.*, 2013). Since that time, AHPND has resulted in significant decreases in shrimp production, up to 20% globally. (Hong *et al.*, 2016). Typically, 20 to 35 days after stocking shrimp fry in shrimp ponds, AHPND causes mortality rates of up to 100%. (Eshik *et al.*, 2017). Unlike non-pathogenic types, the pathogenic bacteria have distinctive extrachromosomal plasmids. Pir A and Pir B toxins are the primary virulence factors for AHPND, and they are encoded by the additional chromosomal plasmid seen in pathogenic *Vibrio* species (Xiao *et al.*, 2017). A study was conducted by Lai *et al.*, 2015, revealing the presence of Pir A and Pir B toxins in protein lysates from the stomach, hepatopancreas, and hemolymph of diseased shrimps. Main Clinical signs of infected shrimp are empty stomach, pale discoloration, and hepatopancreatic (HP) atrophy, as well as an empty or interrupted midgut (Devadas *et al.*, 2019). Disease infected shrimp frequently accumulating at the pond bottom are some of the clinical indications.

Hepatopancreatic tubules develop hemocytic aggregations and melanized granulomas along with opportunistic bacterial invasions (Tran *et al.*, 2013). Whiteleg shrimp (*Penaeus vannamei*) and black tiger shrimp (*Penaeus monodon*) are found to be sensitive species to AHPND. (Hong *et al.*, 2016). It causes serious economic losses. AHPND outbreaks across the major whiteleg shrimp farms in Peninsular Malaysia's five major states led to significant economic degradation estimated at USD 0.1 billion in 2011 (Shinn *et al.*, 2018).

In Bangladesh, shrimp sector mostly depends on wild PL stock. In Cox's Bazar, a few nurseries for wild tiger shrimp, PL were started in the late 1980s. The first commercial hatchery of Bangladesh (Pioneer Hatchery Ltd.) was established at Kalatali, Cox's Bazar in 1992. Now there are almost 60 hatcheries in Cox's Bazar region with a production of 19.83 billion per year. Shrimp farms all over the country mostly dependent on those hatcheries. However, outbreaks of disease are reducing the production of farmed shrimps. A crisis in the shrimp hatcheries over the last few years is due to an increase in the virulence of pathogens especially viral and bacterial diseases. Viral diseases are severe problem for shrimp culture sector worldwide.

The spread of WSSV and AHPND have the global impact on commercial as well as ecological settings. These diseases are a serious drawback for shrimp production. Various studies were conducted to investigate different virus at different time in brood, PL, nauplii (Haque *et al.*, 2020; Hossain *et al.*, 2015; Iqbal *et al.*, 2011) But still, there is very little information about infection in hatchery samples including live feed and water. Present study is conducted on the investigation of WSSV and AHPND causative agent in *Penaeus monodon* brood (male, female), PL, nauplii, zoea, artemia, squid, algae and mussel by PCR technique. Therefore, by conducting this study, we have tried to reveal the possible sources and the transmission of the diseases.

1.2. Objectives of the study

The objectives of this study were-

- To detect the WSSV and AHPND causative agent in *Penaeus monodon* from commercial shrimp hatcheries.
- To assess the quality of brood and PL stock of shrimp hatchery.

CHAPTER TWO

(LITERATURE REVIEW)

Literature Review

2.1. Black tiger shrimp (*Penaeus monodon*)

Black tiger shrimp (*Penaeus monodon*) is a marine crustacean. It is locally known as *Bagda*. It is dispersed nearby the Indo-Pacific territory extending from northern Australia to the eastern coast of Africa, the Arabian Peninsula, Southeast Asia, and the Pacific Ocean (Waqairatu *et al.*, 2012).

Scientific classification of *Penaeus monodon*

Kingdom: Animalia

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Order: Decapoda

Suborder: Dendrobranchiata

Family: Penaeidae

Genus: *Penaeus*

Species: *P. monodon* (Fabricius, 1798)

Young black tiger shrimp can be found in estuaries, lagoons and mangroves and are extremely tolerant to saline levels ranging from 2 to 30 ppt. Adults migrate into deeper water and reside on bottom that are rocky or muddy, with a depth range of 0 to 110 meters. These shrimps may burrow into the substrate during the day and come out at night to feed. They can only survive at 28–33-degree Celsius temperature (FAO, 2012). The wide range of fluctuations of physicochemical conditions, such as water temperature and salinity make the fingerlings more tolerant.

Mating occurs throughout the year under natural conditions. Females start to spawn mostly at the offshore. Their life cycle starts in coastal seawater, and the stages—egg, nauplii, protozoa, and mysis—spent in the water (Fig .1) (Motoh, 1985).

The larvae usually consume zooplankton, mainly crustaceans, and very small worms. Post larvae and adults of this species are omnivorous and consume algae, aquatic plants, mollusks, squid, aquatic insects, and worms (Motoh, 1985).

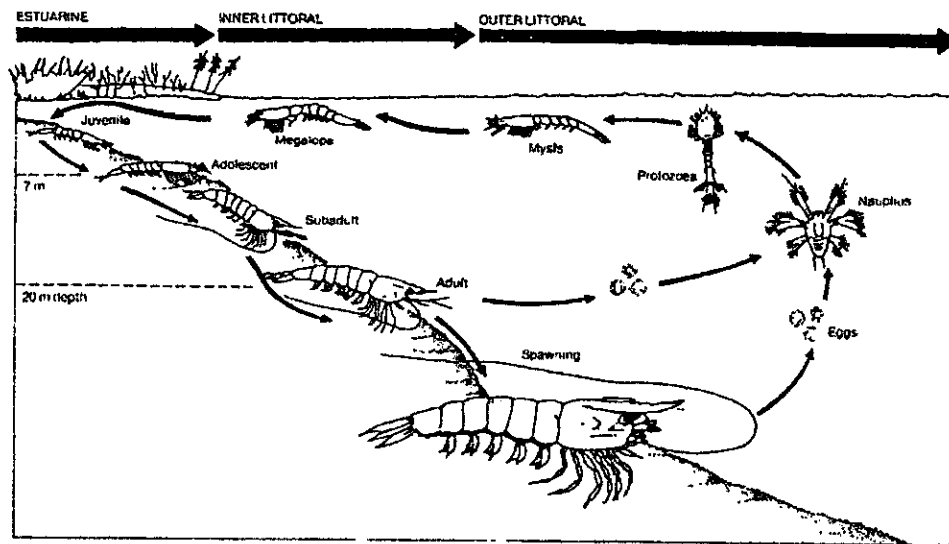


Fig. 1. Schematic representation of the life history of black tiger shrimp (*Penaeus monodon*).

2.2. Economic importance of shrimp in Bangladesh

Ray *et al.*, (2021) conducted study on the role of shrimp farming in the socioeconomic development of Bangladesh. They revealed that shrimp farming helped increasing the income level from 26 % to 36 % for the income range groups of 101–150 USD and over 150 USD, respectively. According to their study, 60% of participants preferred shrimp farming on freshwater fish culture, particularly for better production, 78% of participants firmly adopted that shrimp farming was more profitable than rice culture.

According to Hossain and Hasan, (2017), 160 million people of the country supports more than 11% of their income and means of subsistence through this sector. Over the past 30 years, total farmed shrimp production of Bangladesh has climbed from 14773 tons in 1986 to 132730 tons in 2016, an almost nine-fold increase. Over the last 15 years, the amount of shrimp produced and exported from farms has increased at a pace of around 20 percent annually.

2.3. Diseases of shrimp

Lightner and Redman, (1998) described different shrimp diseases and their diagnostic methods. They found that the majority of significant illnesses in cultured penaeid shrimp have bacterial or viral causes, while a few significant diseases are brought on by fungal and protozoan agents. Major diseases of Indo-Pacific and East Asian penaeid shrimp (White spot group, yellow head virus, BMN group, MBV group, IHHNV, HPV, REO group) were mentioned by them. According to them, the interaction of host, pathogen, and environment is the main cause of diseases and by excluding pathogen, disease outbreak can be reduced.

Flegel (2012) revealed that major epidemic of shrimp caused by the following viruses, such as Taura syndrome virus (TSV), infectious hypodermal and hematopoietic virus (IHHNV), white spot syndrome virus (WSSV), hepatopancreatic parvovirus (HPV), yellow head virus (YHV), and monodon baculovirus (MBV). Authors mentioned that these viruses caused 15 billion US dollars of losses to shrimp farmers for the past 15 years. About 80% of such loss attributed to Asia alone.

Yu *et al.*, (2022) conducted study on bacterial and parasitic disease of shrimp. They revealed that shrimp production has drastically reduced and causing domestic economic loss. According to them, Shrimp parasitic diseases like *Aphanomyces astaci* (crayfish plague) and bacterial diseases like Acute Hepatopancreatic Necrosis Disease (AHPND) and Necrotizing Hepatopancreatitis (NHP-B) are also arising and changing into new forms and causing a serious economic damage.

2.4. White Spot Syndrome Virus (WSSV) in shrimp

Iqbal *et al.*, (2011) conducted a study to observe seasonal prevalence of WSSV on brood, nauplii and PL in Bangladesh. They observed that WSSV incidence in brood stock, nauplii, and PL was high during the rainy season and low during the winter when water current, salinity, and tidal flashes were more or less consistent in Bay of Bengal. According to them, the average highest prevalence occurs in July (40%) and lowest in September (0%). They revealed that rainfall, seawater flow, tidal flushing and salinity variations appear to be vital triggering factors for the proliferation of this virus into shrimp.

Debnath *et al.*, (2012) also carried study on WSSV prevalence on brood, nauplii and PL. They found that WSSV showed more prevalent in tiger shrimp brood, nauplii, and post larvae in the months of March and April then it started to decrease. According to them WSSV fluctuation patterns for nauplii correlated with brood stock patterns with average lowest (0%) prevalence in September and highest (40%) in July.

According to Megahed *et al.*, (2019) WSSV is the enveloping, elliptical virions which is 250 and 380 nm long and 80 to 120 nm wide, with a tail-like appendage at one end. According to them, WSSV affect a wide range of host including shrimp, lobster, crab etc.

2.4.1. WSSV detection in *Penaeus monodon*

Kanchanaphum *et al.*, (1998) conducted research of the hypothetical spread of the white spot syndrome virus from crab to shrimp. They claimed that shrimp possessed strong viral infections for up to 45 days while remaining healthy, as shown by normal histology, particularly in *in situ* DNA hybridization, and PCR amplification.

Lightner and Redman, (1998) mentioned several diagnostic methods to identify the viral pathogen like gross clinical signs, direct microscopy, histopathology, electron microscopy, culture and biochemical enhancement, bioassay serological methods, hematology, clinical DNA probes, PCR, and tissue culture. Among them, the molecular method (gene probes and PCR) was considered the most accurate and standard method to detect WSSV in shrimp.

Ayub *et al.*, (2008) used nested and one-step PCR to study the prevalence of white spot syndrome viral infection. They conducted one-step and nested PCR using pairs of primers called 146F1/146R1 and 146F2/146R2, amplifying the 1447 bp and 941 bp fragments, respectively. They observed that WSSV were positive for both one-step and nested PCR. By using nested PCR, some of the samples that appeared to be healthy were also discovered WSSV infected.

Debnath *et al.*, (2014) conducted an experiment and revealed a comparison between deep zone broods and shallow zone broods. They found that there had a substantially greater rate of WSSV infection in shallow zone broods. They suggested that without further management measures, vertical transmission of WSSV could be reduced by 46% with the exclusive use of tiger shrimp broods harvested from the deep zone.



Hossain *et al.*, (2015) investigated the prevalence and distribution of WSSV in cultured shrimps of the coastal regions in Bangladesh. They collected 60 samples from Satkhira, Khulna, Bagerhat and Cox's Bazar district. They used conventional PCR by using VP28 and VP664 gene-specific PCR primer. They found 60% sample were positive.

Haque *et al.*, (2020) detected White Spot Syndrome Virus (WSSV) in Shrimp PL from Bangladesh using PCR technology. They discovered an overall prevalence rate of WSSV 16.93% in the collected PL samples. According to them, though the prevalence rate of WSSV in shrimp PL was 16.93%, it would play a dangerous effect on shrimp aquaculture of Bangladesh. According to their research, segregating WSSV-infected shrimp before releasing them into culture ponds or *ghers* is crucial for reducing or preventing WSD infection in shrimp farming.

Talukdar *et al.*, (2021) conducted a study to identify WSSV prevalence and found that Khulna region had a 100% positive rate for WSSV in all seasons and had the highest incidence. WSSV loads ranged from 5.62×10^9 to 2.01×10^{15} copies/g in shrimp tissue. They reported that 15 typical samples were 100 percent identical to the 2018 WSSV strain of India, according to the VP28 gene sequence.

2.4.2. Genome analysis of WSSV

WSSV is a bacilliform and double stranded virus. The length of the total viral genome is about 292.976 kb and it is a circular shaped virus (van Hulten *et al.*, 2001). The WSSV genome exhibits a consistent 58.9% A/T composition over the whole genome. Complete genome sequences of 12 strains of WSSV have been done till now. The genome of these isolates' ranges from 293 to 307 kb (Megahed *et al.*, 2019).

Complete genome sequences of 12 isolates have been done till now including four from China (GenBank accession numbers AF332093, KT995472, KT995470 and KT995471) (Yang *et al.*, 2001; Li *et al.*, 2017), one from respectively of Thailand (AF369029) (van Hulten *et al.*, 2000), Taiwan (AF440570) (Tsai *et al.*, 2000), Korea (JX515788), India (MG702567) (Vinaya *et al.*, 2018), Brazil (MF784752) (Danta *et al.*, 2018), Mexico (KU216744) (Rodriguez *et al.*, 2016), Australia (MF768985) (Oakey *et al.*, 2018) and Ecuador (MH090824) (Restrepo *et al.*, 2018). The WSSV genome exhibits a consistent 58.9% A/T composition over the whole genome. WSSV has a wide range of host (Karunasagar *et al.*, 1997).

2.4.3. WSSV pathogenicity and host range

Karunasagar *et al.*, (1997) studied on histopathological and bacteriological study of white spot syndrome virus of *Penaeus monodon*. They noticed that the diseased shrimp had white spots on its carapace and post-abdominal segments and histopathologically, the stomach epithelial cells had also intranuclear inclusion. They discovered that shrimp that were dead had mild to severe septicemia.

Otta *et al.*, (1999) experimented to assess the effectiveness of polymerase chain reaction (PCR) for detecting the white spot syndrome virus (WSSV) in *Penaeus monodon* post larvae and brood stock then in possible Indian crustacean carriers. They found WSSV prevalence among cultured and captured crab species indicating that wild marine crabs could be reservoirs of WSSV infection.

Rajendran *et al.*, (1999) conducted research to reveal the host range and histopathology of WSSV. They examined by injecting WSSV in shrimp and noticed that there was a cumulative mortality of 100% after 5-7 days of infection. They also noticed that two different mud crab species, *Scylla sp.*, endured the infection for 30 days without displaying any obvious signs of illness. The freshwater shrimp, *Macrobrachium rosenbergii*, and all three species of lobster, *Panulirus sp.*, survived the infection for 70 days without exhibiting any symptoms. They mentioned that crabs, prawns, and lobsters may serve as latent carriers or reservoir hosts of WSSV.

Hasan *et al.*, (2022) conducted a study to investigate the occurrence of WSSV in different *ghers* of Bangladesh and the virulence of the circulating phylotypes. They gathered 120 crab (*Scylla sp.*) samples and 360 shrimp (*Penaeus monodon*) samples from coastal region of Cox's Bazar and Satkhira, respectively. These findings showed that a single nucleotide aa change in VP28 could have an impact on the relative virulence features of WSSV based on mortality rate, viral load, and expression of host immune genes in experimentally infected shrimp PL.

2.4.4. Endocytosis Pathway of WSSV

Verbruggen *et al.*, (2016) conducted a study on endocytosis pathway of WSSV on host body. According to them the top panel shows viral entry occurs into the host cell. When WSSV proteins engage with host receptors, Clathrin-mediated endocytosis is induced. After that, WSSV moves via endosomes. As cells mature, the pH drops, signaling viruses to leave the

endosomes. VP28 and Rab7 are most likely interacting at this time. It is unclear how WSSV penetrates the nuclear envelope. Host transcription factors bind the WSSV genome once they entered into the nucleus and start the production of viral genes. The lower panel shows contacts between the host cell and the WSSV intracellularly. The availability of host machinery, such as processivity factors, is necessary for WSSV DNA replication. Through E2F1, WSSV can stop the cell cycle in the S-phase.

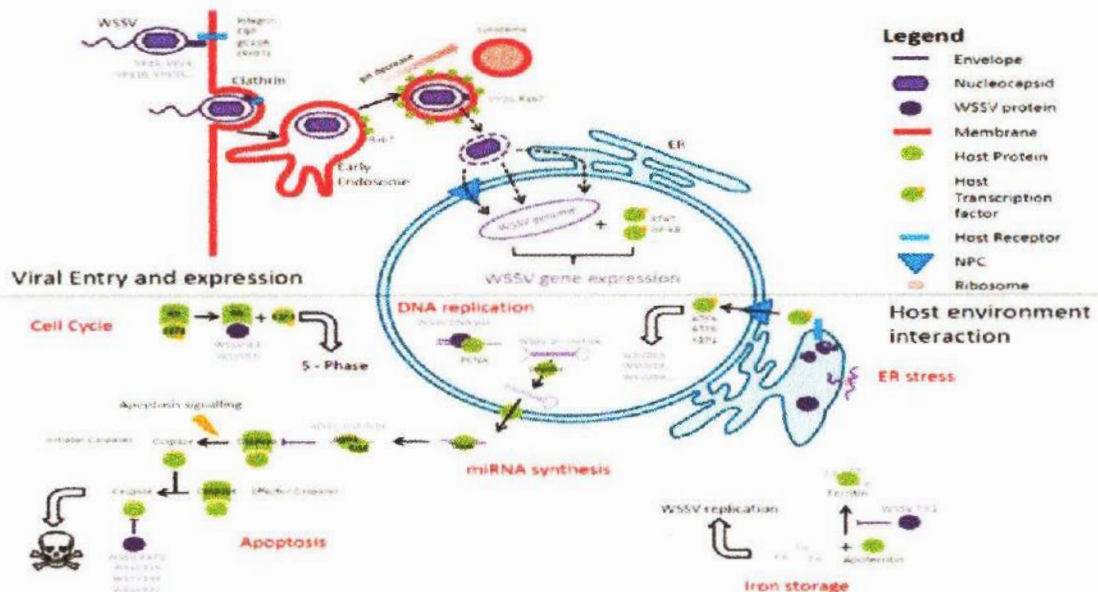


Fig 2. Endocytosis pathway of WSSV in shrimp

The activation of unfolded protein response (UPR) pathways, for example, can result from ER stress brought on by a high quantity of viral protein synthesis. Viral genes' expression can be stimulated by transcription factors of the UPR, which may then impede translation through eIF2. Iron is one of the critical elements needed for WSSV replication. WSSV has the ability to impede iron's binding to ferritin, preventing the host from withholding iron. Viral proteins reduce effector activity or miRNA-mediated suppression of initiator.

2.5. Acute Hepatopancreatic Necrosis Disease (AHPND) in shrimp

Flegel and Lo, (2013) worked on the detection of AHPND causative agent. For the amplification of AHPND bacterial DNA fragments, two primer sets—AHPND primer set 1 (AP1) and AHPND primer set 2 (AP2)—were utilized in their experiment. The two forward (F) and reverse (R) primer sets' sequences were as follows: AP1R-5'- GCAAACATATCGC

GCAGAACACC -3'; AP1R, 5'-GCAAACATATCGCGCAGAACACC -3'; AP2F-5'- TCA CCCGAATGCTCGCTTGTGG -3' and AP2R-5'- CGTCGCTACTGTCTAGCTGAAG -3'.

Joshi *et al.*, (2014) conducted study on revealing the differences in virulence and induction of AHPND for different isolates. They identified four independent bacterial isolates as *V. parahaemolyticus* by BLAST analysis and by gene-specific marker detection of a lecithin dependent hemolysin (LDH) which were considered to be specific for the species. They revealed that four independent isolates showed differences in virulence and induction of AHPND.

Lightner *et al.*, (2012) worked on the prevalence of EMS on shrimp. They found that 20 to 30 days after post larvae are stocked into ponds, typically disease symptoms that are linked to mortality start to show up. Santos *et al.*, (2020) conducted another study on the diagnosis and potential treatments for Acute Hepatopancreatic Necrosis Disease (AHPND). They used histopathology and observation of clinical signs as the preliminary tests of AHPND. Then they use polymerase chain reaction

2.5.1. Genome analysis of AHPND causative agent

The AHPND causing *V. parahaemolyticus* plasmid has a 69 kb region that encodes the *pirA* and *pirB* toxin genes. The strain VP_{AHPND} has one or more extrachromosomal plasmids (pVA1) with post segregational killing systems (PSK) that direct plasmid-encoded homologs of the binary toxins PirA and PirB.

The first genome sequencing report of Bangladesh was conducted to MSR16 and MSR17 AHPND positive *V. parahaemolyticus* strains isolated from shrimps. These strains are found in shrimp farms in southwest region of Bangladesh. In the MSR16 strain, the genomes were noticed to assemble into 108 contigs, and in the MSR17 strain, 66 contigs. The entire genome size of MSR16 is 5.4 Mbp, while that of MSR17 is 5.2 Mbp. For the MSR16 and MSR17 strains, the total GC content were, respectively, 45.09 and 45.19 percent (Ahmmed *et al.*, 2019).

2.5.2. Pathogenicity and host range of *Vibrio parahaemolyticus*

Zorriehzahra and Banaederakhshan, (2015) conducted a study on the pathogenicity of *Vibrio parahaemolyticus* on shrimp. They revealed the clinical signs which contain pale discoloration and atrophy of hepatopancreas with irregular black bands. According to them other clinical

signs were pale and empty stomach and gut, reduced growth, flexible shell and black discoloration. They also noticed that lethargy, swimming lethargically along the dikes, spiral swimming and reduced feeding among the affected shrimp.

Black tiger shrimp (*Penaeus monodon*) and whiteleg shrimp (*Penaeus vannamei*) are reported to be species susceptible to AHPND (Hong *et al.*, 2016).

An endocytosis pathway was proposed by Kumar *et al.*, (2020) for acute hepatopancreatic necrosis disease (AHPND). (i) The shrimp stomach is made up of epithelial cells, a chitinous layer/mucus lining, peritrophic membrane, and a lumen that houses a healthy community of gut microbiota under normal, healthy conditions. The digestive system's regular operation is aided by the metabolites (circles and triangles) that are released by the gut bacteria and shrimp cells.

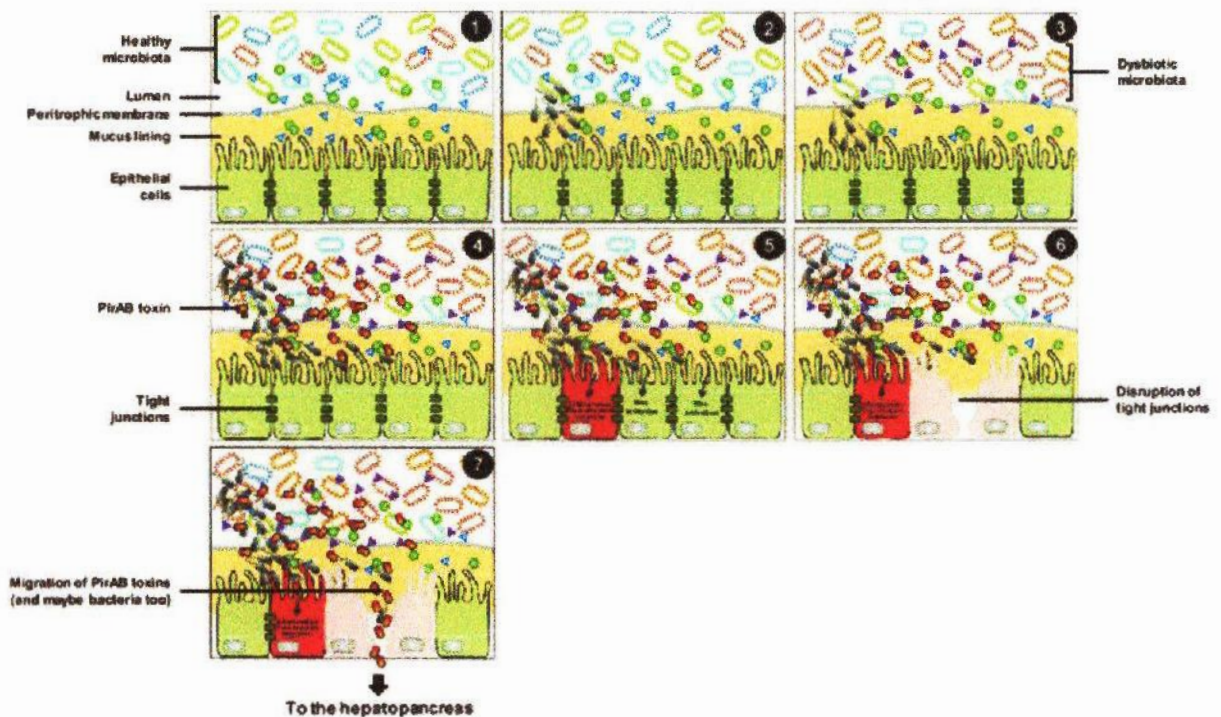


Fig 3. Endocytosis pathway and pathogenesis of *Vibrio parahaemolyticus* in shrimp

(ii) *Vibrio parahaemolyticus*, which causes AHPND, enters the shrimp's stomach orally. (iii) The once-healthy gut microbiota becomes dysbiotic as a result of the pathogenic, AHPND-causing bacteria releasing their own metabolites into the lumen. (iv) The *V. parahaemolyticus* releases PirA and PirB VP toxins into the lumen upon replication and colonization. (v) The

immunological response and activation of the Rho pathway are brought on by the PirA and PirB VP toxins. (vi) When the Rho-signaling pathway is activated, the stomach epithelial cells' tight connections are broken down, enabling intercellular gaps to emerge. (vii) Through the intracellular openings, PirA and PirB VP toxins and *V. parahaemolyticus* bacteria enter the hepatopancreas (Kumar *et al.*, 2020).

2.6. Economic vulnerability of WSSV and AHPND

In 1994, WSSV outbreak happened in cultured Penaeid shrimp of semi-intensive farms of Cox's Bazar region. The disease spread to Khulna region, the southwest part of the country, affecting approximately 90% of extensive shrimp farms and causing a 20% drop in national shrimp production. For this reason, Shrimp exports dropped 25,742 tons to 18,630 tons in 1997–1998. They subsequently rebounded in 1999–2000, only to fall by 25% in 2001 but the production was again badly affected by WSSV associated with other viral and bacterial pathogens (Alam *et al.*, 2007).

Kua *et al.*, (2016) worked on the reporting of EMS on shrimp at Malaysia. They reported that during the initial stages of shrimp production in 2010 and the beginning of 2011, there was a serious outbreak of AHPND in the middle regions of Peninsular Malaysia. According to their study, AHPND outbreaks across the major whiteleg shrimp farms in Peninsular Malaysia's five major states led to significant economic losses estimated at USD 0.1 billion in 2011 (Shinn *et al.*, 2018).

Alam *et al.*, (2007) revealed that among other coastal region Satkhira (79%) region was mostly affected by WSSV. According to them, in 2007, 90% of the shrimp extensive farm were affected by WSSV which caused a 20% loss in national shrimp production whereas 1996, WSSV alone caused a 44% production loss.

2.7. Present status of shrimp sector including hatchery in Bangladesh

Rahman *et al.*, (2012) investigated the Prevalence of Pathogenic Bacteria in Shrimp samples collected from Hatchery, Local Markets, and the Shrimp Processing Plant and found bacterial pathogens in those samples. They collected 8 samples of which, one from the hatchery, 3 from local markets, and 4 processed export quality samples. They found pathogenic bacteria from 6 samples and two processed export quality samples showed no presence of pathogen but they

showed antibacterial activity against some bacteria. The author concludes that proper handling and maintenance are necessary for the shrimp industry in Bangladesh.

Debnath *et al.*, (2016) conducted a study on the status and prospects of the culture of shrimp, mainly *Penaeus monodon* which makes an important contribution to Bangladesh's aquaculture industry. They recommended that shrimp hatcheries are the foundation on which the shrimp industry is built.

Ali *et al.*, (2022) studied on to characterize, assess the economic performance and identify challenges for intensive shrimp farming in Bangladesh. Our findings suggest that there is an urgent need for human capacity development for shrimp farm owners, workers and technicians. It is also important to improve access to quality inputs, rapid and affordable diagnostics and other technical services.

Dhar *et al.*, (2020) conducted a study that assessed the economic, environmental, and sustainability issues of organic shrimp farming in Bangladesh. Their study revealed that the productivity of organic shrimp per hectare was 383 pounds and the benefit-cost ratio was 1.91 which meant that organic shrimp farming was highly profitable in Bangladesh. Their study also showed that organic shrimp farming has the potential to mitigate the adverse effects on the environment caused by traditional shrimp farming. Their study also confirmed the sustainability of organic shrimp production from the standpoint of consuming energy, protecting the environment, economic feasibility, and social/political equity.

CHAPTER THREE

(MATERIALS AND METHOD)

Materials and Method

3.1. Study area and sample collection

One hundred eighteen samples including brood, zoea, mysis, PL, nauplii, artemia, algae, squid and mussel were collected from 10 hatcheries (Platinum, Modern Hatchery, New Madina, NR Hatchery, Satota, Quality, Sonali, Golden Aqua & Golden Aquaculture Ltd., Balaka ARC Hatchery) located at Kolatoli and Sonapara hatchery zone of Cox's Bazar district in December 2020. Five male fresh brood shrimp, eighteen female fresh brood, thirty PL, seven zoea, eleven mysis, thirty artemia, fourteen algae, one nauplii, one squid and one mussel were collected from those hatcheries. and transported to Khulna University by ice cool boxes. Pleopod, exoskeleton etc. were collected from the brood for WSSV and gut, hepatopancreas etc. were collected for AHPND. A minor number of other hatchery samples, including Pl, zoea, nauplii, Mysis, artemia, squid, and mussels, were also collected. All the collected samples preserving in 70% ethanol were transported to the Molecular and Genetic lab of Fisheries and Marine Resource Technology discipline of Khulna University. DNA was extracted from all samples and stored at -20°C at the Molecular and genetics lab of Khulna University (Courtesy of World fish, Khulna).

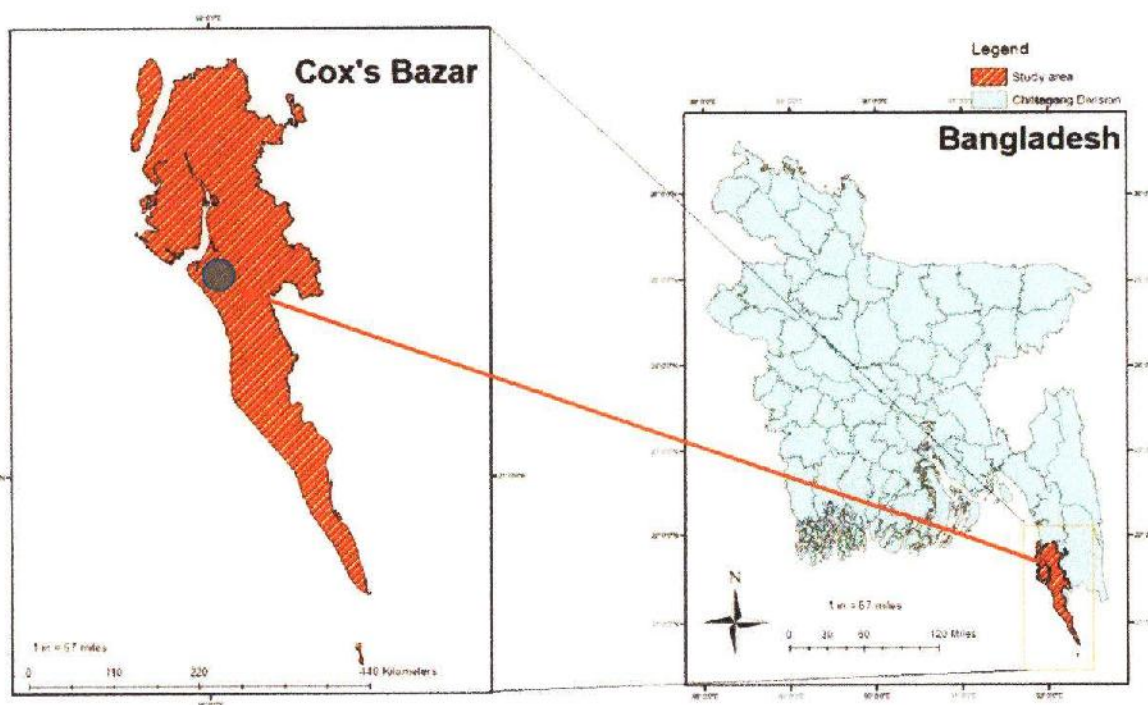


Fig. 4. Sampling area of Cox's Bazar district. Pointer indicates the possible sampling area.

3.2. DNA extraction for WSSV and *Vibrio parahaemolyticus*

For DNA isolation we used GeneJet DNA purification kit. At first, 20 mg samples were taken in a 1.5 ml tube for grinding. Then 180 µl digestion solutions was taken and grinded it properly. After that 20 µl of proteinase K solution was added and mixed thoroughly by vortexing or pipetting to obtain a uniform suspension. Thereafter we incubated the sample for 3 hours at 56°C. After that 20µl of RNase solution was added and mixed by vertexing then incubated for 10 min at room temperature. After incubation we added 200 µl of lysis solution, mixed thoroughly by vortexing for 15 s until a homogenous mixture is obtained. Then 400µl of 50% ethanol was mixed by pipetting. After that 500µl of wash buffer 1 was added and centrifuged for 1 minute at 12000 rpm and discarded the flow through and recapped collection tube. Then we added 500µl of wash buffer 2 and centrifuge for 3 minutes at 12000 rpm, then the flow through was discarded again and collection tube was recapped. At last, we centrifuged for 1 min at 12000 rpm then we discarded the column and caped properly the 1.5 ml microcentrifuge tube and stored the isolated DNA at -20°C or -70°C until use.

3.3. PCR detection of WSSV

3.3.1. Primer selection

Two pairs of primers were used for this study (146F1, 146R1, 146F2 and 146R2) (Lo *et al.*, 1996b) (Table 1). The primers were designed for targeting the WSSV genome.

Table 1. List of primers used to detect WSSV prevalence rate (%) in shrimp hatcheries

Primers	Sequences	Size of amplicon
WSSV 146F1	5'-ACTACTAACTTCAGCCTATCTAG-3'	1447bp
WSSV 146R1	5'-TAATGCGGGTGTAATGTTCTTACGA-3'	
WSSV 146F2	5'-GTAAGTCCCCCTCCATCTCCA-3'	941bp
WSSV146R2	5'-TACGGCAGCTGCTGCACCTTG T-3'	

The primer pairs were designed from the following genome sequences. The location of the 1447 bp first primer sequence is 273311 bp to 274757 bp, while the location of the 941 bp nested PCR primer is 273562 bp to 274502 bp (Fig. 5). The primer was chosen from the 'ORF 167' of the complete genome of WSSV.

White spot syndrome virus, complete genome

GenBank Accession: AF369029.2

273301-

CAGTCGACAGACTACTAAGCTTACGCTATCTAGTAAACAAGCTAAAAGATTCGACGGAGTTGACCCAGCCTT
CCCTGCCGXXXTCACCTGCGCTTCTACCTCATGCTTCTTCCATGGATTCCCATACAAAGTCATCTTTCATGGA
CAACATCAAATTGCACATGACTGATACTCAATGCTTCTTCAAGAACATTGAACGATTGAGAAATTCTTGGGA
AGATATGGGGACGAATACGCCATGTCCACAAAGCAAAATTGTAAGTGGGCTTCCATCTCCACCACACTTTTA
CTCCCTCAGATAACGAGCATCTGGTATCTCTTTCGATTTCGCGCCGCCCAGAAAGTCTCCATGGAAGAAATTAG
AGCCACACCCATCAGGCCAACAAAGCTTATTAGTGACAAACATTACGTGATGAACATGTCGAAGATCGATTCT
AGAGTAACAGGATCTTCCCTCCTTAAGAAGGTTAGCGAATGGACTGAAATGAGAAATGAACTCCAACTTTAATG
GAACATTTGAACCATCAAGACTCGCCCTCTCCAACCTTGGCATGACAACGGCAGGAGTCAACCTCGACGTTAT
TGTCAAACCAATAATGCAAGAAAGTGTACTAGGAATATTGGAATGTCATCGCCAGCACGTGTGCACCGCCGAC
GCCAAGGGAACTGTGCTTCAGCCATGCCAGCCGTCTTCCAGGCAACCGATGGAAACGGTAACGAATCTGAA
CTGATCCAGAATGCTCTGCCAAGGAACAGATACATCCAAAAGAGCACAAATGAACGCTCAAACCTGTCGTGTTTG
CTAATGTTTTGGAACAACCTATCGCCGATCTTGGAAAGGTTATCGTGAACGAACCTGGCCGGCACCATCGCTGA
ATCTGTACCAGAAAGCGTATATGAAAACACCAAGGAAATGATTGATAGACTAGGCTCTGACGACCTCTTCAAA
TCTAATAATAATGGAGGAGTAGAATCAATGGATTATGAAGATAGCGAAACAACATCCAACAATGGTCCCGTC
CTCATCTCAGAAGCCATGAAGAATGCCGTCTATCACACACTAATTTCCGGCAAGGCAGCTCGCCCGGAAAATG
TACCATTGCGCTCATGCGCCAGCGGCCCTCTCGCCTTTGATTTCTTCTGTCAAAGGGAGATACATTGGAAGAA
AAGAACGXXGAACAAGGTGCAGCAGCTGCCGTAATCTCTACCTATTCTTCTCTTCTAACACTACTCTTCGTAA
GCATTTGGCTCGAGTTTTCGAAGCCATCTCTAAGCAAGTAAGTATGCTGAATTCAAGGATATCTCTCAACGAT
ATCGAACGTAATATTTCTTCTGACTATACTAACTGTCCACCAAATACTAACCAAAATGCCTTTGCTCTAGCTAT
CAAGAGAGAATTCAGCAGAATTGTTTCTTCTTAACCATTTTCGTAAAGAACATTACACCCGCATTAGTCGACC
CTAAAGGCGCGTTACACGAGAAAGTAGCCATCTA-274798

Fig. 5. Nucleotide sequences of partial WSSV genome. The highlighted sections indicate primer sequences and its corresponding position which is used for detecting WSSV in the hatcheries.

3.3.2. Sequence analysis and phylogenetic tree construction for WSSV isolates

The genome sequences of 10 different WSSV isolates were compared which were obtained from GenBank in NCBI using NCBI-BLAST including the sequence of primer which amplified at 941 bp. Then the phylogenetic tree was constructed by was using MEGA ver. 1.1 software and neighbor-joining method. The isolates which were used to construct the phylogeny were WSSV isolate CN_95_DFPE (Cruz-Flores *et al.*, 2020), *Procambarus clarkii* virus isolate 1601, WSSV isolate LC1 (Parrilla-Taylor *et al.*, 2018), WSSV strain PC (Jiang *et*

al., 2017), WSSV complete genome (Marks *et al.*, 2005), WSSV isolate DBA1182 (Vinaya Kumar *et al.*, 2018), WSSV isolate WSSV_CIBA_002, WSSV isolate DV1 (Parrilla-Taylor *et al.*, 2018), WSSV isolate WSSV-AU, and WSSV isolate IN_AP4RU. All the sequences already present in NCBI GenBank database under the accession numbers MN840357.1, MH663976.1, MG432481.1, KX686117.1, AF369029.2, MW248107.1, MH883318.1, MG432477.1, MF768985.1 and MG702567.1, respectively.

3.3.3. WSSV primary PCR

The protocol of WSSV detection using the PCR approach was performed using the method obtained in the OIE manual (OIE, 2003). Total DNA were isolated from the samples using GeneJet DNA purification kit. The solution of 25 µl composed of 9.5 µl RNase free water, 12.5µl taq green PCR master mix, 0.5µl WSSV 146F1 primer, 0.5µl WSSV 146R1 primer and 2 µl sample. PCR cycle was then carried out. The first cycle was 1 denaturation cycle which was carried out at 94°C for 4 min, 55°C for 1 min and 72°C for 2 min. then 39 elongation cycle was carried out at 94°C for 1 min, 55°C for 1 min and 72°C for 2min. then 1 termination cycle was at 72°C for 5 min. After all cycle it was held at 4 °C.

3.3.4. WSSV nested PCR

The solution of 25 µl composed of 9.5 µl RNase free water, 12.5µl taq green PCR master mix, 0.5µl WSSV 146F2 primer, 0.5µl WSSV 146R2 primer and 2 µl samples. Then PCR cycle was then carried out. The first cycle was 1 denaturation cycle which was carried out at 94°C for 4 min, 55°C for 1 min and 72°C for 2 min. then 39 elongation cycle was carried out at 94°C for 1 min, 55°C for 1 min and 72°C for 2min. then then 1 termination cycle was at 72°C for 5 min. After all cycle it was held at 4 °C. After getting the PCR product we run the PCR product for Gel Electrophoresis analysis and only primers showing the positive bands 941bp amplicon for in the WSSV positive controls.

3. 4. PCR detection of AHPND causative agent

3.4.1. Primer selection

Two pairs of primers were used for this study (AP41, AP4R1, AP4F2 and AP4R2) (Dangtip *et al.*, 2015) (Table 2). The primer was designed for targeting the *Vibrio parahaemolyticus* genome.

Table 2. List of primers used to detect AHPND prevalence rate (%) in shrimp hatcheries

Primers	Sequences	Size of amplicon
AP4-F1	5'-ATGAGTAACAA TATAAACATGAAAC-3'	1269bp
AP4-R1	5'-ACGATTTCGACGTTCCCCAA-3'	
AP4-F2	5'-TTGAGAATACGGGACGTGGG-3'	230bp
AP4-R2	5'-GTTAGTCATGTGAGCACCTTC-3'	

The location of the corresponding 1269bp first PCR primer is 58054 bp to 59322 bp and for 230 bp nested PCR primer the location is 58181 bp to 58410 bp (Fig. 6).

***Vibrio parahaemolyticus* strain 20160303005-1 plasmid pVPSD20165, complete sequence**

NCBI Accession no: NZ_CP034304.1

58031-

GTGCAATTTAATAGGAGAACATC**ATGAGTAACAATATAAACATGAAAC**TGACTATTCTCACGATTGGACTGT
CGAACCAAACGGAGGCGTCACAGAAGTAGACAGCAAACATACACCTATCATCCCGGAAGTCGGTCGTAGTGT
AGACA**TTGAGAATACGGGACGTGGG**GAGCTTACCATTCAATACCAATGGGGTGCGCCATTTATGGCTGGCGGC
TGGAAAGTGGCTAAATCACATGTGGTACAACGTGATGAACTTACCATTTACAACGCCCTGATAATGCATTCT
ATCATCAGCGTATTGTTGTAATTAACAATGGCGCTAGTCGTGGTTTCTGTACAATCTATTACCACTAA**GAAGGT**
GCTCACATGACTAACGAATACGTTGTAACAATGTCATCTTTGACGGAATTTAACCCCTAACAAATGCTCGTAAAA
GTTATTTATTTGATAACTATGAAGTTGATCCTAACTATGCTTTCAAAGCAATGGTTTCATTITGGTCTTTCAAATA
TTCTTACGCGGGTGGTTTTTATCAACGTTATGGAATATCTTTGGCCAAATACGCCAAATGAGCCAGATATT
GAAAACATTGGGAACAATTACGTGACAGAA'TCCAAGATTTAGTAGATGAATCGATTATAGATGCCATCAATG
GAATATTGGATAGCAAAAATCAAAGAGACACGCGATAAAATTCAAGACATTAATGAGACTATCGAAAACITCG
GTTATGCTGCGGCAAAAGATGATTACATTGGTTTAGTTACTCATTACTTGATTGGACTTGAAGAGAACTTTAAG
CGCGAGCTAGACGGTGATGAATGGCTTGGTTATGCGATATTGCCTCTATTAGCAACAACGTGAAGTCTTCAA
TTACTTACATGGCTTGTGGTCTGGATTATAAGGATGAATTCGGTTTCACCGATTCTGATGTGCATAAGCTAACA
CGTAATATTGATAAGCTTTATGATGATGTATCGTCTTACATTACAGAACTCGCTGCGTGGGCTGATAACGACTC
TTACAATAATGCAAACCAAGATAACGTGTATGATGAAGTGATGGGTGCTCGTAGTTGGTGTACGGTTCACGGC
TTTGAAACATATGCTTATTTGGCAAAAAATCAAAGAGTTGAAAAAAGTTGATGTGTTTGTTCACAGTAATTTAAT
TTCATATTCACCTGCTGTTGGTTTTCCTAGTGGTAATTTCAACTATATTGCTACAGGTACGGAAGATGAAATAC
CTCAACCATTAAACCAAATATGT**TTGGGGAACGTCGAAATCGT**ATTGTAAAAATTGAATCATGGAACAGTAT
TGAAATACA-59360

Fig. 6. Nucleotide sequences of partial *Vibrio parahaemolyticus* genome. The highlighted sections indicate primer sequences and its corresponding position used for detecting AHPND in the hatcheries.

3.4.2. Sequence analysis and Phylogenetic tree construction for *Vibrio parahaemolyticus* strain

The genome sequences of 10 different *V. parahaemolyticus* isolates plasmid including *Vibrio parahaemolyticus* strain 20160303005-1 were used to establish the phylogenetic relationship among the AHPND positive *V. parahaemolyticus* strains. MEGA ver. 1.1 with neighbor-joining method were used to construct the phylogeny. The strains which were used to construct the phylogeny were *Vibrio parahaemolyticus* strain 20160303005-1, *Vibrio parahaemolyticus* strain HY3, complete cds, *Vibrio owensii* strain SH14 plasmid pVHvo, *Vibrio campbellii* strain 170502, *Vibrio parahaemolyticus* strain R14, *Vibrio parahaemolyticus* strain LA8, complete cds, *Vibrio sinoaloensis* strain K61, *Vibrio harveyi* strain K9, *Vibrio alginolyticus* strain K18 and *Vibrio parahaemolyticus* isolate STCR005. These sequences used to construct the cladogram present in NCBI GenBank database under the accession numbers CP034304.1, MH718270.1, CP045861.1, CP033136.1, CP028145.1, MW355910.1, MN846077.1, MN846071.1, MN846074.1 and MH685878.1, respectively.

3.4.3. AHPND first PCR

The solution of 25 µl composed of 9.5 µl RNase free water, 12.5µl taq green PCR master mix, 0.5µl AP4-F1 primer, 0.5µl AP4-R1 primer and 2 µl samples. PCR cycle was then carried out. The first cycle was 1 denaturation cycle which was carried out at 94°C for 2 min. then 39 elongation cycle was carried out at 94°C for 30 sec, 55°C for 30 sec and 72°C for 90 sec. then then 1 termination cycle was at 72°C for 2 min. After all cycle it was held at 4 °C.

3.4.4. AHPND nested PCR

Total DNA were isolated from the samples using GeneJet DNA purification kit. The solution of 25 µl composed of 9.5 µl RNase free water, 12.5µl taq green PCR master mix, 0.5µl AP4-F1 primer, 0.5µl AP4-R1 primer and 2 µl samples. PCR cycle was then carried out. The first cycle was 1 denaturation cycle which was carried out at 94°C for 2 min. then 39 elongation cycle was carried out at 94°C for 30 sec, 55°C for 30 sec and 72°C for 90 sec. then then 1 termination cycle was at 72°C for 2 min. After all cycle it was held at 4 °C.

3.5. Evaluation of PCR products by gel electrophoresis

To prepare the agarose gel, 2g agarose was boiled with 100 ml TAE buffer and 10 μ l SYBR Safe dye for 2 min. The boiled agarose was poured into a mold and placed with a comb to load the sample. After preparing the gel 4-5 μ l PCR product of each sample was loaded in one of the wells of the comb. 100 bp DNA ladder was used as a marker. After loading the sample gel was exposed to the electric field in the range of 80-100 volts for 45 min. Only primers showed the positive bands of 941bp amplicon for the WSSV positive controls and positive samples; 230bp amplicon for the AHPND positive controls and positive samples.

CHAPTER FOUR

(RESULTS)

Result

4.1. Genomic Structure of WSSV

WSSV is a bacilliform and double stranded virus. The length of the total viral genome is about 292.976 kb and it is a circular shaped virus (van Hulten *et al.*, 2001). The WSSV genome exhibits a consistent 58.9% A/T composition over the whole genome. Complete genome sequences of 12 strains of WSSV have been done till now. The genome of these isolates' ranges from 293 kb to 307 kb (Megahed *et al.*, 2019). Here, we have conducted nucleotide sequences comparison among 10 different isolates and strains of WSSV (Table 3). The genome sizes of WSSV isolate CN_95_DFPE (Cruz-Flores *et al.*, 2020) *Procambarus clarkii* virus isolate 1601, WSSV isolate LC1 (Parrilla-Taylor *et al.*, 2018), WSSV strain PC (Jiang *et al.*, 2017), WSSV complete genome (Marks *et al.*, 2005), WSSV isolate DBA1182 (Vinaya Kumar *et al.*, 2018), WSSV isolate WSSV_CIBA_002, WSSV isolate DV1 (Parrilla-Taylor *et al.*, 2018), WSSV isolate WSSV-AU, and WSSV isolate IN_AP4RU are 305 kb, 287 kb, 278 kb, 300 kb, 292 kb, 281 kb, 270 kb, 290 kb, 285 kb and 280 kb, respectively.

Table 3. Nucleotide sequences comparison of 10 WSSV isolates and strains

Countries	Strains & Isolates	Genome size	Accession no.
USA	WSSV isolate CN_95_DFPE	305 kb	MN840357.1
China	<i>Procambarus clarkii</i> virus isolate 1601	287 kb	MH663976.1
Mexico	WSSV isolate LC1	278 kb	MG432481.1
China	WSSV strain PC	300 kb	KX686117.1
Netherland	WSSV complete genome	292 kb	AF369029.2
India	WSSV isolate DBA1182	281 kb	MW248107.1
India	WSSV isolate WSSV_CIBA_002	270kb	MH883318.1
Mexico	Shrimp WSSV isolate DV1	290kb	MG432477.1
Australia	WSSV isolate WSSV-AU	285 kb	MF768985.1
India	WSSV isolate IN_AP4RU	280 kb	MG702567.1

*kb represents kilo base pair.

4.2. Phylogenetic relationship of WSSV with other isolates

A total of 10 isolates and strains of WSSV plasmid including WSSV complete genome (GenBank accession-AF369029.2) were selected for creating the phylogenetic relationship among the WSSV isolates. The Phylogenetic analysis was conducted by using MEGA ver. 11 software and neighbor-joining method (Fig. 7). The corresponding sequence of WSSV genome which was used for primer selection were found in the following isolates and strains. The sequences of the following isolates and strains were extracted from GenBank. WSSV complete genome Netherland and WSSV isolate IN AP4RU India formed a separate cluster and showed their relatedness. WSSV strain PC China, *Procambarus clarkii* virus isolate 1601 China, WSSV isolate CN_95_DFPE USA, WSSV isolate LC1 Mexico, WSSV isolate DV1 Mexico and WSSV isolate CIBA 002 India located in the same cluster. WSSV isolate DBA1182 India and WSSV isolate AU Australia presents formed another cluster.



Fig. 7. Phylogenetic analysis of WSSV isolates. MEGA ver. 11 and neighbor joining method with 100 bootstrap values (numbers = bootstrap percentage values) were used to construct the phylogeny based on the genome sequences of 10 WSSV isolates.

4.3. PCR detection of WSSV

The extracted DNA was used to detect the prevalence of WSSV by polymerase chain reaction using GeneJet DNA purification kit. The primer set 146-F1 (5'-ACTACTAACTTCAGCCTA TCTAG-3') and 146-R1 (5'-TAATGCGGGTGTAATGTTCTTACGA-3') for first PCR and 146-F2 (5'-GTAAGTGGCCCTTCCATCTCCA-3') and 146-R2 (5'-TACGGCAGCTGC TGCACCTTG T-3') for nested PCR were used to amplify the DNA sequence by following the previous methods (Lo *et al.*, 1996) which generated amplicons of 941 bp for nested PCR product. The PCR amplified products were examined by 2% agarose gel containing ethidium bromide and visualized under UV illuminator and amplified product was compared with a 100 bp DNA ladder.

4.3.1. PCR detection of WSSV in Sonali Hatchery: We collected 17 random sample from Sonali Hatchery including brood (male, female), PL, zoea, mysis, artemia, naupli, squid, mussel and algae. Seven out of 17 samples including 1 artemia, 4 female brood, 1 squid and 1 mussel of the Sonali hatchery were found WSSV positive by the conventional nested PCR (Fig. 8). Almost 41.18% samples were found positive in Sonali hatchery.

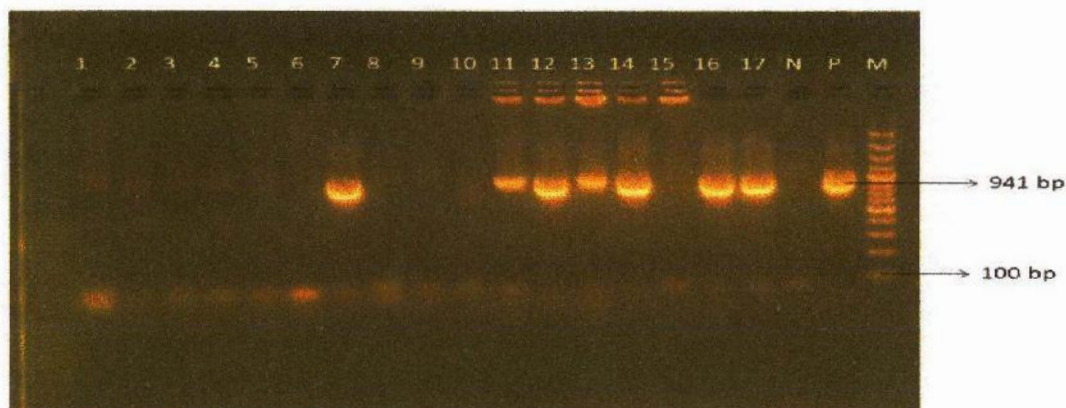


Fig. 8. Agarose gel electrophoresis of the PCR product of WSSV in Penaeid shrimp of Sonali hatchery: (amplified 941bp with WSSV 146F2/WSSV 146R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-17 represents samples (Lane 1-3: PL; Lane 4-6: zoea; Lane 7-9: artemia; Lane 10: algae; Lane 11-15: brood (female); Lane 16: squid; Lane 17: mussel). Amplification of the 941bp fragment were observed only in the positive sample and positive control. Samples 7, 11, 12, 13, 14, 16 and 17 show 941 bp amplification.

4.3.2. PCR detection of WSSV in Satata Hatchery: Twenty-one random samples were collected from Satata hatchery and then PCR detection was conducted. The Satata hatchery's seven out of 21 samples including two male brood and five female brood were discovered positive by the traditional PCR (Fig. 9). In Sonali hatchery 33.33% samples were WSSV positive.

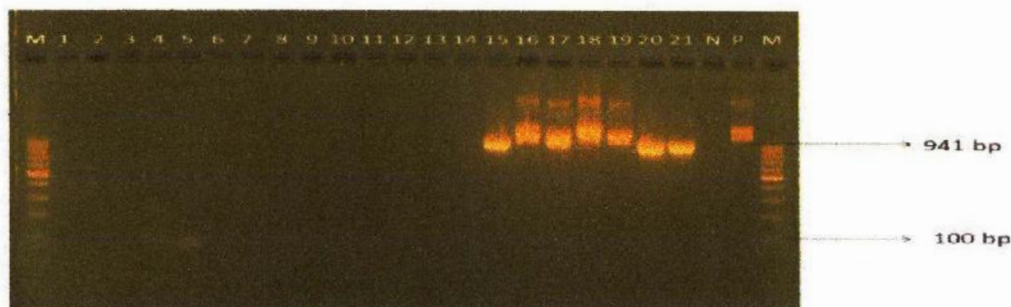


Fig. 9. Agarose gel electrophoresis of the PCR product of WSSV in Penaeid shrimp of Satata hatchery: (amplified 941bp with WSSV 146F2/WSSV 146R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-21 indicates sample (Lane 1-3: PL; Lane 4-6: zoea; Lane 7-9: mysis; Lane 10-12: artemia; Lane 11-14: algae; Lane 15-19: brood (female); Lane 20-21: brood(male)). Amplification of the 941bp fragment were observed only in the positive sample and positive control. samples 15, 16, 17,18,19,20 and 21 number show 941 bp amplification.

4.3.3. PCR detection of WSSV in Golden Aqua Ltd Hatchery: Seventeen sample of Golden hatchery were collected randomly and molecular detection was carried out. One female brood, 3 PL and 2 mysis were found positive out of 17 sample in Golden aqua hatchery through conventional PCR (Fig. 10). About 35.29% samples were WSSV positive in this hatchery.

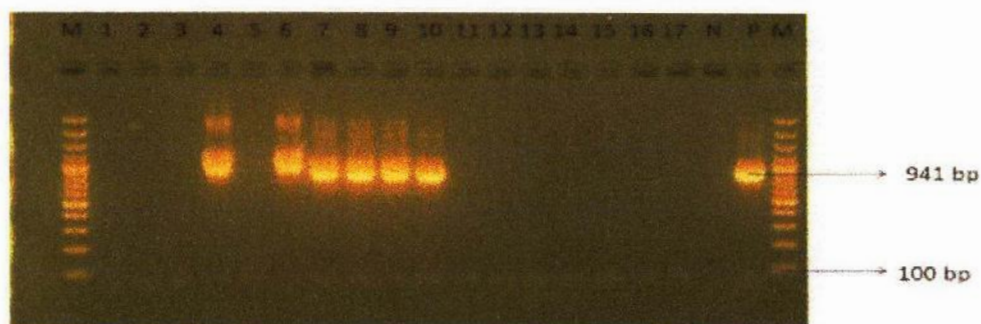


Fig. 10. Agarose gel electrophoresis of the PCR product of WSSV in Penaeid shrimp Golden Aqua hatchery: (amplified 941bp with WSSV 146F2/WSSV 146R2 nested primer). Lane 1-17 indicates sample (Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-5: brood (female); Lane 6-8: PL; Lane 9-10: mysis; Lane 11-13: artemia; Lane 14-16: algac; Lane 17: mysis). Amplification of the 941bp fragment were observed only in the positive sample and positive control. Samples 4, 6,7,8,9 and 10 no. show 941 bp amplification.

4.3.4. PCR detection of WSSV in Balaka Hatchery: From Balaka hatchery 11 samples were collected randomly and then PCR detection was conducted. In Balaka hatchery, one PL sample were found positive out of 11 sample through the conventional PCR method (Fig. 11). Only 9.09% samples were positive in Balaka hatchery.

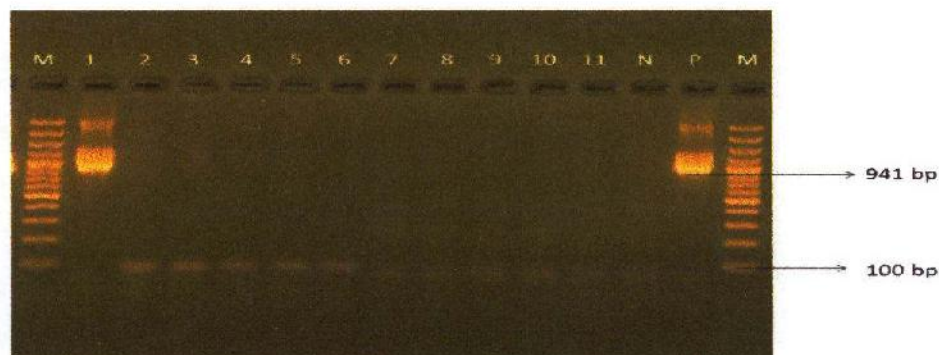


Fig. 11. Agarose gel electrophoresis of the PCR product of WSSV in Penaeid shrimp of Balaka: (amplified 941bp with WSSV 146F2/WSSV 146R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-11 indicates sample (Lane 1-3: PL; Lane 4-5: mysis; Lane 6-8: artemia; Lane 9-11: algae). Amplification of the 941bp fragment were observed only in the positive control. Sample 1 shows 941 bp amplification.

4.3.5. PCR detection of WSSV in NR Hatchery: Six sample of NR hatchery were collected randomly and molecular detection was carried out. In NR hatchery, no sample were found positive through the conventional nested PCR method (Fig. 12).

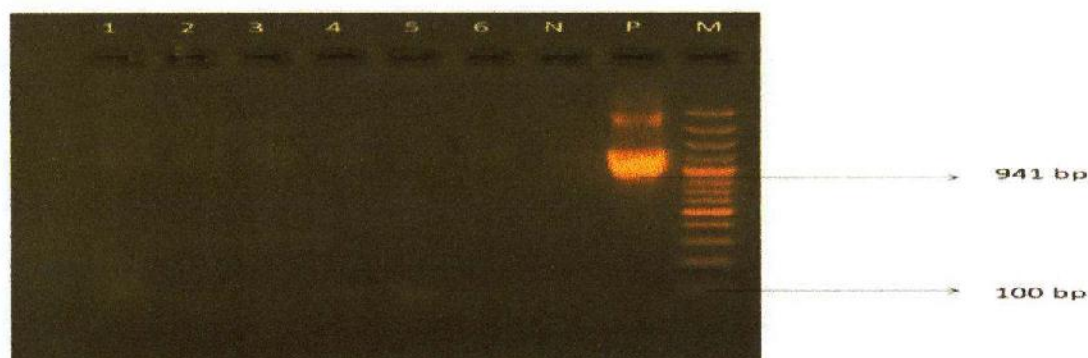


Fig. 12. Agarose gel electrophoresis of the PCR product of WSSV in Penaeid shrimp of NR hatchery: (amplified 941bp with WSSV 146F2/WSSV 146R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-6 indicates samples (Lane 1-3: PL; Lane 4-6: artemia). Amplification of the 941bp fragment were observed only in the positive control.

4.3.6. PCR detection of WSSV in ARC Hatchery: From ARC hatchery 12 samples were collected randomly and then PCR detection was conducted. One PL sample was found positive out of 12 sample through the conventional PCR method in the ARC hatchery (Fig. 13). Only 8.33% samples were positive in this hatchery.

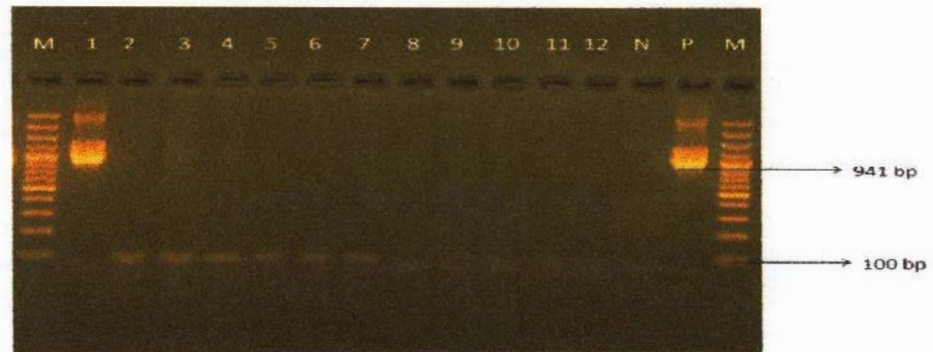


Fig. 13. Agarose gel electrophoresis of the PCR product of WSSV in Penaeid shrimp of ARC hatchery: (amplified 941bp with WSSV 146F2/WSSV 146R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-12 indicates samples (Lane 1-3: PL; Lane 4-6: artemia; Lane 7-9: mysis; Lane 10-12: algae). Amplification of the 941bp fragment were observed only in the positive control.

4.3.7. PCR detection of WSSV in Platinum Hatchery: Fifteen random sample were collected from Platinum hatchery and then PCR detection was conducted. Nine out of 15 samples including 2 Artemia, 1 female brood, 1 nauplii, 1 PL, 1 zoea and 3 male brood of the Platinum hatchery were found WSSV positive by the conventional PCR (Fig. 14). The highest 60% samples were found positive in this hatchery.

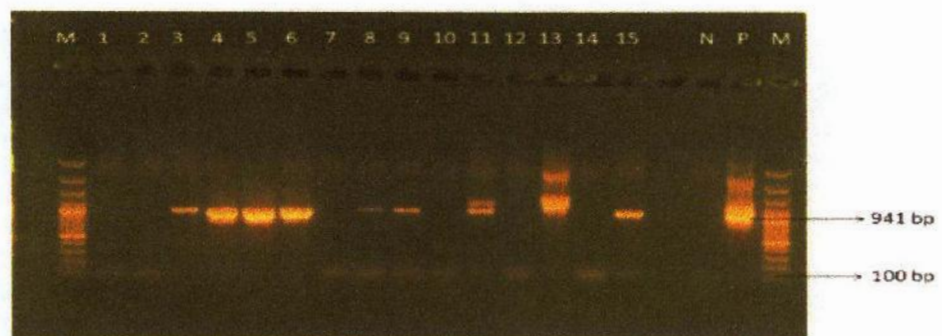


Fig. 14. Agarose gel electrophoresis of the PCR product of WSSV in Penaeid shrimp of Platinum hatchery: (amplified 941bp with WSSV 146F2/WSSV 146R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-15 denotes samples (Lane 1-3: PL; brood (female); Lane 4: zoea; Lane 5-6: male, Lane 7-9: artemia; Lane 10: algae; Lane 11: nauplii; Lane 12-14: brood (female); Lane 15: brood (male)). Amplification of the 941bp fragment were observed only in the positive sample and positive control. Sample 3, 4, 5, 6, 8, 9, 11, 13, 15 show 941 bp amplification.

4.3.8. PCR detection of WSSV in Quality Hatchery: From Quality hatchery 7 samples were collected randomly and then PCR detection was conducted. One PL sample and 2 artemia were found positive out of 7 sample through the conventional PCR method in the Quality hatchery (Fig. 15). About 42.86% WSSV prevalence was noticed in this hatchery.

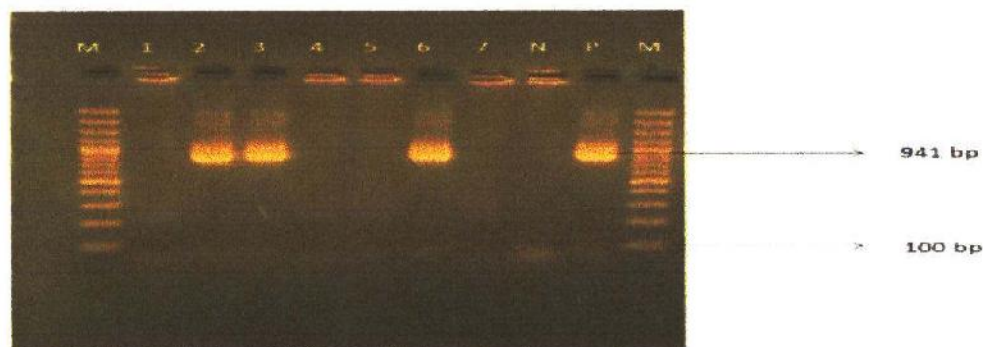


Fig. 15. Agarose gel electrophoresis of the PCR product of WSSV in Penaeid shrimp of Quality hatchery: (amplified 941bp with WSSV 146F2/WSSV 146R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-7 denotes samples (Lane 1: algae; Lane 2-4: artemia; Lane 5-7: PL). Amplification of the 941bp fragment were observed only in the positive sample and positive control. Sample 2, 3 and 5 show 941 bp amplification.

4.3.9. PCR detection of WSSV in New Madina Hatchery: Six random sample were collected from Platinum hatchery and then PCR detection was conducted. In New Madina hatchery, no samples were found positive through the conventional PCR method (Fig. 16).

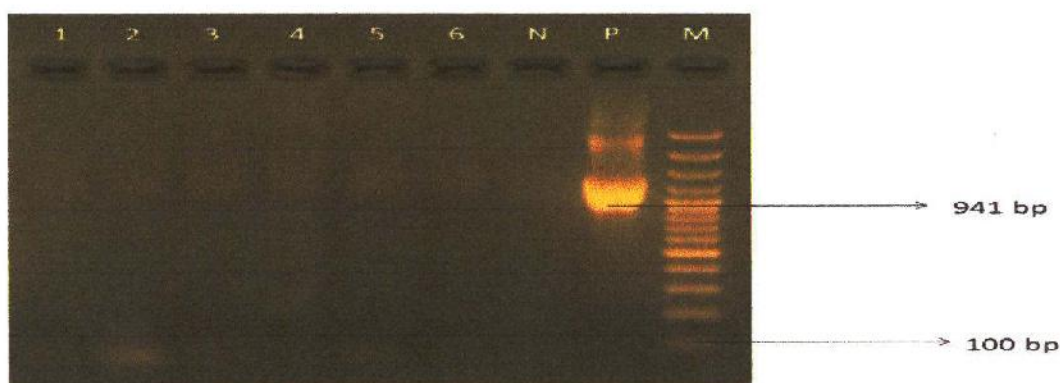


Fig. 16. Agarose gel electrophoresis of the PCR product of WSSV in Penaeid shrimp of New Madina hatchery: (amplified 941bp with WSSV 146F2/WSSV 146R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-6 denotes samples (Lane 1-3: artemia; Lane 4-6: PL). Amplification of the 941bp fragment were observed only in the positive control.

4.3.10. PCR detection of WSSV in Modern Hatchery: From Modern hatchery 6 samples were collected randomly and then PCR detection was conducted. In Modern hatchery, all samples were negative through the conventional PCR method (Fig. 17).

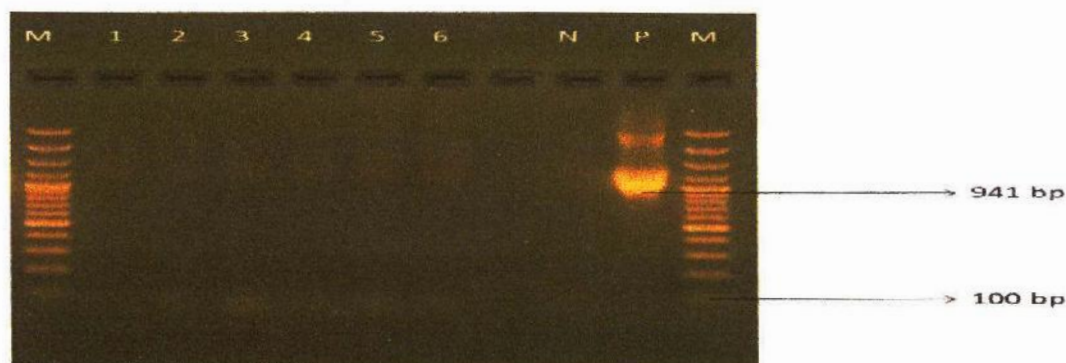


Fig. 17. Agarose gel electrophoresis of the PCR product of WSSV in Penaeid shrimp of Modern hatchery: (amplified 941bp with WSSV 146F2/WSSV 146R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-6 denotes samples (Lane 1-3: artemia; Lane 4-6: PL). Amplification of the 941bp fragment were observed only in the positive control.

4.3.11. WSSV prevalence rate (%) at different hatcheries

From the analysis, it was found that in Platinum Hatchery the highest 60% samples were WSSV positive. In case of Sonali, Satata, Golden Aqua, Balaka, NR, ARC, Quality, New Madina and Modern Hatchery WSSV percentage are 41.18%, 33.33%, 35.29%, 9.09%, 0%, 8.33% 42.86%, 0% and 0%, respectively among the total sample of the respective hatchery (Fig. 18).

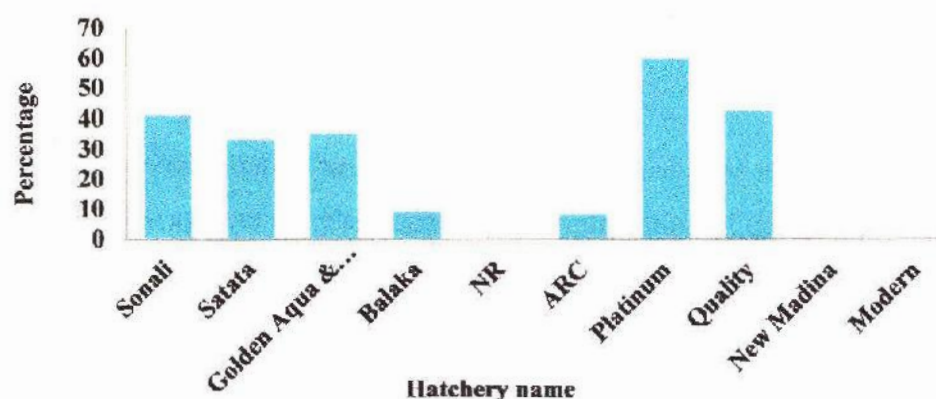


Fig. 18. WSSV prevalence rate (%) at different hatcheries (Sonali, Satata, Golden, Balaka, NR, ARC, Platinum, Quality, New Madina and Modern).

Among the total (118) samples, 34 samples (28.81 %) were WSSV positive in the hatcheries of Cox's Bazar (Fig. 19). According to the investigation 100% male brood, nauplii, squid and mussel were positive and in case of female brood, PL, zoea, mysis, artemia, algae and nauplii the WSSV prevalence were 61.11%, 23.33%, 14.29%, 18.18%, 16.67% and 0% respectively (Fig. 20).

Prevalence rate (%) of WSSV infection

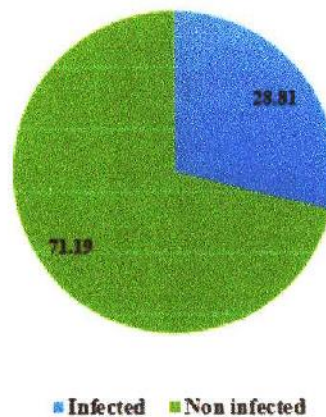


Fig. 19. Percentage of WSSV infection in the hatcheries of Cox's Bazar

The highest WSSV prevalence were found in brood, nauplii, squid and mysis samples.

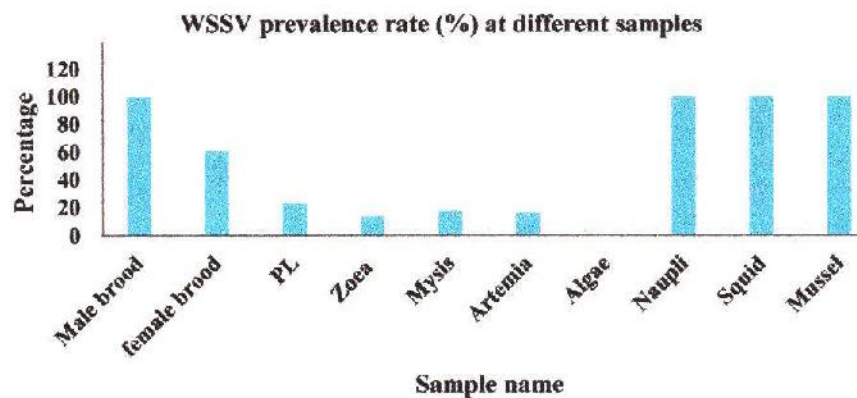


Fig. 20. WSSV prevalence rate at different sample. Total 34 out of 118 samples are positive for WSSV. Male brood, nauplii, squid and mussel are 100% positive and female brood, PL, zoea, mysis, artemia, and algae shows the WSSV prevalence rate 61.11%, 23.33%, 14.29%, 18.18%, 16.67% and 0%, respectively.

4.5. Genomic structure of *Vibrio parahaemolyticus*

The AHPND causing *V. parahaemolyticus* plasmid has a 69 kb region that encodes the pirA and pirB toxin genes. The strain VP_{AHPND} has one or more extrachromosomal plasmids (pVA1) with post segregational killing systems (PSK) that direct plasmid-encoded homologs of the binary toxins PirA and PirB. The first genome sequencing report of Bangladesh was conducted to MSR16 and MSR17 AHPND positive *V. parahaemolyticus* strains isolated from shrimps. These strains are found in shrimp farms in southwest region of Bangladesh. In the MSR16 strain, the genomes were noticed to assemble into 108 contigs, and in the MSR17 strain, 66 contigs. The entire genome size of MSR16 is 5.4 Mbp and MSR17 is 5.2 Mbp. For the MSR16 and MSR17 strains, the total GC content were, respectively, 45.09 and 45.19 percent (Ahmmed *et al.*, 2019). Here, we have conducted nucleotide sequences comparison among 10 different *Vibrio sp.* (Table 5). The nucleotide sequence sizes of Vp_{AHPND} isolate including *Vibrio parahaemolyticus* strain 20160303005-1, *Vibrio parahaemolyticus* strain HY3,

Table 5. Nucleotide sequencing information of different *Vibrio sp.* strains

Countries	Strain	Sequence size	Accession no.
China	<i>Vibrio parahaemolyticus</i> strain 20160303005-1	74488 bp	CP034304.1
China	<i>Vibrio parahaemolyticus</i> strain HY3, complete cds	1961 bp	MH718270.1
China	<i>Vibrio owensii</i> strain SH14 plasmid pVHvo	69148 bp	CP045861.1
China	<i>Vibrio campbellii</i> strain 170502	69012bp	CP033136.1
USA	<i>Vibrio parahaemolyticus</i> strain R14	74457 bp	CP028145.1
Vietnam	<i>Vibrio parahaemolyticus</i> strain LA8, complete cds	1374 bp	MW355910.1
Vietnam	<i>Vibrio sinaloensis</i> strain K61	1317bp	MN846077.1
Vietnam	<i>Vibrio harveyi</i> strain K9	1317bp	MN846071.1
Vietnam	<i>Vibrio alginolyticus</i> strain K18	336bp	MN846074.1
Costa Rica	<i>Vibrio parahaemolyticus</i> isolate STCR005	284bp	MH685878.1

* bp denotes base pair

Vibrio owensii strain SH14 plasmid pVHvo (Lui *et al.*, 2015), *Vibrio campbellii* strain 170502 (Kanrar *et al.*, 2018), *Vibrio parahaemolyticus* strain R14, *Vibrio parahaemolyticus* strain LA8

complete cds, *Vibrio sinaloensis* strain K61, *Vibrio harveyi* strain K9, *Vibrio alginolyticus* strain K18 and *Vibrio parahaemolyticus* isolate STCR005 range from 74488 bp to 284 bp.

4.6. Phylogenetic relationship of *Vibrio parahaemolyticus* with other isolates

Publicly available *V. parahaemolyticus* genomes were downloaded from NCBI. A total of 10 *V. parahaemolyticus* isolates plasmid including *Vibrio parahaemolyticus* strain 20160303005-1 plasmid pVPSD2016-5 were used to establish the phylogenetic relationship among the AHPND positive *V. parahaemolyticus* plasmid (Fig. 21). The phylogenetic relationship among these strains has been done by MEGA ver. 11. This cladogram showed that *Vibrio parahaemolyticus* strain 20160303005-1 plasmid pVPSD2016-5 has a close relationship with other strains. All the strains formed a cluster with 81-100% similarity except the USA isolate *Vibrio parahaemolyticus* strain R14.

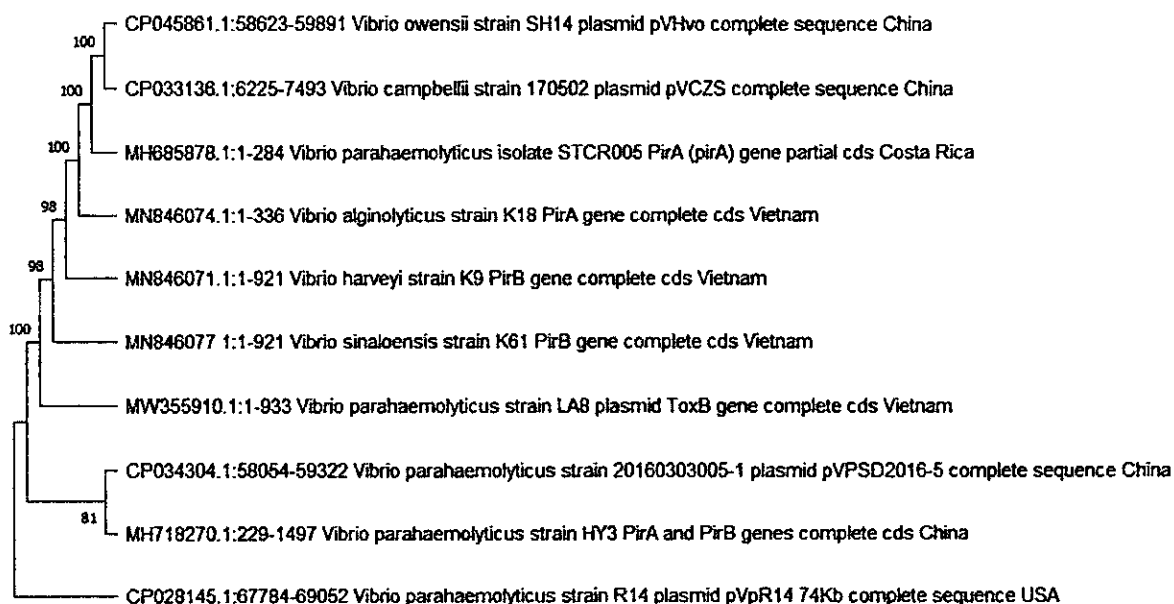


Fig. 21. Phylogenetic relationship among plasmid sequences from VP_{AHPND} isolates. MEGA ver. 11 and neighbor joining method with 100 bootstrap values (numbers = bootstrap percentage values) were used to construct the phylogeny based on the genome sequences of 10 VP_{AHPND} strain.

4.7. PCR detection of AHPND

The extracted DNA was used to detect the presence of AHPND positive samples by polymerase chain reaction using GeneJet DNA purification kit. The primer sequence of 1st PCR was AP4-F1 5'-ATGAGTAACAATATAAAACATGAAAC 3' and AP4-R1 5' ACGATTTCGACGTTCCCCAA-3' and the primer sequence of nested PCR was AP4-F2 5' TTGAGAATACGGGACGTGGG-3' and AP4-R2 5'GTTAGTCATGTGAGCACCTTC-3'. These primer set were used to amplify the DNA sequence by following the previous methods (Dangtip *et al.*, 2015) which generated amplicons at 230bp for nested PCR product. The PCR amplified products were analyzed by 2% agarose gels containing ethidium bromide and visualized under UV illuminator and amplified product was compared with a 100 bp DNA ladder.

4.7.1. PCR detection of AHPND in Sonali Hatchery: We collected 17 random sample from Sonali Hatchery including brood (male, female), PL, zoea, mysis, artemia, nauplii, squid, mussel and algae. In Sonali hatchery, all samples were found negative for AHPND (Fig. 22).

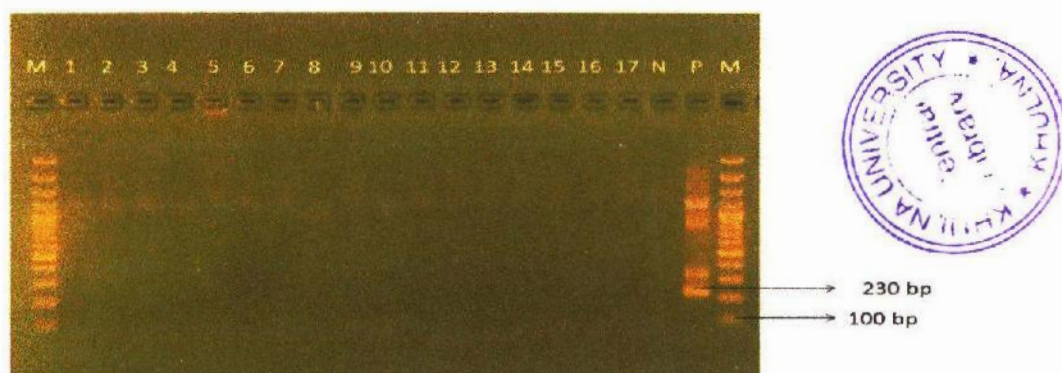


Fig. 22. Agarose gel electrophoresis of the PCR product of AHPND in Penaeid shrimp of Sonali hatchery: (amplified 230bp with AP4F2/ AP4R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-17 indicates sample (Lane 1-3: PL; Lane 4-6: zoea; Lane 7-9: artemia; Lane 10: algae; Lane 11-15: brood (female); Lane 16: squid; Lane 17: mussel). Amplification of the 230 bp fragment were detected only in the positive control.

4.7.2. PCR detection of WSSV in Satata Hatchery: Twenty-one random sample were collected from Satata hatchery and then PCR detection was conducted. No AHPND positive sample were found in Satata Hatchery through conventional PCR method (Fig. 23)

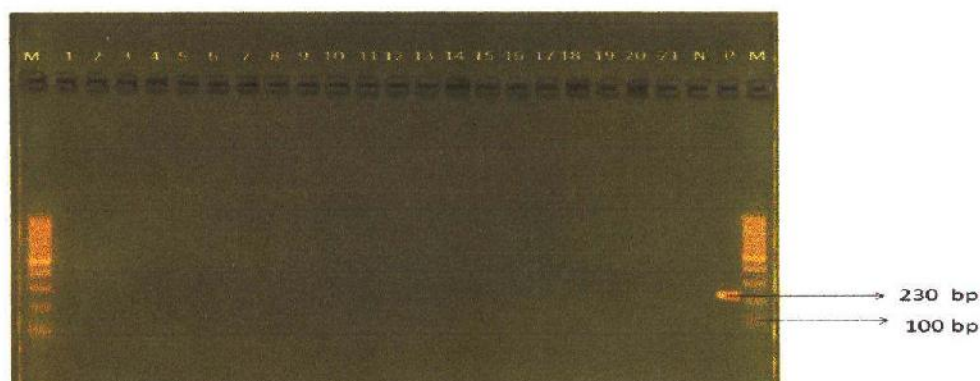


Fig. 23. Agarose gel electrophoresis of the PCR product of AHPND in *Penaeid* shrimp of Satata hatchery: (amplified 230bp with AP4F2/ AP4R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-21 indicates sample (Lane 1-3: PL; Lane 4-6: zoca; Lane 7-9: mysis; Lane 10-12: artemia; Lane 11-14: algae; Lane 15-19: brood (female); Lane 20-21: brood(male)). Amplification of the 230bp fragment was observed only in the positive control.

4.7.3. PCR detection of AHPND in Golden Aqua Ltd. Hatchery: Seventeen samples of Golden hatchery were collected randomly and molecular detection was carried out. In Golden hatchery, no sample were found positive for AHPND through the conventional PCR method (Fig. 24).



Fig. 24. Agarose gel electrophoresis of the PCR product of AHPND in *Penaeid* shrimp of Golden Aqua hatchery: (amplified 230bp with AP4F2/ AP4R2 nested primer). Lane 1-17 indicates sample (Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-5: brood (female); Lane 6-8: PL; Lane 9-10: mysis; Lane 11-13: artemia; Lane 14-16: algae; Lane 17: mysis). Amplification of the 230bp fragment was detected only in the positive control.

4.7.4. PCR detection of AHPND in Balaka Hatchery: From Balaka hatchery 11 samples were collected randomly and then PCR detection was conducted. No samples were positive for AHPND in Balaka Hatchery through the conventional PCR method (Fig. 25).

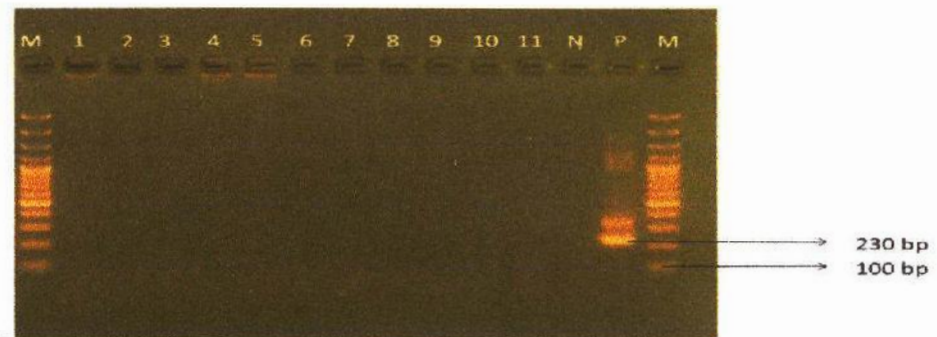


Fig. 25. Agarose gel electrophoresis of the PCR product of AHPND in Penaeid shrimp of Balaka hatchery: (amplified 230bp with AP4F2/ AP4R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-11 indicates sample (Lane 1-3: PL; Lane 4-5: mysis; Lane 6-8: artemia; Lane 9-11: algae). Amplification of the 230bp fragment was observed only in the positive control.

4.7.5. PCR detection of AHPND in NR Hatchery: Six sample of NR hatchery were collected randomly and molecular detection was carried out. No positive band were found for the sample in NR Hatchery through the conventional PCR method (Fig. 26).

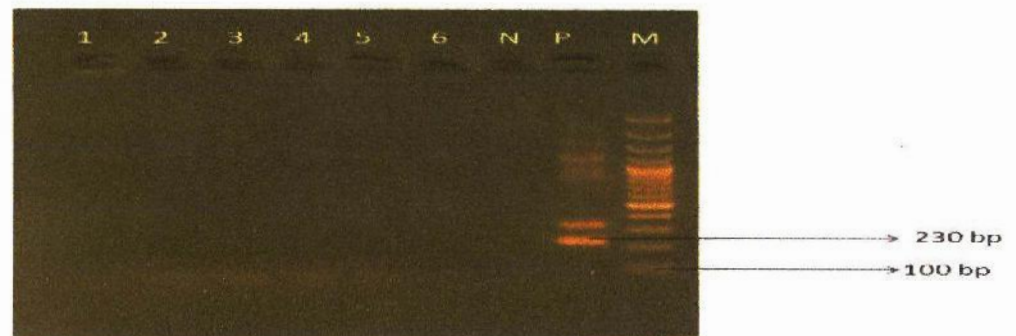


Fig. 26. Agarose gel electrophoresis of the PCR product of AHPND in Penaeid shrimp of NR hatchery: (amplified 230bp with AP4F2/ AP4R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-6 indicates samples (Lane 1-3: PL; Lane 4-6: artemia). Amplification of the 230bp fragment was detected only in the positive control.

4.7.6. PCR detection of AHPND in ARC Hatchery: From ARC hatchery 12 samples were collected randomly and then PCR detection was conducted. In case of ARC Hatchery all samples were negative for AHPND through the conventional PCR method (Fig. 27).

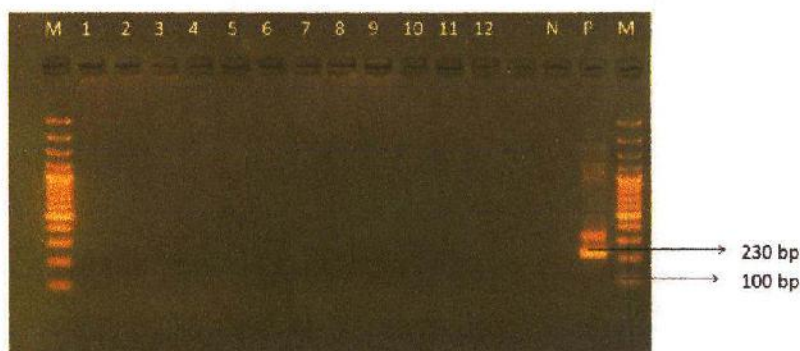


Fig. 27. Agarose gel electrophoresis of the PCR product of AHPND in Penaeid shrimp of ARC hatchery: (amplified 230bp with AP4F2/ AP4R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-12 indicates samples (Lane 1-3: PL; Lane 4-6: artemia; Lane 7-9: mysis; Lane 10-12: algae). Amplification of the 230bp fragment was detected only in the positive control.

4.7.7. PCR detection of AHPND in Platinum Hatchery: Fifteen random sample were collected from Platinum hatchery and then PCR detection was conducted. Six out of 15 samples including 2 male brood, 1 zoea, 2 artemia, and 1 algae of the Platinum hatchery were found positive for AHPND by the conventional PCR (Fig. 28).

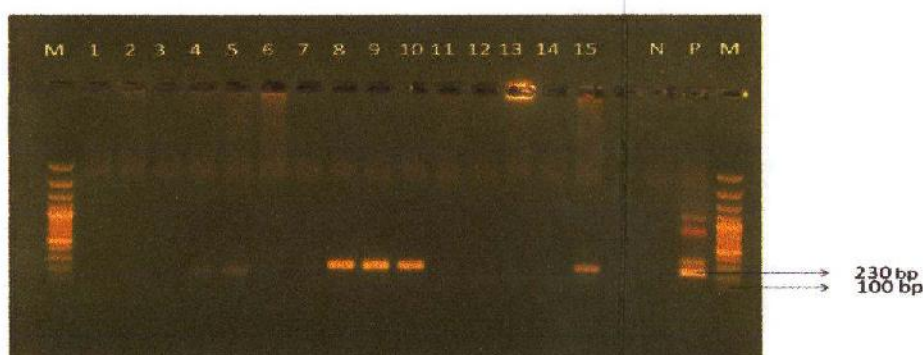


Fig. 28. Agarose gel electrophoresis of the PCR product of AHPND in Penaeid shrimp of Platinum hatchery: (amplified 230bp with AP4F2/ AP4R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-15 denotes samples (Lane 1-3: PL; brood (female); Lane 4: zoea; Lane 5-6: male, Lane 7-9: artemia; Lane 10: algae; Lane 11: nauplii; Lane 12-14: brood (female); Lane 15: brood (male)). Amplification of the 230bp fragment was detected only in the positive sample and positive control. Samples 4, 5, 8, 9, 10 and 15 no. show amplification at 230 bp.

4.7.8. PCR detection of AHPND in Quality Hatchery: From Quality hatchery 7 samples were collected randomly and then PCR detection was conducted. All samples were negative for AHPND in Quality Hatchery (Fig. 29).

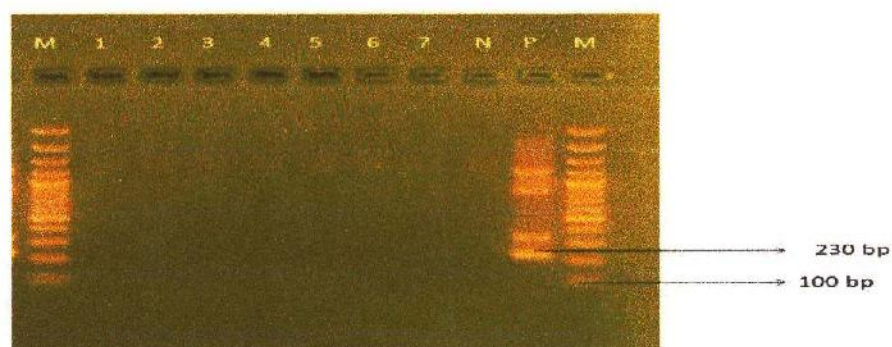


Fig. 29. Agarose gel electrophoresis of the PCR product of AHPND in *Penaeid* shrimp of Quality hatchery: (amplified 230bp with AP4F2/ AP4R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-7 denotes samples (Lane 1: algae; Lane 2-4: artemia; Lane 5-7: PL). Amplification of the 230bp fragment was detected only in the positive control.

4.7.9. PCR detection of AHPND in New Madina Hatchery: Six random sample were collected from Platinum hatchery and then PCR detection was conducted. In case of New Madina Hatchery, no sample were found positive for AHPND through the conventional PCR method (Fig. 30)

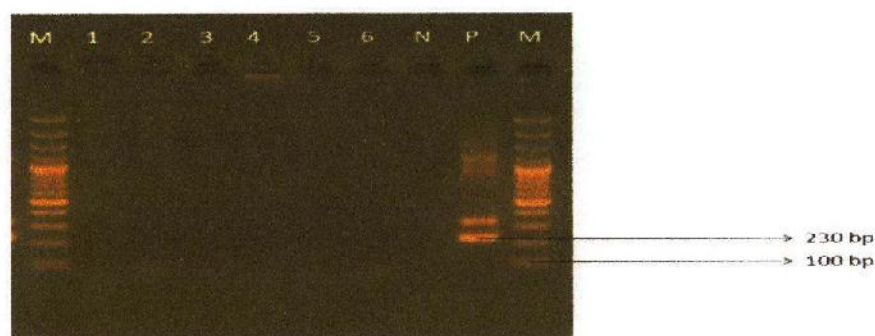


Fig. 30. Agarose gel electrophoresis of the PCR product of AHPND in *Penaeid* shrimp New Madina hatchery: (amplified 230bp with AP4F2/ AP4R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-6 denotes samples (Lane 1-3: artemia; Lane 4-6: PL). Amplification of the 230bp fragment was detected only in the positive control.

4.7.10. PCR detection of AHPND in Modern Hatchery: From Modern hatchery 6 samples were collected randomly and then PCR detection was conducted. In Modern Hatchery, all samples were AHPND negative (Fig. 31).

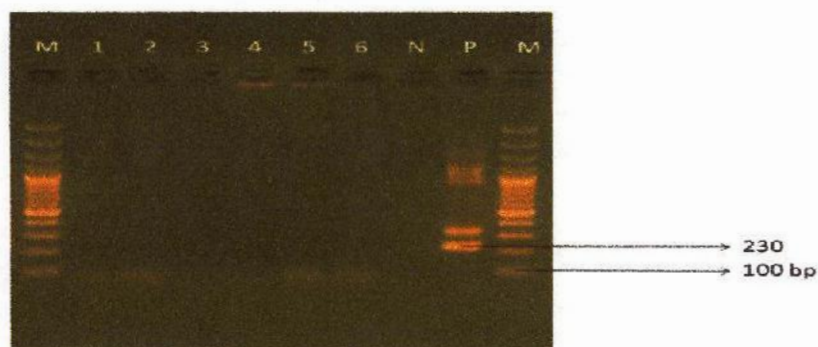


Fig. 31. Agarose gel electrophoresis of the PCR product of AHPND in Penaeid shrimp of Modern hatchery: (amplified 230bp with AP4F2/ AP4R2 nested primer). Lane N and Lane P denote negative and positive control, respectively. M denotes DNA ladder. Lane 1-6 denotes samples (Lane 1-3: artemia; Lane 4-6: PL). Amplification of the 230bp fragment was detected only in the positive control.

4.7.11. Hatchery wise AHPND positive sample

Among all the Hatcheries, only 40% samples of platinum Hatchery were found positive for AHPND (Fig. 32).

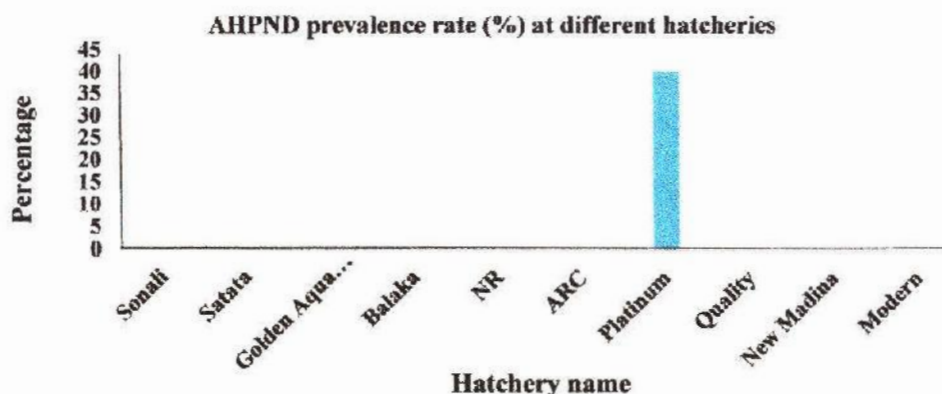


Fig. 32. AHPND prevalence rate (%) at different hatcheries

But in case of Sonali, Satata, Golden Aqua, Balaka, NR, ARC, Quality, New Madina and Modern Hatchery no AHPND positive sample have been found (Fig. 32). This result indicates the biosecurity management of these hatcheries are strong enough to resist the disease infection.

4.8. Prevalence and distribution of AHPND in different hatcheries of Cox's Bazar

After collecting samples from hatcheries extraction and purification were conducted and DNA was separated using 1% agarose gel electrophoresis to detected another disease causative agent *Vibrio parahaemolyticus*. For this purpose, we selected the primer pairs AP4-F1, AP4-R1 and AP4-F2, AP4-R2 (Table 6). Amplification of the DNA was done by AP4F1 and AP4R1 primer for first PCR and AP4F2 and AP4R2 primer for nested PCR through a conventional nested PCR process.

Table 6. AHPND positive sample in different hatcheries

Hatchery	Brood		PL	Zoea	Mysis	Artemia	Naupli	Squid	Mussel	Algae
	male	female								
Sonali	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Satata	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Golden aquaculture	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Balaka	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
NR	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ARC	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Platinum	2	ND	ND	1	ND	2	ND	ND	ND	1
Quality	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
New Madina	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Modern	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

*ND denotes not detected

The positive control showed band in 230bp for nested PCR product. Total 6 out of 118 samples showed positive for AHPND. Therefore, only 5.08% samples were AHPND positive (Fig. 33). The prevalence rate of AHPND in different hatcheries were lower. Among the samples 2/5 of male brood, 1/7 zoea, 2/30 artemia, 1/1 Algae. No female brood, PL, mysis, naupli, squid and mussel were positive for AHPND (Table 6).

So, it was found that 40% male brood, 14.29% zoea, 6.67% artemia, 7.14% algae were found AHPND positive respectively (Fig. 34).

Prevalence rate (%) of AHPND in samples

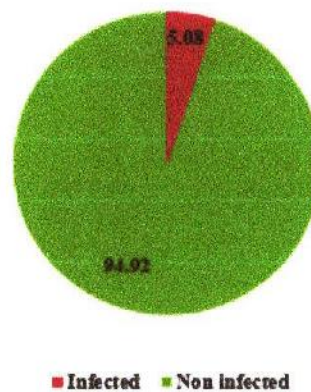


Fig. 33. Overall AHPND prevalence rate (%) in the hatcheries

The presence of AHPND prevalence in artemia and algae indicates the horizontal transmission of bacterial infection.

AHPND prevalence rate (%) at different samples

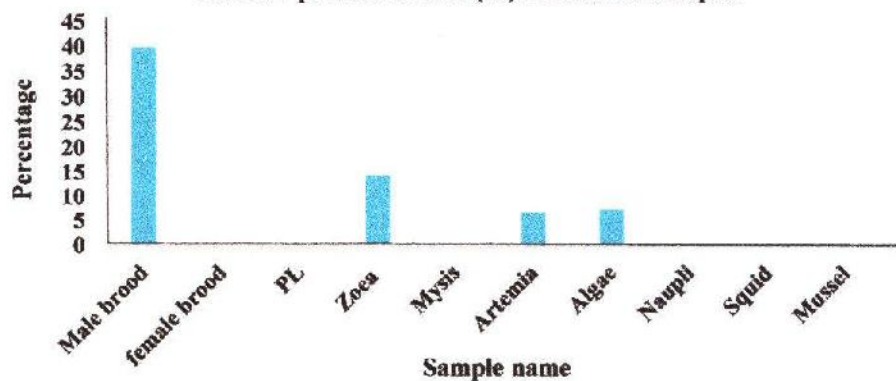


Fig. 34. Percentage of AHPND positive sample. Total 6 out of 118 samples are positive for AHPND. Male brood, zoea, artemia and algae are 40%, 14.29%, 6.67% and 7.14% prevalence of AHPND. Female brood, PL, mysis, nauplii, squid and mussel are negative for AHPND.

CHAPTER FIVE

(DISCUSSION

Netherland and WSSV isolate IN AP4RU India formed (Fig. 7) a separate cluster and showed their relatedness. WSSV strain PC China, *Procambarus clarkii* virus isolate 1601 China, WSSV isolate CN_95_DFPE USA, WSSV isolate LC1 Mexico, WSSV isolate DV1 Mexico and WSSV isolate CIBA 002 India located in the same cluster. WSSV isolate DBA1182 India and WSSV isolate AU Australia formed another cluster. Basically, these isolates distributed in south east Asian, east Asian and American countries and these strains showed 98-100% Blast similarity. Talukder *et al.*, (2021) also noticed that VP28 gene of WSSV showed 100% Blast similarity with the strain MH883319. Hossain *et al.*, (2015) reported 99% similarity with the reference sequence (Accession no-EF534254), which was discovered in Kerala, India. He reported that sequencing results demonstrating Bangladesh and the examined strains had a same WSSV ancestry.

For the purpose of monitoring and managing WSSV, numerous diagnostic techniques have been developed. Among these diagnostic techniques, nested PCR has been discovered to be the most sensitive. Hameed *et al.*, (2005) utilized three sense primers F1, F2 and F3, respectively and one antisense primer R1 to detect WSSV in *Penaeus monodon*. The designated primer sets had same annealing temperature (55°C). In another study, a set of species-specific primer WSSV-VP28 F and R was used by Talukder *et al.*, (2021). In this study, we collected 118 samples from 10 hatcheries of Cox's Bazar district including brood (male, female), PL, zoea, mysis, artemia, algae, nauplii, squid and mussel. We used conventional nested PCR method to detect WSSV by using 146-F1, 146-R1 and 146-F2, 146-R2 primers pairs (Lo *et al.*, 1996) which showed band at 1447bp and 941bp for single and nested PCR, respectively. We discovered that both primer combinations were effective at amplifying predicted size fragments from WSSV isolates from Bangladesh. We have found that about 28.81% sample are WSSV positive which is lower compared to 30% infection described by Ayub *et al.*, (2008) from five hatcheries of Cox's Bazar, lower than the prevalence of 75% reported by Otta *et al.*, (1999) from India's west coast as well as lower in comparison to the 39.4% prevalence reported by Uma *et al.*, (2005) from the south coast of India. We also found that 100% male brood, nauplii, squid and mussel are positive and in case of female brood, PL, zoea, mysis, artemia, algae and nauplii the WSSV prevalence were moderate to low respectively. Therefore, this study suggests that WSSV affect all stages of life cycle of shrimp. DNA virus generally transmitted vertically from brood stock to the offspring through eggs via nauplii. This result also suggests that squid, mussel and other hatchery samples also a potential host for WSSV and horizontal transmission from these samples infect the PL. In this study, it was found that in Platinum Hatchery 60%

sample were WSSV positive, whereas Sonali, Satata, Golden Aqua, Balaka, ARC and Quality hatcheries were found lower infection. In some hatcheries WSSV are negative. Reason behind this variation may be the different sources of brood stock in these hatcheries and the level biosecurity management. According to the research by Durand and Lightner, (2002) a significant cause is the requirement of minimum dosage of WSSV to transmit WSD in shrimp via immersion which is 10⁵ copies.

Shrimps with clinical signs of severe hepatopancreatic atrophy have been linked to the infectious condition known as acute hepatopancreatic necrosis disease (AHPND) (Tran *et al.*, 2013). The AHPND-causing *V. parahaemolyticus* plasmid has a 69 kb region that encodes the *pirA* and *pirB* toxin genes. In Bangladesh, the first genome sequence of VP_{AHPND} was conducted to AHPND positive *V. parahaemolyticus* strains MSR16 and MSR17 which was led by Ahmmed *et al.*, (2019). In Blast analysis, *Vibrio parahaemolyticus* strain 20160303005-1 plasmid pVPSD2016-5 (Accession no- CP034304.1) showed complete matching (Fig. 36) with *Vibrio parahaemolyticus* strain HY3 (Accession no- MH718270.1), *Vibrio owenssi* strain SH14 (Accession no-CP045861.1), *Vibrio campbellii* strain 170502 (Accession no- CP0333136.1), *Vibrio parahaemolyticus* strain-R14 (Accession no-CP028145.1), *Vibrio parahaemolyticus* strain-LA8 (Accession no-MW355910.1), *Vibrio sinaloensis* strain (Accession no-MN846077.1), *Vibrio harveyi* strain (Accession no-MN846071.1), *Vibrio alginolyticus* strain (Accession no-MN846074.1) *Vibrio parahaemolyticus* isolate STCR005 (Accession no-MH685878.1) (NCBI).

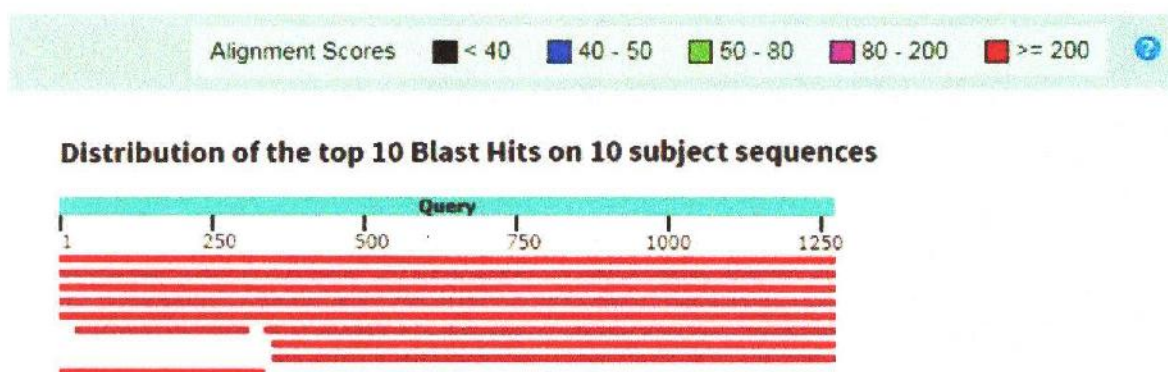


Fig. 36. Alignment of 10 different sequences with *Vibrio parahaemolyticus* strains. Graphic summary of the Blast analysis of 10 sequences shows matching among these sequences.

The phylogenetic analysis among these sequences shows the relationship among these isolates. The cluster shows that the strains are related with 100 bootstrap values. The strains from Southeast Asia, East Asia, and the USA make a cluster and show 98-100% (Fig. 21). USA

strain *Vibrio parahaemolyticus* R14 (Accession no-CP028145.1) made a separate branch from the Asian strain. The infectious type distributed in east and Southeast Asia, and American countries. Kongrueng *et al.*, (2014) adopted a PCR technique that was specifically designed to target the distinctive DNA sequences by utilizing the primer AP2 and AP3 obtained from the toxin gene that caused AHPND. In addition, AP3 primer was utilized by Soto-Rodriguez *et al.*, (2015) to identify disease causing *V. parahaemolyticus* strains. Dangtip *et al.*, (2015) revealed a new primer AP4 which was more sensitive. Another study was conducted by Eshik *et al.*, (2017) to detect the existence of AHPND positive *V. parahaemolyticus* using AP3 and AP4 nested PCR assay and found four isolates were AHPND positive. In the present study, we used AP4F1/AP4R1 and AP4F2/AP4R2 primer pairs for one step and nested PCR respectively and found that only 5.08% samples were AHPND positive in the hatcheries of Cox's Bazar which is much lower than 30.56% prevalence reported by Kongrueng *et al.*, (2014), also a lower prevalence than the 90% reported by Soto-Rodriguez *et al.*, (2015) and also lower than 49.04% positive reported by Dangtip *et al.*, (2015). Eshik *et al.*, (2017) found 14.29% AHPND positive sample at 2017 collecting sample from Khulna, Satkhira and Bagerhat district. However, this study revealed that the prevalence of AHPND is comparatively low than other countries. It also revealed that male brood are highly positive (40%). Zoea, artemia and algae were found moderately AHPND positive. Though the infection level is lower, hatchery operators should follow the biosecurity aspect to stabilize this condition. The discovery of viral and bacterial infection in fingerlings, live supplementary feed, and brood stock from those hatcheries suggests both vertical and horizontal transmission take place to cause diseases. This diseased shrimp could cause exceptionally high death rates after it is placed on the farm, which would make commercial shrimp farms less profitable. So, authorities should test the hatchery components through PCR for conformation of diseases status and to provide pathogen free PL. This study suggests that the advancement of fast investigative tests for the early diagnosis of WSSV and EMS/AHPND infection, is still needed and should be pursued.

CHAPTER SIX

(CONCLUSION)

Conclusion

Shrimp sector is one of the rapid developing food producing sectors in the world. Outbreak of White Spot Syndrome Virus (WSSV) has been the main problem since 1995 along with AHPND which has been causing huge economic losses in the shrimp culture industry since 2009. WSSV is the main concern in shrimp aquaculture in Bangladesh which causes huge mortalities in cultured shrimp. AHPND is also an emerging disease though the prevalence is comparatively low. Randomly selected *P. monodon* broods, PL and other hatchery components from the hatcheries show a significant level of viral and bacterial contamination, hence infected broods and PL, might be a source of infectious viral and bacterial diseases in hatcheries and shrimp culture farms. Both vertical and horizontal transmission occurs in these diseases. Artemia, mysis, algae, mussel, squid etc. are the potential host of WSSV and *Vibrio parahaemolyticus*. The present work suggests that the differentiation of WSSV and AHPND infected shrimp PL before releasing to the grow out pond or *gher* is very important to lessen the WSD and AHPND infection in shrimp culture of Bangladesh. To minimize the problem, regular disease monitoring and effective preventative measures should be taken. The hatchery managers should follow the biosecurity aspects to conduct hatchery operation. Accordingly, the production of WSSV as well as other harmful virus and bacteria free PL could emergently increase the shrimp production in Bangladesh. This study suggests conducting further research for integrated management of these dreadful viral and bacterial diseases in shrimp aquaculture by controlling disease intrusion into brood stock, PL and other hatchery components.



CHAPTER SEVEN

(REFERENCES)

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APPENDICES

Appendix

List of photographs

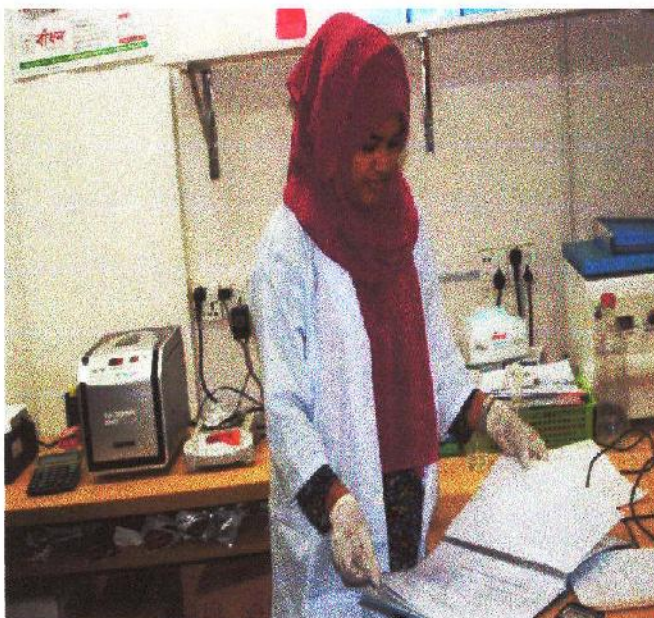


Photo 1. Checking of the protocol



Photo 2. Thawing of taq DNA polymerase



Photo 3. Pipetting

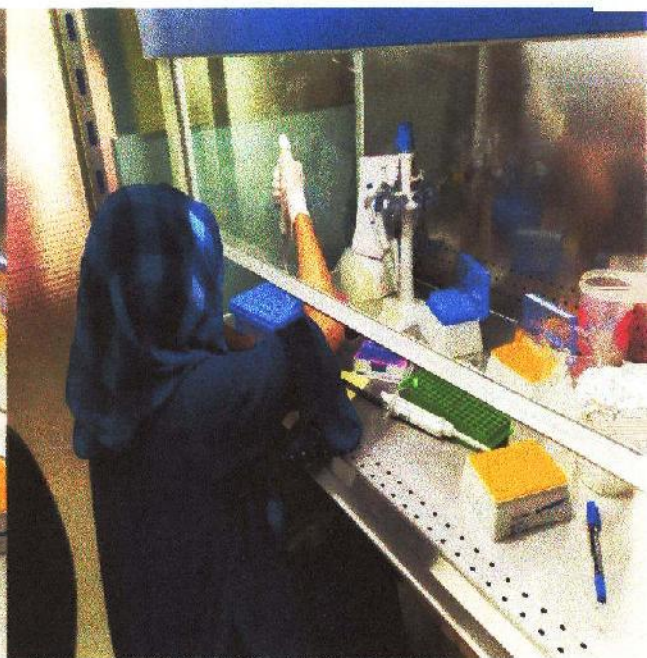


Photo 4. Master mix preparation



Photo 5. PCR tube preparation preparation



Photo 6. PCR Master mix



Photo 7. Weighting of 2% Agarose

Photo 8. Gel run Preparation