MOLECULAR AND HISTOPATHOLOGICAL STUDIES OF WHITE SPOT SYNDROME VIRUS INFECTION IN TIGER SHRIMP (*Penaeus monodon*)

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CERTIFICATE

This is to certify that thesis entitled, **"MOLECULAR AND HISTOPATHOLOGICAL STUDIES OF WHITE SPOT SYNDROME VIRUS INFECTION IN TIGER SHRIMP (*Penaeus monodon)*"** submitted to the Faculty of Agriculture, Shere-e-Bangla Agricultural University, Dhaka-1207, in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (MS) IN BIOTECHNOLOGY**, embodies the result of a piece of bona fide research work carried out by **MD. JUWEL HASAN**, Registration No. **19-10398** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

I further certify that such help or source of information, as has been availed of during the course of this investigation has duly been acknowledged.

Dated: December, 2021

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Dedicated To

My

Beloved Parents

ABBREVIATIONS AND ACRONYMS

Abbreviation	Elaboration
WSSV	White Spot Syndrome Virus
WSD	White Spot Disease
ds	Double Strand
Taq	*Thermophilus aquaticus*
Kb	Kilo Base Pair
OIE	Office International des Epizooties
PL	Post Larvae
PCR	Polymerase Chain Reaction
et al.	And others (at elli)
IHHNV	Infections Hypodermal And Hematopoietic Necrosis Virus
SHP	Slough Hepatopancreas
EMS	Early Mortality Syndrome
HPV	Hepatopancreatic Parvovirus
RFLP	Restriction Fragment Length Polymorphism
DHP	Degenerated Hepatopancreas
TBE	Tris Boric Acid EDTA
UV	Ultra Violet
bp	Base pair
SDS	Sodium Dodecyl Sulphate
NaCl	Sodium chloride
rpm	Rotation per minute
CHX	Cycloheximide
TL	Tubule Lumen
HP	Hepatopancreas
BEPB	Bangladesh's Export Promotion Bureau
(Ac-ELISA)	Antigen-Based Enzyme linked Immunosorbent Assay
ddH_2O	Distilled Deionized Water
EDTA	Ethylene Diamine Tetra Acetic Acid
TSV	Taura Syndrome Virus
YHV	Yellow Head Virus

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LIST OF CONTENTS

Chapter No.	Title	Page
	ABBREVIATIONS AND ACRONYMS	i
	ACKNOWLEDGEMENTS	ii
	LIST OF CONTENTS	iv
	LIST OF TABLES	vi
	LIST OF FIGURES	vii
	LIST OF APPENDICES	viii
	ABSTRACT	ix
CHAPTER I	**INTRODUCTION**	01
CHAPTER II	**REVIEW OF LITERATURE**	10
	2.1 Molecular diagnosis of WSSV in shrimp by PCR	11
	2.2 Histopathological studies of WSSV in shrimp	21
CHAPTER III	**MATERIALS AND METHODS**	26
	3.1 Sub-experiment-I: Detection of the WSSV Infection in tiger shrimp (*Penaeus monodon*) through one-step and two-step PCR.	27
	3.1.1 Sampling area and sample collection	27
	3.1.2 Major instruments and appliances	28
	3.1.3 Isolation of genomic DNA	28
	3.1.3.1 Reagent required for DNA isolation	28
	3.1.3.2 Sample preparation	30
	3.1.3.3 Procedure of DNA isolation	30
	3.1.4 Principles of PCR	31
	3.1.4.1 Reagents preparation for PCR	31
	3.1.4.2 Primer design	32
	3.1.4.3 PCR reaction mixture	32

LIST OF CONTENTS (Contd.)

Chapter No.	Title	Page
	3.1.4.3 (A) PCR reaction mixture for one-step PCR	33
	3.1.4.3 (B) PCR reaction mixture for two-step PCR	33
	3.1.4.4 Polymerase Chain Reactions	34
	3.1.5 TBE (10X) buffer preparation	35
	3.1.6 Agarose gel preparation	36
	3.1.7 Electrophoresis procedure	37
	3.1.8 Interpretation of PCR result	37
	3.2 Sub-experiment-II: Histopathological study to observe the change in tissue due to WSSV infection	38
	3.2.1 Collection of shrimp samples and on-site processing	38
	3.2.2 PCR ponfirmation for WSSV infection	39
	3.2.3 Histopathological study	40
	3.2.3.1 Fixation and preservation of samples	40
	3.2.3.2 Steps of histopathological procedure	40
CHAPTER IV	**RESULTS AND DISCUSSION**	45
	4.1 Confirmation of WSSV infection in the studied shrimp by one-step and two-step PCR assay	46
	4.2 Histopathological assay of WSSV infected shrimp	55
CHAPTER V	**SUMMARY AND CONCLUSION**	60
CHAPTER VI	**RECOMMENDATIONS**	63
	REFERENCES	65
	APPENDICES	77

LIST OF TABLES

Table No.	Title	Page
1	Sample collection from different locations of Satkhira and Bagerhat district.	28
2	Preparation of extraction buffer/lysis buffer	29
3	Primer sequences used for the amplification of white spot syndrome virus (WSSV) and tiger shrimp DNA	32
4	Reaction mixture for one-step PCR reaction	33
5	Reaction mixture for second step of two-step PCR reaction	33
6	Thermal profile for one-step PCR	34
7	Thermal profile for two-step PCR	35
8	Preparation of Davidson's fixative solution.	39
9	Time schedule in automatic tissue processor	41
10	Staining procedure for hematoxylin and eosin (H & E) stains	42
11	The overall PCR result of all collected sample after one-step and two-step PCR	49
12	Prevalence rate of all collected samples after one-step and two-step PCR	49

LIST OF FIGURES

Figure No.	Title	Page
01	Flow chart of 1.5% agarose gel preparation.	36
02	Samples of WSSV non-infected (control) shrimp and (a) and infected shrimp with white spot on carapace (b).	38
03	Injection and preservation of sample in Davidson fixative solution	39
04	Steps of histopathological procedure	43
05	Detection of white spot syndrome virus in *Penaeus monodon* collected from three farms of Satkhira district (6 samples from each farm) by one-step PCR with primer pairs 146F1/146R1 and 146F2/146R2	47
06	Detection of white spot syndrome virus in *Penaeus monodon* collected from three farms of Bagerhat district (6 samples from each farm) by one-step PCR with primer pairs 146F1/146R1 and 146F2/146R2	48
07	Detection of White spot syndrome virus (WSSV) in *Penaeus monodon* by two-step PCR with inner primer pair 146F2/146R2	49
08	PCR confirmation of WSSV infection for histopathological study by one-step PCR and two-step PCR using primar pairs 146F1/146R1 and 146F2/146R2	55
09	Histopathological (H & E) stained WSSV infected and non-infected hepatopancraeas of *Penaeus monodon*	56
10	Histopathological (H & E) stained WSSV infected and non-infected muscle of *Penaeus monodon*	57

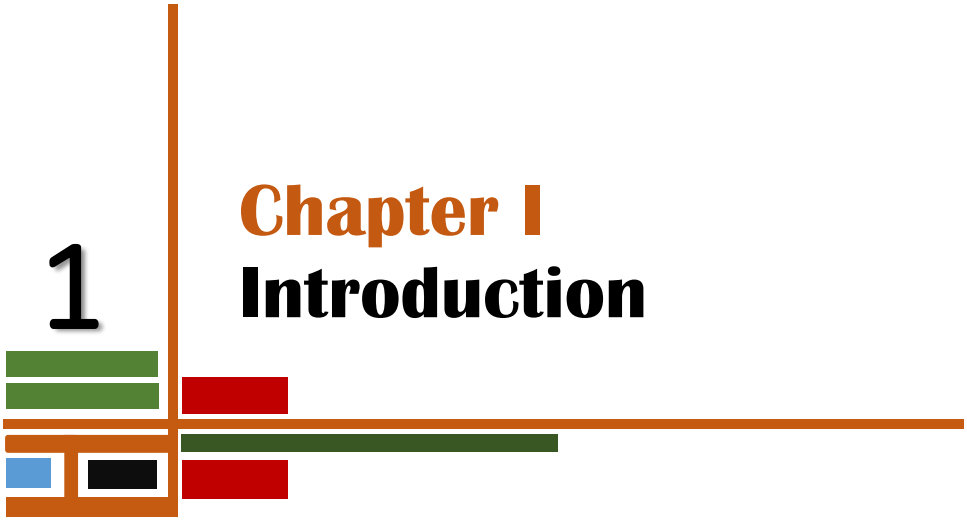
LIST OF APPENDICES

Appendix No.	Title	Page
1	Calculation to prepare lysis buffer for DNA isolation	78
2	Preparation of 10X TBE buffer for gel electrophoresis	79
3	Calculation to prepare of 1X TBE buffer from 10X stock solution	79
4	Equalization of different values of DNA concentration to prepare working solution	80

MOLECULAR AND HISTOPATHOLOGICAL STUDIES OF WHITE SPOT SYNDROME VIRUS INFECTION IN TIGER SHRIMP (*Penaeus monodon*)

ABSTRACT

Since the 1980s, shrimp farming has been rapidly expanded and has become the third largest export earnings source, contributing about 3.57% to national GDP of Bangladesh. Now this sector facing a serious problem with a disease named White Spot Disease (WSD) caused by dreadful White Spot Syndrome Virus (WSSV), which leads to 100% mortality in the shrimp farm within 3-5 days. This study showed PCR detection and prevalence rate of WSSV infection in *Penaeus monodon* and further confirmed by histopathological study to observe the pathological changes in the tissue. The prevalence of this virus has been studied in six selected farms located in the Bagerhat and Satkhira districts of Bangladesh. Both one-step and two-step PCR were used to detect using two pairs of primers, namely, 146F1/146R1 and 146F2/146R2, amplifying the 1447 bp and 941 bp fragments, respectively. Out of 36 randomly selected shrimp samples, 25 samples were WSSV-positive other 11 samples were negative by one-Step PCR. The negative eleven samples were further subjected to two- step PCR and resulted only 3 samples being negative. The prevalence rates of WSSV infection were 83.33% and 55.56% in Satkhira and Bagerhat district, respectively, confirmed by one-step PCR and the prevalence rate of WSSV infection was 83.33% and 100% in Satkhira and Bagerhat district, respectively, confirmed by two-step PCR. However, the overall prevalence rate increased from one-step to two-step PCR from 69.44% to 91.67%. Between two methods two-step PCR was more efficient than one-step PCR. The shrimp specimens showing clinical signs of WSSV were positive by both one-step and two-step PCR. Some of the apparently healthy samples were also found to be positive for WSSV by two-step PCR. Histopathological studies of hepatopancreas of two-step PCR confirmed WSSV-positive samples resulted in degenerated tubule lumen, degenerated and sloughing hepatopancreas with necrosis and infected muscle tissue resulting in fragmentation and separation of muscle fibers. Furthermore, it is suggested that more molecular studies about WSSV and disease management is required to increase the shrimp production in Bangladesh.



Chapter 1
Introduction

CHAPTER I
INTRODUCTION

In Bangladesh, shrimp farming has rapidly expanded since the 1980s and has become the third largest export earnings source after jute sand jute manufacture contributing about 3.57% to the national GDP and approximately 8.5 million Bangladeshi peoples, particularly coastal regions directly depend on this sector for their livelihood. According to the Department of Fisheries (DoF), 36 species of marine shrimp were identified. Among them, the main cultured species is tiger shrimp (*Penaeus monodon*) cultured in the southeast region of Bangladesh. Total shrimp and prawn production of Bangladesh has been increased from 1.40 Lakh MT in 2000-01 to 2.52 Lakh MT in 2020-21 where the total production area is 263,025 ha with its present growth rate of 5.45% of total fish production (Fisheries Statistical Yearbook 2020-21). Recently, Bangladesh exported black tiger shrimp worth USD 268.95 million (EUR 237.7 million) in the first six months of the 2021-2022 fiscal year (July-December 2021), an increase of 38.2 percent in the same period in 2020 (Bangladesh's Export Promotion Bureau, 2021-2022). It has been expected to be the highest compared with the country's shrimp export value in the fiscal year 2020-2021. Even being the third largest export item of Bangladesh all over the other products, quantitative data shows that Bangladesh captured only 2.5 percent of world shrimp.

However, a significant obstacle to the swift growth and extension of the industry is the reduction of shrimp production every year due to the incidence of a large variety of pathogenic bacteria and viruses particularly White Spot Syndrome Virus (WSSV) which results in huge economic loss every year all over the world (Paul and Vogl, 2012). Approximately 60% of losses in the shrimp industry occurred due to viral diseases and approximately 20% due to bacterial diseases recorded by Flegel, T.W. (2012). More than 20 different viruses were identified from wild and cultured shrimp among six devastating viral diseases listed by OIE (Talukder *et al*., 2020). Several new viral pathogens have appeared successively, resulting in mass mortalities and bullying the economic sustainability of the industries in Asia and the Americas since 1981. With the extension of the commercial shrimp industry, the number of viral pathogens of penaeid shrimp has also increased, reaching as high as 22 (Walker *et al.*, 2009). High mortality rates due to different viral and bacterial diseases reduces the production and

export as well (Uddin *et al.,* 2013). Among the more lethal viruses passing on a disease to penaeid shrimp, the white spot syndrome virus (WSSV), a rapidly reproducing and extremely virulent pathogen in shrimp, has emerged all over the world shrimp industry as one of the most prevalent and common.

White spot syndrome (WSS) is measured as a countless threat to the farming of tiger shrimp (*Penaeus monodon*). The pathogen of WSS is a kind of DNA virus called white spot syndrome virus (WSSV) (Ayub *et al.,* 2008). WSSV is a non-occluded, double-enveloped, and double-stranded rod-shaped DNA virus that belongs to the family, Nimaviridae (Chou *et al.,* 1998). WSSV with a thread-like polar extension at one end of its body, became the sole species of a new family Nimaviridae (Paz, 2010). The genome of WSSV constitutes a circular, supercoiled, double-stranded (ds) DNA size of approximately 300 kilobase pairs (kb) (Durand *et al.,* 1997). Among Asian countries, it was first detected in Taiwan in 1992 (Chou *et al.,* 1998), and then it spread out to Japan and other Asian countries. In America, it was first identified in 1995 in a South Texas shrimp farm, and it was suggested that the most probable route for its introduction was through an Asian imported frozen shrimp commodity during export (Hasson *et al.,* 2006).

The first gross signs and histopathology of white spot disease (WSD) have been reported in India and Korea from diseased shrimp in all kinds of aquaculture systems, whether extensive, semi-intensive or, intensive (Karunasagar *et al.,* 1997). Infected shrimps exhibit lethargic behavior, reduction in food consumption, reduced appetite, reduced preening activities, anorexia, loose cuticle, and reddish to pink body discoloration. One of the distinct characteristics of the white spot syndrome virus is white spots on the exoskeleton especially, on the carapace and last segment of the abdomen. These spots are due to the calcified deposits that range in size from up to 1 cm or more in diameter (Chou *et al.,* 1998). In the case of cultured shrimp, the cumulative mortality of WSSV infection can increase up to 100 % within 3–10 days of infection (Lightner, 1996). But in the case of *Penaeus vannamei* no prominent white spots are observed on the carapace of affected animals, though shrimp become lethargic, stop feeding and swim erratically periphery of the pond and die. It also has been reported that mass mortalities start 30–40 days after stocking (Perez *et al.,* 2005). It has a wide host range which includes all cultured, wild marine shrimps, crabs, lobsters, crayfishes, squilla, copepods, and freshwater cultures species (Chang *et al.,*

1998) and targets various tissues including subcuticular epithelium, hematopoietic tissues, gills, lymphoid organs, antennal gland, connective tissue, ovary, and the ventral nerve cord (Wongteerasupaya *et al.,* 1995). WSSV is actually harmful to most of the commercially important species of penaeid shrimps that caused white spot disease including *P. monodon, P. vannamei, P. indicus, P. japonicus, P. chinensis, P. penicillatus, P. azteus,* and *P. merguiensis* (OIE, 2003).

WSSV can transmit from diseased shrimp to healthy shrimp in the natural environment mainly by vertical and/or horizontal transmission. The disease transmit horizontally by ingestion of dead infected shrimp, by contact with water containing infected animals or free virus particles. However, previously infected broodstock or spawners and post larvae are also considered a major sources of infection for the shrimp industry. Potential secondary sources responsible for WSSV horizontal transmission include human activities, seabirds or other animal's immigration, infected pond sediments, infected frozen food products, contaminated aquaculture tools or instruments, and infected shrimp by-products which are untreated derived from processing plants (Lightner *et al*., 1997). Infection primarily occurs by the gills, but may occur through other body surfaces as well (Chang *et al.,* 1996; Chou *et al.,* 1995; Chou *et al.,* 1998). Vertical transmission may occur from the mother shrimp to offspring by viral particles during spawning time and when larvae ingested the first feeding, although it is uncertain whether the WSSV virions are either present inside the shrimp eggs or not (Peng *et al.,* 2001). Transferring wild-caught brood stocks to cultured post larvae (PL) that are used to stock in the rearing ponds is considered as one of the major routes of viral introduction (Ayub *et al.,* 2008).

In the context of Bangladesh, penaeid shrimp is regarded as a highly valued product with high demand in the national and international markets. That's why its production and export play a leading role in the economy of Bangladesh. Until the occurrence of WSSV in the country, the shrimp farming industry of Bangladesh had been reflected as a lucrative industry compared with other industries. After WSSV made its first occurrence in the southeastern region of Bangladesh in 1994 and spread of the disease in cultured stock of tiger shrimp in semi-intensive farms of Cox's Bazar, resulted in losses of 50–60%. Later, it severely outbreaks in small-scale farms practicing low stocking density, causing great economic loss. The scientist of Bangladesh studied hard and did much research to detect the prevalence of the WSSV virus easily within a day of infection with the scientist of other countries (Ayub *et al.,* 2008). Studies have shown

that the formation of white inclusions can also be associated with bacterial infections in the cuticle, without the presence of WSSV infection (Wang *et al.,* 2000). The white spots associated with bacterial infection produced closely resemble as caused by WSSV. They are related to the presence of rod-shaped bacteria shown by scanning electron microscopy. Thus, white spots presenct in the cuticle are not sufficient for the diagnosis of WSSV. So, it is extremely important to understand that diagnosis for WSSV infection cannot be based only on the gross signs of white inclusions in the cuticle.

Histopathology is one of the pioneer methods for the detection of WSSV in shrimp (Wang *et al.,* 1997). There are also many other techniques that are established including conventional PCR (Takahashi *et al.,* 1996), real-time PCR, isothermal amplification, immunological methods (Takahashi *et al.,* 1996) RT-PCR methods (Talukder *et al.,* 2021) and monoclonal antibody-based detection methods are most commonly used currently. Another method known as histopathology has been the gold coin for the identification of WSSV in cases when clinical signs and histopathological changes are observed. Though transferring wild caught brood stocks to cultured post larvae (PL) that are used to stock in the rearing ponds is considered one of the major routes of viral introduction, the brooders before being used in the hatchery or PL before stocking for the detection of viral infection could help the farmers to take some measurements to overcome this type of problems (Hameed *et al.,* 2005).

PCR techniques are most commonly used all over the world to detect WSSV in farmed shrimp, wild and laboratory postlarvae, carrier animals, water, and sediments (Lightner, 1996, Hossain *et al.,* 2001, 2004; Hameed *et al.,* 2005; Natividad *et al.,* 2008). But the identification of WSSV in water and plankton samples by PCR does not indicate that it is infectious. The presence of WSSV in plankton samples detected by PCR showed that there is no occurrence of WSD outbreaks (Corsin *et al.,* 2005). PCR techniques followed either a conventional amplification with a forward/reverse primer set (Lo *et al.,* 1996a; Takahashi *et al.,* 1996; Kim *et al.,* 1998) or a nested amplification (Lo *et al.,* 1996b; Lo and Kou 1998) to detect WSSV in shrimp. Nested PCR was used to analyze an increased level of primer sensitivity test compared with conventional PCR. Real-time PCR is a more sensitive highly simple, sensitive, reliable, and powerful tool that has been used for the quantification of infectious hypodermal and hematopoietic necrosis viruses in infected penaeid shrimp tissue (Tang and Lightner, 2001). Nested or two-step PCR provides a huge advantage with a high level of sensitivity compared with

single-step PCR. When a shrimp exhibits clinical signs and symptoms of WSSV in body tissue, it is easily identified by single step PCR (Bir *et al.*, 2017). Two-step nested PCR has a disadvantage with the risk of introducing contamination when a second PCR reaction is initiated using an amount of the amplicon from the first step PCR. Rolfs *et al.* (1992) have used a single tube, non-stop, semi-nested PCR where two or three primer pairs with different melting points are simultaneously added with all reaction components of PCR volume and then test the samples in a single tube to rapidly detect WSSV in shrimp.

However, one-step PCR and *in situ* hybridization is used to detect the presence of WSSV in shrimp that show gross signs of the disease with high infection but two-step PCR techniques can detect the presence of WSSV in lightly infected brood stock, post-larvae and juveniles (Lo *et al.*, 1997, Deng *et al.*, 2000). As light infection with WSSV is one of the major causes of WSS outbreaks in shrimp, two-step PCR techniques are a powerful tool to identify and take remedial measures before severe outbreaks. Since different environmental stress can trigger light infections to acute ones and cause incidences of disease outbreaks in shrimp ponds and these environmental stress could not change within a short period of time two-step PCR will be very helpful (Peng *et al.*, 1998, Hsu *et al.*, 1999, He *et al.*, 2000). Therefore, early-stage diagnosis of WSSV infection in the brood stocks and stocking of WSSV-free PL is one of the most significant tactics to monitor and prevent WSSV occurrence in shrimp farming industries (Haque *et al.*, 2020). Some commercial kits including EBTL, DiagXotics, IQ2000, BIOTEC, based on *in situ* hybridization, PCR and immunodetection, are also used for the detection of WSSV in shrimp (Ayub *et al.*, 2008).

The most sensitive diagnostic methods like PCR are helpful even when histopathological changes are not seen after a histopathological study. But histopathology is one of the pioneer methods for the detection of WSSV in shrimp (Wang *et al.*, 1997). Although PCR techniques have proved themselves as highly sensitive for the detection of WSSV, there are still limitations to their widespread application such as the requirement of special equipment, expensive reagents and well-trained personnel (Walker and Mohan 2009). WSD identification using clinical signs and then confirmed by PCR assay and histopathology method is now a strong tool in biotechnology (Pazir *et al.*, 2011). Histopathology is the diagnosis tools and study of diseases of the tissues and involves examining tissues and/or cells under a microscope go through several processes to observe the change in tissue level compared with

healthy tissue. Histopathology can analyze the susceptibility of WSSV to live species of shrimp associated with histological and morphological manifestation and the ultra-structural details of the virus experimentally in the infected shrimp (Rajendhran *et al.,* 1999). Furthermore, Histopathology is used to determine the condition of cells, tissues, and organs that are infected by not only virus or bacteria but also ectoparasites. The main purpose of the histopathological study is to compare the condition of healthy tissue to shrimp tissue samples infected with ectoparasites, bacteria, viruses or, other pathogens. Firstly, it needs to be deliberated in diagnosing the disease to see clinical signs which include behavior, external and internal characteristics and pathological changes.

White Spot Syndrome Virus mostly infects the tissues that originate from both ectoderm and mesoderm. Histopathological signs of WSSV infection include enlarged nuclei in tissues and hypertrophy which are found on the tissues including subcuticular epithelium, gills, lymphoid organs, antennal gland, hematopoietic tissues, connective tissue, ovary and the ventral nerve cord (Wongteerasupaya *et al.,* 1995). The enlarged nuclei containing basophilic inclusions at the early-stage of infection or light infection show marginated chromatin parted from a reddish inclusion by a ring of the unstained nucleoplasm. These are known as Cowdry A-type inclusions (Flegel, 2006). During early-stages of infection, the stomach, gills, cuticular epidermis and the connective tissue of the hepatopancreas develop infection by WSSV. At later infection stages, the lymphoid organ, antennal gland, muscle tissue, hematopoietic tissue, heart, hindgut and parts of the midgut also get infected by the virus severely. The compound eyes and the nervous system becomes infected very late. At late stages of infection, necrosis become prominent in the stomach, gills, cuticular epidermis, lymphoid organ, hematopoietic tissue and antennal gland and are all heavily infected with WSSV (Chang *et al.,* 1998; Lo *et al.,* 1997). Thus, the subcuticular epithelium in tissue sections is the most convenient tissue for diagnosis WSSV in shrimp. A significant reduction in the total hemocyte count is detected after shrimp are infected with WSSV heavily (Braak *et al.,* 2002). Histopathological observations of WSSV showed basophilic Cowdry type A inclusion bodies in all tissues such as gills, haematopoietic tissue, cuticle epithelium, lymphoid organ and connective tissue that indicates shrimp infected by WSSV with high infection.

Hepatopancreas (HP) is one of the major part of the shrimp body covered by cephalothorax and is used as an indicator to identify whether shrimps health condition is either infected or not. It is composed of branched tubule and different types of epithelial cell lining (Wu and Yang, 2011). The HP shape and colour of the shrimp can be used as a sign of the shrimp's health condition and can also recognize the severity of problems that affected the shrimp. The HP can be used directly on the head under a light source to observe the health condition and can also recognize the severity of problems. In the case of early mortality syndrome (EMS) infection, the infected shrimp also exhibited an abnormal hepatopancreas including shrunken, small, swollen and discoloured. Histopathology of infected *P. vannamei* contained the EMS trace limited to the hepatopancreas with lack of mitotic activity in E cells of hepatopancreas, the dysfunction of central hepatopancreas B, F and R cells and massive sloughing of central HP tubule epithelial (Lightner *et al*., 2012). Prachumwat *et al*. (2012) also recognized dysfunction of the tubule epithelial cells through histopathological studies. Histopathology on EMS infected shrimp at the pond level discovered that connective tissue of HP capsule was pale to white in colour, significant atrophy of HP, black spot or streaks at the HP, HP does not squash easily between thumb and finger and mortality started as early as 10 days of stocking after the clinical sign (Lightner *et al*., 2012). However histologically, the hepatopancreas tissue of shrimp showed vacuolization of B cells, without inclusion bodies, but histopatholgical changes caused by IHHNV including eosinophilic Cowdry type A inclusion bodies and hepatopancreas tissue of WSD infected shrimps showed severe vacuolization due to high activity which may be on ectodermal, mesodermal and on endodermal tissue (Pazir *et al.*, 2011).

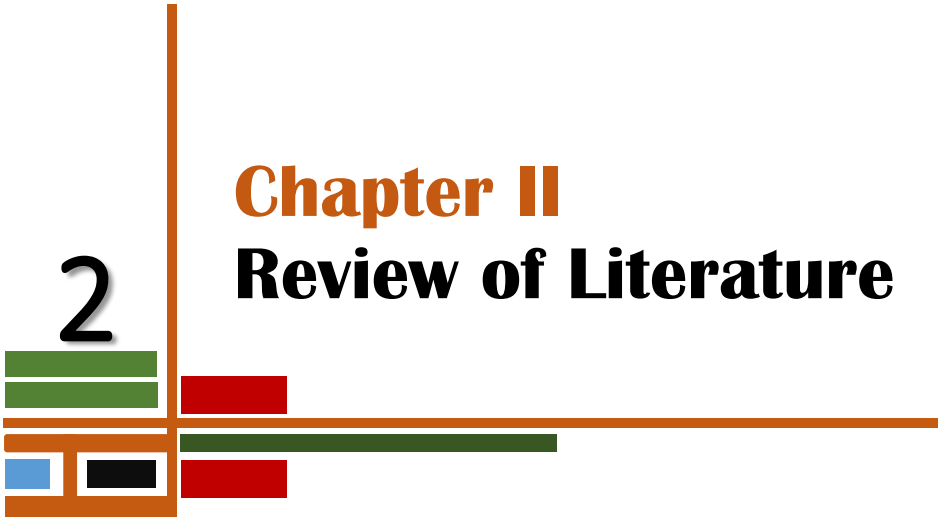
Therefore, healthy HP of shrimp will illustrate the good condition of the shrimp because of the important role of HP in shrimp survival (Manan *et al.*, 2015). Sometimes histopathological study showed the presence of large, usually single, basophilic intranuclear inclusion bodies in the distal tubules, which directed to the displacement of the nucleoli when squashed hepatopancreatic tissue mount with stained with malachite green (wet-mount technique). This type of infection revealed and known as Hepatopancreatic parvovirus (HPV) which was detected from *Penaeus monodon* (Catap and Pitogo, 2004).

Due to the compound structures of white spots which are composed of melanized material from tegumental glands, deteriorated opaque cuticular lesions embedded in the

cuticle, underlying lysed cellular debris and blocked cuticular exudates deposited at the cell-cuticle interface. However, in spite of these reasons, the white spots are mainly embedded in the shell and they are still visible after the underlying tissues have been taken from the inner surface of the shell histopathology is a good tool to detect WSSV till now (Wang *et al.*, 1999). Histopathologically the disease syndrome of *P. monodon* is very close to what has been observed for *P. japonicus, P. chinensis* (Momoyama *et al.*, 1994; Wongteerasupaya *et al.*, 1995), so without studies on viral DNA it is impossible to say whether the same virus has been spreading within the shrimp.

Considering the above overall introduction, the present study was planned to attain the following objectives:

i. To identify White Spot Syndrome Virus (WSSV) in shrimp from muscle or pleopod tissue by PCR.
ii. To know the prevalence rate of WSSV infection in the studied shrimp farms.
iii. To observe the pathological change in the tissue by histopathological process.



Chapter II
Review of Literature

CHAPTER II

REVIEW OF LITERATURE

A considerable literature is available on detection of WSSV of shrimp in Asian countries. A number of work have been carried out to study WSSV through different PCR techniques in different time with histopathological and histological studies. An attempt is made to review the available literature that's are relevant with this present study in this chapter are presented below.

2.1. Molecular Diagnosis of WSSV in Shrimp by PCR

Nunan *et al.* (1998) detected white spot syndrome virus in imported frozen shrimp by polymerase chain reaction. PCR confirmation of the presence of WSSV in the frozen, purchased products indicated that exotic shrimp pathogens can be transmitted via imported frozen products.

Otta *et al.* (1999) carried out a research to assess polymerase chain reaction (PCR) for the identification of white spot syndrome virus (WSSV) in postlarvae and broodstock of panaeid shrimp *Penaeus monodon* and in potential crustacean carriers from India. Primer pairs awere synthesize for WSSV affecting *Penaeus japonicus* (WSSV PJ) and *P. monodon* (WSSV PM) were used. Both the primer pairs resulted positive assay results for crustaceans screening gross signs of WSSV infection. However, all the healthy PL and brood stock gave negative results with both primer pairs by non-nested PCR. But some of the normal specimens were positive for WSSV by nested PCR using WSSV PM Primers. These results reported that WSSV carriers are communal in normal shrimp stocks and other crustaceans in India.

Hsu *et al.* (1999) recommended to avoid false negatives in WSSV PCR broodstock tests screening detection should be delayed up to spawning. In addition, with PCR detection system discarding all egg batches from brood stock which are l-step PCR positive after spawning. Moreover, it can be possible with adequate intensive care to use eggs from 2-step PCR positive brood stock for production of WSSV-free or lightly infected post larvae. It reveal that 2-step PCR implies lower viral load that can be cured by appropriate monitoring.

Durand *et al.* (2000) reported that white spot syndrome virus could be introduced in rozen commodity shrimp that has been confirmed by PCR. Imported frozen tails of

Penaeus monodon were tested for WSSV by polymerase chain reaction (PCR) and reverse transcriptase (RT) PCR analysis. Around 80% samples tested and detected WSSV. About 60% sample of frozen shrimp showed strong positive tests for WSSV by PCR, and these were selected for bioassay with specific pathogen-free *P. vannamei,* which were represented as the indicator shrimp for infectious virus WSSV. *P. vannamei* were exposed to WSSV positive sample by injection. Infectious WSSV resulted in 100% mortality in the indicator *P. vannamei* in four of nine bioassays.

Zhan *et al.* (2000) detected White Spot Syndrome Virus (WSSV) from hemolymph of infected shrimp. After extraction of DNA from shrimp, WSSV genome were cloned by EcoR I-digestion; three fragments were utilized as non-radioactive probes. The in situ hybridization of probes with sections detected the virus in the gill, stomach, epidermis, and connective tissue and so on, but not identified in healthy shrimp tissues.

Tang and Lightner (2000) quantified white spot syndrome virus DNA through a competitive polymerase chain reaction. To quantify the number of WSSV in diseased shrimp, a preliminary estimation of the target WSSV DNA concentration with a 10-fold dilution ($10^1 - 10^7$ copies) of the internal standard in a competitive PCR was conducted. It has been reported that the concentration of WSSV genome in hemolymph decreased from infected shrimp to be $10^5 - 10^6$ copies/µl hemolymph. Then two-fold serial dilutions of internal standard amplified together with constant aliquots 1 l. µl of hemolymph has been done. The PCR products were detected two bands, the larger amplicon 341 bp. from WSSV genome and the smaller amplicon 289 bp. from the internal standard. It has also been reported that WSSV amplicon concentration progressively high as the concentration of internal standard DNA be lower.

Kiatpathomchai *et al.* (2001) concluded through a nested PCR technique that it is simple and convenient and can detect as little as 5 fg WSSV DNA (20 viral particles) in extracts of post larval samples or extracts of pleopods and haemolymph from larger shrimp very easily. Furthermore, the technique uses internal control primers that produce a shrimp characteristic fragment for healthy samples and samples with a low quantity of viral particles in order to assure reliability and reproducibility of the PCR assays. The research conducted by using 1 sense primer and 3 antisense primers that yield up to 3 PCR products (1100, 526 and 250 base pairs based on the severity of infection. Unambiguously, heavy infections carries more than 2×10^4 viral particles of

WSSV produce all 3 fragments, while moderate infections around 2×10^3 viral particles produce 2 (526 and 250 bp) and light infections from 20 to 200 viral particles produce 1 (250 bp).

Tan *et al.* (2001) conducted a quantitative study of an experimental WSSV infection in grow out *P. monodon.* Where gills, abdominal integument and abdominal muscle were selected for viral load quantification. They observed that gill tissue has the highest viral load followed by integument and muscle.

Hossain *et al.* (2001) detected new hosts including marine crustaceans and in non-cultured crustaceans from shrimp farms for white spot syndrome virus of shrimp using nested polymerase chain reaction. The results indicate that wild-caught marine shrimp carry WSSV. This virus might be detected in apparently healthy marine crabs. The virus could also be detected in asymptomatic *Macrobrachium rosenbergii* cultured inland far away from coastline. Detection of carrier animals, two-step nested PCR is required.

Chakraborty *et al. (*2002) reported the prevalence of WSSV in wild crustaceans including shrimp, crabs, squilla and prawn collected from landing center, local fish market of India tested by first-step and second-step nested PCR. They revealed that wild crustaceans including shrimp, crabs, squilla and prawn can carry WSSV confirmed by two-step PCR. First-step and second-step nested PCR tools is used as a wonderful techniques to detect WSSV infection.

Liu *et al.* (2002) established a result while working with WSSV detection in shrimp by means of monoclonal antibodies (MAbs) that were screened by r-28 antigen-based enzyme-linked immunosorbent assay (Ac-ELISA), specific to an envelope protein (28 kDa) and was further re-checked by a polymerase chain reaction (PCR) and Western blot. Approximately 400 pg of purified WSSV sample from shrimp and 20 pg of r-28 could be detected by Ac-ELISA, which is akin in sensitivity to PCR assay but more sensitive than Western blot in the detection of purified WSSV virus.

Thakur *et al.* (2002) estimated the prevalence of white spot syndrome virus (WSSV) by polymerase chain reaction in *Penaeus monodon* post larvae at time of stocking, India. A maximum of the 73 batches of PL stocked at various farms in the west coast of India and then tested for the presence of WSSV by two-step nested PCR. About thirty-six (49%) of the 73 batches confirmed positive for WSSV either by one-step alone (3 batches) or after two-step nested PCR (33 batches). Sub-samples each confirmed to

quantify the proportion of infected PL within batches documented that WSSV prevalence was very high in positive batches by one-step PCR and low in two-step PCR-positive batches that implies two-step PCR resulted positive sample with lower viral load.

Vaseeharan *et al.* (2003) reported that a total of 280 postlarvae collected from nine different hatcheries and 350 juvenile shrimps collected from 18 different culture farms were tested for WSSV. The prevalence rate was around 53% from these cultured samples which were tested by single-step PCR.

Felix *et al.* (2003) studied on relative efficiency of three primer set for detecting white spot syndrome virus of shrimp (*P. monodon*). Three primer pairs viz. the Taiwanese (146F/146R), the Philippines (NLF/NLR) and the Indian (IWSVF/IWSVR) primer pairs were used for the detection of WSSV in samples obtained from three different geographic locations. They observed that all the three primers pairs gave a positive result for the isolation of WSSV from the different geographic region by using the optimized parameters.

Yoganandhan *et al.* (2003) conducted a study to detect white spot syndrome virus (WSSV) in shrimp samples by PCR and histopathological observations. Histopathological observations revealed the presence of intranuclear inclusion bodies in hill tissue, eyestalk, appendages and connective tissue at 36 post infection and stomach at 48 post infection. The author also observed eyestalk samples can be used for non-lethal screening of P. indicus to detect WSSV in positive samples as early as 12 h post infection by PCR or 36 h post infection by histopathology.

Khadijah *et al.* (2003) explored whether specific-pathogen-free (SPF) shrimps are asymptomatic haulers of white spot syndrome virus (WSSV) by a WSSV-specific DNA microarray to measure WSSV gene expression in SPF and WSSV-infected shrimps. Three WSSV genes were establish to be relatively highly stated in SPF shrimps. Reverse transcription-PCR using nested primers as well as real-time detection confirmed that these genes have no measureable counterparts in Gen Bank. PCR amplified from genomic DNA of SPF shrimp, supporting the additional suggestion that these shrimps are asymptomatic carriers of WSSV.

Otta *et al.* (2003) worked in India for detection of monodon baculovirus and white spot syndrome virus in apparently healthy *Penaeus monodon* post larvae from different

hatcheries by nested polymerase chain reaction (PCR). MBV and WSSV could be detected in 54% and 75% of the samples respectively by nested PCR. However, only 15% of samples were MBV positive and 19% being WSSV positive by non-nested reaction. The results indicates simultaneous presence of WSSV and MBV in most of samples at various degrees of infection. Only 14% of the samples analyzed were negative for both viruses.

Hossain *et al.* (2004) detected WSSV in cultured shrimps, captured brooders, shrimp postlarvae and water samples in Bangladesh by PCR using different primers. It has been expressed that PCR analysis for broodstock and post larval samples designate the variability in positivity depending on the type of primers used. Almost 100% positivity has been observed in broodstock samples when PCR using primers IK 3–4 was done whereas only 20% are positive when PCR using primers yielding bigger amplicons was accomplished. From this study it can be clearly concluded that sensitivity of PCR detection of WSSV depends on the size of amplicon created which in turn depends on the type of primers used. The primers should be chosen taking into consideration the purpose of diagnosis. If PCR is used as a confirmatory diagnosis of disease, primers of larger fragments can be used but if the purpose is to screen for WSSV and where a low irus load is expected, use of primers yielding smaller amplicons would give more precise results.

Galaviz-Silva *et al.* (2004) made a systematic survey for the first time of the gross signs, histopathology , transmission electron microscopy (TEM) and multiplex PCR in WSSV from 123 shrimp farm located in 20 different coastal provinces in Mexico.

Huang *et al.* (2005) detected four shrimp viruses eg. White spot syndrome virus (WSSV), infections hypodermal and hematopoietic necrosis virus (IHHNV), Yellow head virus (YHV) and Taura syndrome virus (TSV) using PCR and RT-PCR. They also added that these methods can be used to quarantine WSSV, IHHNV, YHV and TSV in live or frozen shrimp, larvae and feed of shrimp in the field of import and export.

Liu *et al.* (2005) reported for the first time the successful use of cycloheximide (CHX) as an inhibitor to block de novo viral protein synthesis at the time of WSSV infection while working on Microarray and RT-PCR screening for WSSV IE (immediate-early) genes in cycloheximide-treated shrimp. Almost sixty candidate IE (immediate-early) genes were isolated using an analysis by microarray technique. RT-PCR revealed that

the genes corresponding to ORF126, ORF242 and ORF418 in the Taiwan isolate were consistently CHX insensitive.

Jian *et al. (*2005) made a comparison among different methods including PCR for white spot syndrome virus (WSSV) detection in *Penaeus vannamei*. In these test, Penaeus *vannamei* were experimentally vaccinated with white spot syndrome virus (WSSV) and confirmed for WSSV at different times post-injection (p.i.) by one-step polymerase chain reaction (PCR), two-step PCR, *in situ* hybridization (ISH) and *in situ* polymerase chain reaction (ISPCR) to compare sensitivity test of the methods. It has been observed that two-step PCR showed the highest sensitivity, followed by ISPCR, one-step PCR and ISH. Although ISPCR revealed WSSV in most of *P. vannamei* that tested positive for WSSV using two-step PCR, any of the shrimp did not examined showed clinical signs of WSSV infection or detectable WSSV with one-step PCR.

Sahul Hameed *et al*. (2005) established a non-stop, single tube and semi-nested PCR procedure for simultaneous detection and grading the level of white spot syndrome infection in penaeid shrimp, *Penaeus monodon*. Three sense primers and one antisense primer were used depending upon the severity of infection. Quantities of WSSV-DNA at 10 pg and more gave three PCR products of 500, 300, 200 bp. A moderate concentration around 100 fg, gave two products of 300 and 200 bp and a low concentration of 1 fg or more gave only one PCR product of 200 bp. This PCR method was assessed for early detection of WSSV in shrimp.

Uma *et al.* (2005) screened the brood stock and post larvae during the month of March 2000 to May 2002 and collected sample randomly from shrimp hatcheries in the Southeast coast of India for WSSV and MBV by PCR. Nested PCR was performed for detection of WSSV whereas wet-mount method and one-step PCR were carried out for MVB prevalence of WSSV ranged from 25 to 50% in the brood stock and 10-13 % in PL by mested PCR.

Makesh *et al.* (2006) detected white spot syndrome virus (wssv) of penaeus monodon by a rapid method named latex agglutination test using monoclonal antibodies and further confirmed by PCR sensitivity test. Diagnostic sensitivity of the test was 70% and 45%, respectively, compared to single-step and nested PCR techniques. The diagnostic specificity of the test was around 82%.

Okumura *et al.* (2006) conducted a simple passive latex agglutination (RPLA) method for detecting white spot syndrome virus in the hemolymph of infected kuruma shrimp (*Penaeus japonicus*) was made. The observed that WSSV ccan be detected from shrimp hemolymph when latex particles blocked with a casein protein were used as a detection reagent.

Guevara & Meyer (2006) said that nested PCR is more useful to monitor commercial shrimp cultures than single-step PCR as it permits knowing how widely dispersed the virus is in asymptomatically populations. The presence of WSSV in individuals of both sex analyses indicated that there is no inclination for this virus to infect either male or female shrimp. Also, no differences in weight were initiate between WSSV infected and non-infected individual shrimps, as well as nested-PCR positive against single-step PCR positive tarisms. WSSV could widely distributed in the shrimp farms. Although no visual symptoms were observed, WSSV confirmed by nested PCR.

Natividad *et al.* (2006) studied simultaneous duplex PCR identification of two shrimp viruses (WSSV and MBV) in post larvae of *Penaeus monodon* in the Philippines. It has been reported that high specificity and sensitivity of the developed duplex PCR deals an efficient, significant and rapid tool for detection penaeid shrimp viruses since both WSSV and MBV can be identified in a single reaction. In addition, due to high sensitivity of the duplex PCR method, it may also be useful in ratifying early stages of WSSV and MBV infection, when the viral load is relatively low or before infection.

Islam *et al.* (2007) worked to develope a PCR based protocol for wssv screening in cultured shrimp in which wssv was determined by a two-step polymerase chain reactions (PCR)-based screening protocol by using two set of oligonucleotide primers. They concluded that a PCR based screening method of WSSV infection for brood and larval stocks of shrimp and other potential virus carriers could be an effective and significant tool in battle against the lethal virus that has almost destructed the shrimp industry of the country.

Ayub *et al.* (2008) reported that the nested PCR was found more sensitive and strong tool than the one-step PCR. These study were conducted to detect the WSSV by both one-step and nested polymerase chain reaction (PCR) involving primers of, 146F1/146R1 and 146F2/146R2, amplifying the1447 bp and 941 bp fragments,

respectively. From 60 randomly collected shrimps, 12 (20%) were found to be positive by one-step PCR, while 18 (30%) were found to be positive by nested PCR.

Patil *et al.* (2008) also made a comparison between monoclonal antibody-based immunodot test and polymerase chain reaction (PCR) assay for managing white spot syndrome virus (WSSV) on shrimp of India. The result was out of 12 grow-out farms F1 to F6 samples that were negative for WSSV by both immunodot test and 1-step PCR. Samples from another farms F7 to F12 were positive for WSSV by both immunodot test and one-step PCR at various times post stocking. In addition, 2 farms F13, F14 immunodot and 1-step PCR results were both negative but In contrast to one-step PCR results, farms F5, F6, F13, and F14 gave positive results for WSSV by two-step PCR, Furthermore, they concluded that an inexpensive immunodot assay and two-step PCR can be used to replace the more expensive one-step PCR assay for accurate result and disease monitoring.

Cavalli *et al.* (2008) evaluated white spot syndrome virus (WSSV) in wild caught shrimp after a major outbreak in shrimp farms in Brazil. During summer and winter samples were collected by one-step and nested PCR All of the samples were negative for PCR method used, representing that prevalence of this disease in wild animals must be very smaller than 5% per sampling period, or smaller than 3% when considering total number of tested shrimp (114 shrimp). So far, it has been concluded that WSSV is not widely outbreak among native shrimp, even after strong disease outbreak in native farms.

Leal *et al.* (2009) detected white spot syndrome virus in filtered shrimp-farm water fractions and experimental assessment of its infectivity in *Penaeus.* They studied on a hypothesis that WSSV may extent through water to neighbor ponds or farms. Daily water exchange and wastewater treatment during white spot disease emergency harvests may preserve WSSV in shrimp farming zones. An on-site experiments were conducted in a WSSV infected farm in three different areas i.e. Guasave, Sinaloa, Mexico. Plankton and shrimp hemolymph were collected for analysis and PCR analyses showed that 72% of the hemolymph pools (26 out of 36) were WSSV positive while only 14% (4 of 28) plankton samples from three ponds were WSSV-positive.

Paz, A. S. (2010) reported that white spot syndrome virus (WSSV), has spread globally as one of the most prevalent, widespread and lethal for shrimp and other crustaceans.

However, there is no treatment available to restrict with the uncontrolled occurrence and outbreaks of the disease but improved progress in molecular biology techniques including PCR has made it possible to get information on the factors, mechanisms and strategies used by this virus to infect and reproduce in susceptible host cells.

Dieu *et al.* (2010) reviewed that the polymerase chain reaction (PCR) has created powerful molecular-based detection methods for epidemiological study that's led to techniques such as Restriction Fragment Length Polymorphism (RFLP) monitored by Amplified Fragment Length Polymorphism (AFLP) as powerful marker practices to examine the historical origins and geographic distribution of eukaryotes as well as viruses. A general point PCR-based markers have the significant benefit of requiring minimal amounts of tissue or sample.

Sethi *et al.* (2011) studied white spot syndrome virus (wssv) in brood stock of *Penaeus monodon* and other crustaceans of Andaman waters and confirmed the prevalence of WSSV was 26.38% in *P.monodon* brood stocks. The PCR product of tiger shrimp, *Penaeus monodon* was negative in first step PCR but become positive in second step nested PCR with infection rate of 55%. They also concluded that among different PCR techniques particularly nested PCR have been found to be most sensitive technique.

Tuyen *et al.* (2014) studied on horizontal transmission dynamics of White spot syndrome virus by cohabitation trials using PCR in juvenile *Penaeus monodon* and *P. vannamei* and the study of the dynamics of disease transmission of WSSV were detected in small, closed populations of Penaeus monodon and *Penaeus vannamei* by pair cohabitation trial using PCR. For *P. monodon* the direct contact transmission rate of WSSV was significantly higher than the indirect environmental transmission rate, but for, *P. vannamei* the opposite was found. WSSV is transmitted very fast in shrimp populations, however, the modes and dynamics of transmission of this virus are not well understood, pair cohabitation trial using PCR may be a significant tool.

Hossain *et al.* (2014) claimed that prevalence study of White Spot Syndrome Virus (WSSV) in cultured shrimp (*Penaeus monodon*) of Bangladesh was very first and WSSV detected by Conventional and real-time PCR. It has been also reported that conventional PCR using VP28 and VP664 gene-specificnprimers 39 of 60 samples were found WSSV positive and correlated well with SYBR green real-time PCR using 71-bp amplicon for VP664 gene PCR data. However, the prevalence rates of WSSV

among the collected sample were Satkhira 79%, Khulna 50%, Bagerhat 38% and Cox's Bazar 25%.

Dutta *et al.* (2015) investigated that WSSV prevalence associated with disease resistance was studied among wild black tiger shrimp, *Penaeus.* The WSSV prevalence in wild *P. monodon* was the highest (56.2%) in Chennai, Tamil Nadu followed by Digha, West Bengal (10.9%), Visakhapatnam, Andhra Pradesh (0.6%) and Chilika, Orissa (0%). Quantitative data recommended that the copy number of WSSV among these four places was 1.4×10^6, 4.6×10^4, 1.6×10^2 and 2.3×10^2 copies μg^{-1} in shrimp tissue respectively. In addition, higher the WSSV prevalence in Chennai, Tamil Nadu and Digha, West Bengal, lower the disease resistant prevalence.

Bir *et al*. (2017) reported that there are several diagnostic procedures have been established for identification of WSSV. These include histopathological techniques, in situ, immunological methods such as Nitrocellose-enzyme immunobLolightnert and Western blot techniques and another more recently highly simple, sensitive and reliable technique such as Polymerase Chain Reaction based methods. Among the various diagnostic methods, PCR affords a high level of sensitivity and specificity in detection of WSSV. Nested or two step PCR has the benefit of aggregate the level of sensitivity over single step PCR. When a shrimp shows clinical signs and symptoms of WSSV, it is easily identified by single step PCR. Nowadays, nested PCR method is renowned to be the most effective pinpointing tool for the detection of WSSV in shrimp.

Saravanan *et al.* (2017) reported that out of 241 shrimp samples, WSSV was detected in 39 samples (16%), in which 11 samples detected with WSSV by first step PCR during investigation and confirmation of white spot syndrome virus (WSSV) infection in different wild caught penaeid shrimps of Andaman and Nicobar Islands, India. Among all the tested penaeid shrimp, WSSV was detected only in 39 samples out of 151 *P. monodon* shrimp samples and not detected in other penaeid shrimps including *P. indicus*, *P. merguiensis* and *Metapenaeus monoceros*. The PCR result was further confirmed by histopathological examination.

Hakami (2018) detected WSSV from farmed shrimps in Saudi Arabia. The collected shrimp samples were exposed to DNA extraction, followed by conventional and real time PCR. The achieved positive sample of PCR products were purified, followed by sequencing. These study found that the collected samples were shown a clear positive

band in conventional and real time PCR analysis. PCR charted by sequencing was useful to detect the presence of WSSV in a shrimp.

Haque *et al.* (2020) conducted a research on PCR based detection of white spot syndrome virus (WSSV) in shrimp post larvae (PL) of Bangladesh. In this investigation, shrimp samples were collected from Cox's and Satkhira of Bangladesh. Samples were tested by conventional PCR using two primer viz. VP664 and VP28 genes specific primers. Among the samples the overall prevalence rate was approximately 16.93% in the collected PL samples. Though the rate of infection in shrimp PL was 16.93%, it can play a deleterious effect in shrimp farms of Bangladesh. They also suggested that the segregation of PCR based WSSV infected shrimp PL before releasing to the culture ponds/ghers is of enormously important to avoid the WSD infection in shrimp farm/ghers.

Talukder *et al.* (2021) reported that WSSV prevalence was highest about 100% positivity with in all seasons in Khulna region and viral loads ranged from 5.62×10^9 to 2.01×10^9 copies/g of tissue. It has been detected 46 WSSV-infected shrimp by conventional PCR, where real-time PCR recognized WSSV-positive 47 out of 49. From theseresults, WSSV prevalence rate was highest during the monsoon season in the Khulna region is around 100% and lowest in Bagerhat is 75%. During this time 80% WSSV incidence was found in Satkhira.

Ador *et al.* (2021) gave an outline of the advantage of PCR diagnosis and it's used in the detection of diseases in aquatic animals. The authors observed the potential obstacles to PCR standardization are sample processing; PCR set-up; amplification strategies; diagnostic controls; diagnostic specificity and diagnostic sensitivity.

2.2. Histopathological Studies of WSSV in Shrimp

Momoyama *et al. (*1994) reported that moribund shrimp showed red coloration or discoloration and small white spots on the body, degenerated cells characterized by hypertrophied nuclei, being stained homogeneously with hematoxylin, were detected in various tissues originated from meso- and ectoderm, such as cuticular epidermis (most frequently), connective tissue, lymphoid organ, antennal gland, hematopoietic tissue and nervous tissue of affected shrimp. The author also observed necrotic cells were also frequently present in many affected shrimp. They further concluded that there is no difference in histopathological characteristics between spontaneously diseased

and experimentally infected shrimp and suggested that this is a new infectious disease in penaeid shrimp of which etiological agent might be a virus called WSSV.

Chou *et al.* (1995) conducted a study on pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp and revealed the hispathological changes in tissues from kuruma shrimp naturally infected with white spot syndrome. They observed degenerated cells characterized by hypertrophied nuclei in the cuticular epidermis and connective tissue, cellular necrosis and detachment of intestinal epithelium tissue, severe atrophy of the hepatopancreas in these diseased shrimp and cellular necrosis and detachment of Intestinal epithelium tissue in diseased shrimp.

Karunasagar *et al.* (1997) conducted a study on WSSV affected shrimp that showed white spots on the carapace and post abdominal segments and histopathologically, hypertrophied nuclei with eosinophilic to basophilic intranuclear inclusion bodies were observed in the epithelial cells of the stomach of affected shrimp. They also observed moderate to heavy septicaemia in moribund shrimp.

Wang *et al.* (1997) conducted a histological studies of white spot disease in the giant tiger shrimp, *Penaeus monodon* (Fabricius), and the kuruma shrimp, *Penaeus japonicus* (Bate), cultured in Taiwan and found degenerated cells, characterized by hypertrophied nuclei, especially in various meso- and ectodermal tissues included cuticular epidermis, connective tissue, lymphoid organ, antennal gland, and haematopoietic, gill and nervous tissue. They also found that infected nuclei were Feulgen-positive and no occlusion body was observed in the necrotic tissue.

Kasornchandra *et al.* (1998) conducted study to observe histopathological changes among diseased shrimps collected from various countries expressed widespread cellular degeneration and severe nuclear hypertrophy in cells of most tissues collected from ectodermal and mesodermal origin. Similar rod-shaped to elliptical virus particles of various sizes surrounded by typical trilaminar envelope were identify in the hypertrophied nuclei of affected cells.

Rajendra *et al.* (1999) revealed that the histopathological manifestation in the gill and foregut tissues of experimentally infected prawns, crabs and lobsters are similar to that of WSSV infection in shrimp. Including gill lamellae observed with acute degeneration, marked hypertrophy of nuclei with intracellular incluions, the cuticular ectodernal layer

of gut showed a large number of prominent deeply stained variablysized eosinophilic to besophilic inclusions.

Wang *et al.* (1999) reported that tissue sections of affected shrimps gills, lymphoid organs, hematopoietic tissue, foregut (including the stomach), cuticular epidermis, connective tissue, striated muscle, heart, haemocytes, antennal gland, nervous tissue and the ovary stained with H&E/phloxine and showed that necrotic cuticular epidermal cells collapsed from the epithelial, ovary showing infected oocytes, follicle cells and haemocytes, mildly affected oocytes show deteriorated nuclei with reduced yolk, while dense inclusions are formed in heavy infections. Hepatopancreatic epithelial cells showed no viral infection, but haemocytes in the hepatopancreas (HP) are heavily infected. HECs are highly vacuolated, resulting in diminution of the tubule lumens. B-cells (B) are rarely seen. Both circular and longitudinal muscles (M) of the midgut are infected but not epithelial cells. The authors further added that the stomach wall of *Penaeus monodon* infected with WSSV characterised by nuclear hypertrophy, nucleolus dissolution, chromatin margination and rarefaction of the central area.

Wang *et al.* (2000) identified a new bacterial white spot syndrome (BWSS) in cultured tiger shrimp *Penaeus monodon*. The affected shrimp expressed white spots similar to white spots which is caused by white spot syndrome virus (WSSV), but the shrimp became active and raised normally without significant mortalities. The author also found that there is no evidence of WSSV infection using electron microscopy, histopathology and nested polymerase chain reaction but BWSS caused damage to the deeper tissues which was limited and lesions are non-fatal.

Catap *et al.* (2004) studied histopathology of shrimp that showed the presence of large, usually single, basophilic intranuclear inclusion bodies in the distal tubules, which directed to displacement of the nucleoli. They also did light microscopy which showed ovoid to spherical inclusion bodies, 5 to 11 µm in diameter and transmission electron microscopy that showed the inclusion bodies were composed of electron-dense granular material and virions, hepatopancreas from experimentally infected postlarvae showing large inclusion bodies within epithelial cells of distal tubules.

Vijayan *et al.* (2003) conducted a histopathological study including histopathological changes in subcuticular epithelial cells of the eye stalk and pleopod were at different time post-challenge on tiger shrimp *Penaeus monodon* to detect WSSV using paraffin

and frozen sections. Histological manifestations such as cellular hypertrophy in the subcuticular epithelial cells of the eyestalk and pleopod might be observed as early as 18 h post infection (p.i.) before the manifestation of clinical signs of the disease. However, no histopathological changes could be observed before 18 h p.i. Hypertrophy of the nuclei in the epithelial cells was noticeable after 24 h p.i. including necrosis, and eosinophilic intranuclear inclusions, characteristic of early stages of WSSV infection were detected between 24–36 h p.i.

Yoganandhan *et al*. (2003) conducted a study to detect white spot syndrome virus (WSSV) in shrimp samples by PCR and histopathological observations. They found that histopathological observations revealed the presence of intranuclear inclusion bodies in hill tissue, eyestalk, appendages and connective tissue at 36 post infection and stomach at 48 post infection. The author also observed eyestalk samples can be used for non-lethal screening of P. indicus to detect WSSV in positive samples as early as 12 h post infection by PCR or 36 h post infection by histopathology.

Pantoja and Lightner (2003) studied on similarity between the histopathology of white spot syndrome virus and yellow head syndrome virus and the authors concluded that severe infection by WSSV may result in systemic necrosis, necrosis of the lymphoid organ, and of the fibrous connective tissue in particular, which is very similar to that caused by YHV.

Rajendran *et al.* (2005) reported that Histological manifestations such as cellular hypertrophy in the subcuticular epithelial cells of the eyestalk and pleopod could be detected before the manifestation of clinical signs of the disease. Hypertrophy of the nuclei, marked necrosis and eosinophilic intranuclear inclusions, were observed.

Flegel (2006) conducted a study to detect multiple viruses in non-diseased, cultivated shrimp and reported that histological signs of WSSV infection include enlarged nuclei in tissues of ectodermal and mesodermal origin.

Doan *et al.* (2009) conducted a study on the pathogenesis of the white Spot Syndrome Virus (WSSV) on juvenile *Penaeus monodon* in Vietnam and concluded that the replication of WSSV in *P. monodon* start in heart, antennal gland, foregut, stomach, gills, cuticular epithelium, hematopoietic tissue, connective tissue, and lymphoid organ.

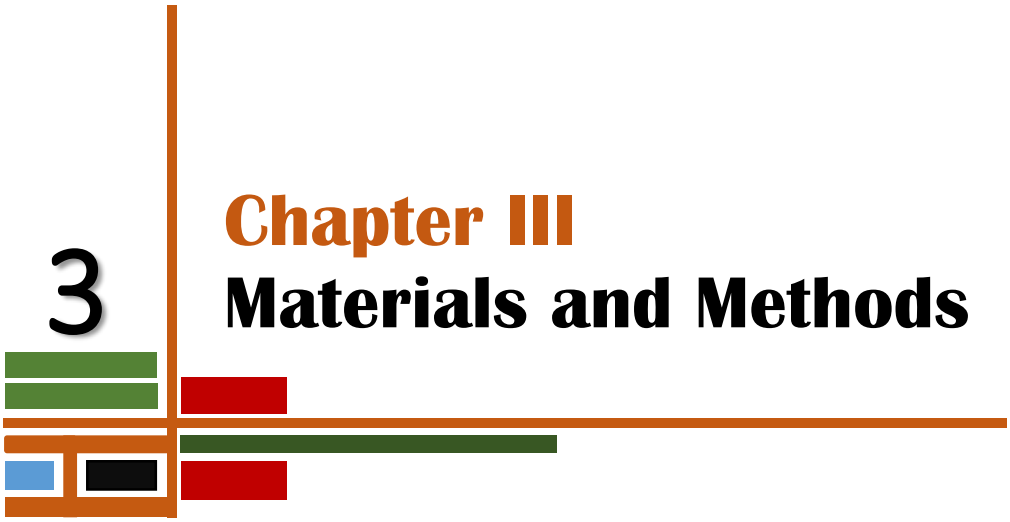
Afsharnasab *et al*. (2009) studied histopathology of white spot syndrome virus in shrimp specific pathogen free *Litopeneaus vannamei* in Iran. They observed that

opaque mussels and the carapace separate easily from cuticle, all tissue except hepatopancreas showed the intranuclear Cowdry type-A inclusion bodies and cuticular epithelium with large basophilc intranuclear inclusion bodies in all WSSV affected tissue.

Pazir *et al.* (2011) reported while working on white spot syndrome virus (WSSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) detection and identification from *Litopenaus vannamei* in Iran. They observed basophilic Cowdry type A inclusion bodies in all tissues such as gills, haematopoietic tissue, cuticle epithelium, lymphoid organ and connective tissue. However histologically, the hepatopancreas tissue indicated vacuolization of B cells, without inclusion bodies. It also has been observed that the histopathological change of the striated muscles abnormality showed severe necrosis.

Shahidy *et al*. (2015) studied to detect WSSV by histopathologically and PCR and revealed tha histopathological inspection of different organs showed intranuclear basophilic inclusion bodies i.e hepatopancrease of *Penaeus semisulcatus* showing hepatopancreatic degeneration and necrosis and basophilic hypertrophied nuclei, heart of *Penaeus Japonicus* showed intranuclear inclusion bodies and inter muscular edema, gills of *Penaeus Japonicus* showed basophilic intranuclear inclusion bodies.

Mannan *et al.* (2015) conducted a study to observed the WSSV infected tissue of hepatopancreas of *P. vannamei* and found that histopathological results showed signs of infected conditions including degeneration of Tubules Lumen(TL), slough hepatopancreas tubules cells (SHP), enlarged of the hepatopancreas nuclei cell and lack of B, F and R epithelial cells in the hepatopancreas tubules. They also commented that HP conditions may be a good indicator to determine the conditions of the shrimp health status which either infected or not.



Chapter III
3 Materials and Methods

CHAPTER III

MATERIALS AND METHODS

The whole study was conducted at the laboratory of Fisheries Biotechnology Division, National Institute of Biotechnology (NIB), Ganakbari, Savar, Dhaka-1349 and partially at the laboratory of Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka-1207 during the period from January 2021 to September 2021. The study was completed under two sub-experiments. The experiments are as follows:

Sub-experiment-I: Detection of the white spot syndrome virus (WSSV) in tiger shrimp (*Penaeus monodon)* through one-step and two-step PCR.

Sub-experiment-II: Histopathological study to observe the change in tissue due to WSSV infection.

3.1. Sub-experiment-I: Detection of the white spot syndrome virus (WSSV) in tiger shrimp (*Penaeus monodon)* through one-step and two-step PCR.

3.1.1 Sampling Area and Sample Collection

Study area covered two districts of Bangladesh which was Bagerhat and Satkhira. Samples were randomly collected from Bagerhat and Satkhira districts of Bangladesh during middle of May, 2021. From each district three different farms with different location were randomly selected for sampling. Almost 6-10 samples were collected from each site and finally 6 samples from each farm were used to perforrm the experiment as shown in Table 1. After collecting samples, they were preserved in the fridge of Shrimp Research Institute, Bangladesh Fisheries Research Institute, Bagerhat until transporting to the laboratories. Samples were randomly collected and some of which showed clinical sign and symptoms of WSSV including white reddish spot on carapace.

Table 1. Sample collection from different locations of Satkhira and Bagerhat districts.

Districts	Upazilas	Farm No.	No. of samples	Total
Satkhira	Satkhira Sadar	Farm No-1	6	18
	Kaliganj	Farm No-2	6	
	Shyamnagar	Farm No-3	6	
Bagerhat	Bagerhat Sadar	Farm No-1	6	18
	Fakirhat	Farm No-2	6	
	Shreehat	Farm No-3	6	

3.1.2 Major Instruments and Appliances Required

As this study was fully laboratory based, a well-developed or well-equipped laboratory was required. To conduct PCR study following instruments and appliances was needed: shaking water bath with a rotator (SBS40), incubator (37 degree celsius), shaking incubator, vortex mixer(SAB), balance (max 220g, div.0,1 mg) orbital shaker, grinder, Thermal cycler with sample block which fits 0.2ml thin-walled tube, high-speed benchtop microcentrifuge (14000 rpm, KUBOTA 6200), micropipette, blue and yellow tips (2 ml), micro tips, eppendorf (1.5 ml) tube, PCR tube (0.2ml, electrophoresis apparatus, UV transilluminator (EZEE Clearview), heating block (IKA Dry Block Heater 2), polaroid camera or digital photo system, PCR cabinet (ESCD/PCR-4A1), autoclave (vision vs-1221), nano drop machine, oven (SHARP), mini spin eppendorf (F 24.2), HistoCore biocut machine (Leica) and disposable gloves.

3.1.3 Isolation of Genomic DNA

Viral DNA was extracted with the genomic DNA of shrimps since shrimp tissues was used for DNA extraction.

3.1.3.1 Reagent Required for DNA Isolation

1. Lysis buffer

A lysis buffer is a buffer solution utilized for breaking open cells used in molecular biology experiments. Before DNA isolation a lysis buffer (SDS/NaCl Extraction

Buffer) must be prepared with certain chemicals with different strength as shown in Table 2. and preparation as per procedure in Appendix 1.

Table 2. Preparation of extraction buffer/lysis buffer

Chemicals	Strength	Volume /1 L	Final Concentration
Tris-HCl pH 8	1 M	200 ml	0.2 M
EDTA pH 8.0	0.5 M	50	0.025 M
SDS	10%	50	0.5%
NaCl	5M	50	0.25 M
ddH2O	-	650	-
	Total	= 1000 ml	

2. Proteinase K

Proteinase K (PK) Solution of Promega was used at a concentration of 20mg/ml in 10mM Tris-HCl (pH 7.5), 1mM calcium chloride and 50% glycerol and kept at room temperature.

3. RNase

10X NEBuffer™ 4 Exonuclease was used for DNA extraction to break the RNA and separate from genomic DNA.

4. Phenol: Chloroform: Isomaylalcohol (25:24:1)

Biotechnology graded [*Amresco*] Phenol: Chloroform: Isomaylalcohol (25:24:1) was used which was saturated with 10mM Tris HCl, pH 8 and 0.1M EDTA and stored at 4 degree celcious.

5. Chloroform: Isomaylalcohol (24:1)

Biotechnology graded [*Amresco*] Chloroform: Isomaylalcohol (24:1) was used which was saturated with 10mM Tris HCl, pH 8 and 0.1M EDTA and stored at 4 degree celcious.

3.1.3.2 Sample Preparation

Samples were taken with the help of autoclaved forceps from the bag and put on a blotting paper to blot extra water. Samples were taken from pleopod and muscle of adult shrimp. Two pieces (20-30 mg) of pleopods were cut into small pieces with autoclaved scissor and taken to 1.5 ml eppendorf tube containing 250 ml of lysis buffer. The samples were then grinded using pellet pestle grinder in the tube and added more 250 ml lysis buffer into that eppendorf tube.

3.1.3.3 Procedure of DNA Isolation

Total genomic DNA was isolated using by Phenol: Chloroform: Isoamylalcohol. For genomic DNA isolation, the following steps were sequentially done one by one carefully to prevent cross contaminations:

1. 20-30 mg of sample containing 450 µl lysis buffer and 20 µl of proteinase k were taken into a 1.5 ml eppendorf tube.
2. For proper mixing the sample in eppendorf tube was taken into vortex mixer.
3. The samples were then kept in water bath at 45℃ overnight until being transparent the sample.
4. After being transparent the sample were cooled at room temperature and vortexed
5. To remove the RNA, 10 µl RNase was added into the tube and taken in incubator at 37℃ for 30 minutes.
6. Equal amount (480 µl) of Phenol: Chloroform: Isoamylalcohol (P.C.I =25:24:1) was added into the eppendorf tube and the tube was shake avertedly for 5 minutes for proper mixing that made white solution.
7. Then the tubes were taken into centrifuge machine and centrifuged at 14000 rpm for 3 minutes.
8. 500 µl of supernatant from surface layer were transferred carefully into a new tube.
9. 500 µl of Chloroform: Isoamylalcohol (C.I =24:1) was added into that eppendorf tubes and the tubes were shaken avertedly that made two layers.
10. Then the tube were again centrifuged at 14000 rpm for 3 minutes.

11. 100-200 µl of sample from surface layer was carefully transferred into a new tube.
12. 100% frozen alcohol was added into the tubes and made the volume 1 ml.
13. The tubes were shaken and freezed at -25℃ for 1 h.
14. Then the tubes were centrifuged at 14000 rpm for 5 minutes.
15. The eppendorf tubes were then empty by removing all solution.
16. 1 ml of 70 % frozen ethanol was added into the each eppendorf tube.
17. Then the tube were centrifuged again at 14000 rpm for 5 minute and the tubes were made empty by removing all solution.
18. The eppendorf tubes were dried by air using PCR cabinet and after complete drying 50 µl TE buffer was added and preserved at -25℃ for later use.

3.1.4 Principles of PCR

The PCR technique a technique which is based on the enzymatic replication of DNA with specific thermal profile. In PCR reaction, a short region of DNA is amplified using primer. DNA polymerase enzyme is used to synthesize new strands of DNA complementary to the template DNA strand. The DNA polymerase enzyme can add a nucleotide to the pre-existing 3`-OH group only. Therefore, a primer is required and more nucleotides are added to the 3' prime end of the DNA polymerase.

3.1.4.1 Reagents Preparation for PCR

i) PCR Premix

PCR premix consists of GoTaq® Master Mixes (2X) includes Taq Polymerase, reaction buffer, dNTPs and$MgCl_2$, Primers (10µM) includes reverse and forward as shown in Table 3, Quick-Load purple 50 bp DNA ladder (120µg/ml), nuclease free water and DNA template.

ii) Gel Electrophoresis Reagents

Gel electrophoresis required Thermo Scientific™ supplied ethidium bromide solution with the strength of 10 mg/mL. Tris-Borate EDTA buffer (TBE buffer) with 10X concentration used for electrophoresis and diluted with water in a ration TBE to water is 1:9 that means 900 ml water is mixed with 100 ml TBE buffer to prepared 1L working solution. 1.5% agarose was used to prepare gel.

3.1.4.2 Primer Design

A total three sets of primer-pairs were used to conduct the PCR test. Among three primer pair, primer pair 146F1/146R1 and 146F2/146R2 were developed from the sequence of a cloned WSSV DNA fragment of 1461 bp by Lo *et al.* (1996) that amplifies 1447 bp and 941 bp fragments, respectively. These two primer pair's 146F1/146R1 and 146F2/146R2 were used in the present study for amplification of the WSSV DNA fragments in shrimp. The other primer pair, 143F/145R, produce a PCR product of 848 bp, developed from a highly conserved region of the 18S rRNA sequence of decapods (Kim and Abele, 1990; Lo *et al*., 1996). Primer pair 143F/145R was used as a positive control to check the quality of the DNA extracted from shrimp samples and the PCR conditions used. The sequences of the three sets of primers are shown in Table 3. These primer pairs were synthesized and supplied by 1st Base Company Ltd., Singapore.

Table 3. Primer sequences used for the amplification of white spot syndrome virus (WSSV) and tiger shrimp DNA

Primer name	Primer sequence (5′–3′)	Size (bp)	References
146F1	ACTACTAACTTCAGCCTATCTAG	1447	Lo *et al.* 1996 (GenBank: MN840357.1)
146R1	TAATGCGGGTGTAATGTTCTTACGA		
146F2	GTAACTGCCCCTTCCATCTCCA	941	
146R2	TACGGCAGCTGCTGCACCTTGT		
143F	TGCCTTATCAGCTNTCGATTGTAG (where N represents G, A, T or C)	848	Kim and Abele 1990; Lo *et al.* 1996
145R	TTCAGNTTTGCAACCATACTTCCC		

3.1.4.3 PCR Reaction Mixture

For PCR diagnosis, reagents of reaction mixture must be prepared just before use in thermo cycler. The extracted genomic DNA from shrimp specimen were used as a DNA template for PCR reaction mixture. Then required PCR mix volume were prepared.

A. One-step PCR Reaction Mixture

Total volume of 25 µl of reaction mixture were prepared with master mix which consist of contain GoTaq® DNA Polymerase, dNTPs, $MgCl_2$ and nuclease free water with both outer and inner primer solution and DNA template shown in Table 4.

Table 4. Reaction mixture for one-step PCR reaction

SL.	Components	Strength	Volume (µl)
01	Master Mix	2X	12.5
02	Forward Primer	100 pmol/µl	1
03	Reverse Primer	100 pmol/µl	1
04	DNA Template	50 ng/µl	2
05	Nuclease Free Water	-	8.5
			Total= 25 µl

B. Two-step PCR Reaction Mixture

As two step PCR was conducted through two different steps followed by Ayub *et al.,* 2008, total 25 µl of reaction mixture were prepared for both first and second step of two step PCR that contain master mix which consist of contain GoTaq® DNA polymerase, dNTPs, $MgCl_2$. For first step PCR, reaction mixture was prepared only with outer primer which is similar to one-step PCR (Table 4) and in case of second step, DNA template was used the amplicon from 1^{st} step PCR and the inner primer with master mix shown in Table 5.

Table 5. Reaction mixture for second-step of two-step PCR reaction

SL.	Components	Strength	Volume (µl)
01	Master Mix	2X	12.5
02	Forward Primer	100 pmol/µl	1
03	Reverse Primer	100 pmol/µl	1
04	Amplicon from 1^{st} step PCR	50 ng/µl	10
05	Nuclease Free Water	-	0.5
			Total= 25 µl

3.1.4.4 Polymerase Chain Reactions

The following amplification conditions applied to Applied Biosystems™ ProFlex™ PCR System, 2x 96-well with particular thermal profile.

A. One-step PCR for the Diagnosis of WSSV

After preparation of reaction mixture that contain master mix, reaction buffer at optimal concentrations, nuclease free water and primer solution both outer and inner primers with DNA template were taken into Quick spin Mini Centrifuge for 15-20 sec to proper mixing the mixture. Then the PCR tube applied to Applied Biosystems™ ProFlex™ PCR System. The reaction mixture was preheated at 95℃ for 3 min for initial denaturation, followed by 35 cycles comprising 1 min denaturation at 95℃, 1 min annealing at 55℃ and 1.5 min elongation or extension at 72℃. A final step consist of 5 min at 72℃ which allow the last cycle to complete extension of all of the amplified fragments. The whole thermal profile is shown in Table 6.

Table 6. Thermal profile for one-step PCR

Steps	Temperature (ºC)	Time (min)	Cycle
Pre-heat	95	3	1
Denaturing	95	1	35
Annealing	55	1	
Elongation	72	1.5	
Final extension	72	5	1

B. Two-step PCR for the diagnosis of WSSV

After finishing the one-step PCR, two-step PCR were performed. Because in two-step PCR, the amplicon of the first step PCR is used as the template DNA for the second step of PCR amplification. For this purpose, the first-step amplification was done by the outer primer pair 146F1/146R1 with particular thermal cycle parameters including initial denaturation at 95 ºC for 3 min, followed by 15 cycles of 95 ºC for 1 min, annealing at 55 ºC for 1 min and elongation at 72 ºC for 1.5 min and a final extension

at 72 ºC for 5 min. After completing one-step PCR, reaction mixture were prepaired again with the amplicon of one-step PCR product. The reaction mixture composed of master mix which consist of contain GoTaq® DNA Polymerase, dNTPs, $MgCl_2$ and reaction buffer at optimal concentrations, nuclease free water and primer solution both reverse and forward (146F2/146R2) and DNA template which was the amplicon from 1st step PCR and spin down for 15 sec. The reaction mixture were pre-heated with initial denaturation at 95℃ for 3 min for initial denaturation, followed by 35 cycles comprising 1 min denaturation at 95℃, 1 min annealing at 55℃ and 1.5 min elongation or extension at 72℃. A final step consist of 5 min at 72℃ which allow the last cycle to complete extension of all of the amplified fragments as per shown in Table 7.

Table 7. Thermal profile for two-step PCR

First step PCR with 146F1/146R1				**Second step PCR with 146F2/146R2**			
Steps	Temperature (ºC)	Time (min)	Cycle	Steps	Temperature (ºC)	Time (min)	Cycle
Pre-heat	95	3	1	Pre-heat	95	3	1
Denaturing	95	1	15	Denaturing	95	1	35
Annealing	55	1		Annealing	55	1	
Elongation	72	1.5		Elongation	72	1.5	
Final extension	72	5	1	Final extension	72	5	1

After completion of PCR, 5µl of 6X loading dye was added to eatch reaction tube and mixed well. Then the sample was ready for electrophoresis and observed under UV transilluminator.

3.1.5 TBE (10X) Buffer Preparation

TBE Buffer, 10X, Molecular Biology Grade – Calbiochem by Sigma-Aldrich was used as buffer for electrophoresis and it was diluted with 1X solution containing 89

mM Tris, 89 mM boric acid and 2 mM EDTA, pH 8.3. Then 10 ml TBE buffer diluted with 90 ml distilled water as shown in Appendix III.

3.1.6 Agarose Gel Preparation

Agarose gel eletrophoresis requires some equipment, chemicals and supplies, these equipment are an electrophoresis chamber and power supply, gel casting tray, composed of UV transparent plastic, sample combs in which molten agarose is poured into it and to form working gel and electrophoresis buffer TBE 10X (Tris HCL, Glacial Acetic Acid, EDTA), ethidium bromide etc.

The procedure for 1.5% agarose gel preparation are following steps:

For 40 ml agarose gel 0.6 g agar powder were measured gel electrophoresis.

⇩

A conical flask was washed before use and measured 40 ml TAE buffer and poured into the flask.

⇩

Then the agar powder was added carefully into the conical flask and was shaken to proper mix.

⇩

The flask were then taken into micro-oven for 1.20 sec to dissolve the agarose powder into TBE buffer.

⇩

4 µl of Ethidium bromide for 40 ml was added into the flask and mixed properly and poured into the tray.

⇩

Kept the tray at least 30 min to form a gel and then the comb were removed carefully and then the sample were loaded.

Figure 1. Flow chart of 1.5% agarose gel preparation.

3.1.7 Electrophoresis Procedure

The electrophoresis procedure followed the steps are as follows:

- At first gel box was filled with 1X TBE until the gel is covered.
- A molecular weight ladder of 5 µl was carefully loaded into the first lane of the gel.
- The samples were then stained with blue dye and carefully load into the additional wells of the gel.
- Run the gel at 80-100 V until the dye line is approximately 75-80% of the way down the gel. A typical run time is about 1-1.30 hours, depending on the gel concentration and voltage.
- After approximately 75-80% of the way down the gel. Turned off the power supply by disconnecting the electrodes from the power source, and then the gel carefully removed from the gel box.
- DNA fragments were then visualized using UV transilluminator. The fragments of DNA are usually referred to as ‘bands’ due to their appearance on the gel.

3.1.8 Interpretation of PCR Result

PCR diagnosis was observed after electrophoresis from the agarose gel through comparing different lane with the molecular marker and made an interpretation with the other study that has been already conducted.

3.2 Sub-experiment-II: Histopathological study to observe the change in tissue due to WSSV Infection.

Penaeid tiger shrimp samples (*Penaeus monodon)* were used to observe the pathological change due to white spot syndrome virus in the tissue including hepatopancreas and muscle by histopathological process. The whole study was conducted at the laboratory of Fisheries Biotechnology Division, National Institute of Biotechnology (NIB), Ganakbari, Savar, Dhaka-1349 and partially at the laboratory of Department of Biotechnology, Sher-e-Bangla Agricultural University, Dhaka-1207.

3.2.1 Collection of Shrimp Samples and on-site Processing

Fresh and live samples were randomly collected from Bagerhat and Satkhira districts of Bangladesh. Total four samples were collected from of which two samples showed signs and symptoms of WSSV confirmed by visual inspection and farmers and another two samples collected from Shrimp Research Institute, Bangladesh Fish Research Institute, Bagerhat in where shrimps are reared in controlled conditions marked as healthy shrimp. After collecting sample, the samples were taken for fixation with Davidson Fixative Solution in the laboratory of Shrimp Research Institute, Bangladesh Fish Research Institute, Bagerhat.

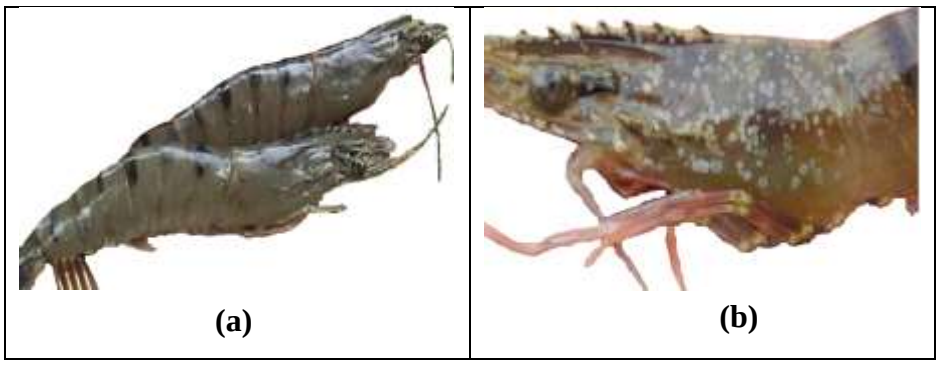


Figure 2. Samples of WSSV non-infected (control) shrimp and (a) and infected shrimp with white spot on carapace (b).

Davidson Fixative solution prepared in that laboratory with formalin, glacial acetic acid and distilled water shown in Table 8 and then injected the solution into the both side of head under cephalothorax and kept 24 hours immersed on that solution as shown in Figure 3. After 24 hours the shrimp were transferred to 70% ethanol until next step of histopathology study in the laboratory.

Table 8. Preparation of Davidson's fixative solution

Necessary Items	Strength	Make 500 ml
Ethanol	95%	165 ml
Formalin	37%	110 ml
Glacial Acetic Acid	100%	57.5 ml
ddH_2O	-	167.5 ml
	Total	=500 ml

Figure 3. Injection and preservation of sample in Davidson fixative solution

3.3.2 PCR Confirmation for WSSV Infection

WSSV infection at different time points was confirmed by PCR technique. Template DNA was extracted following the procedures of Ayub *et al.* (2008) and Vijayan *et al.* (1998) from the pleopods removed from infected and control shrimp. The PCR was carried out according to Takahashi *et al. (*1996) and Ayub *et al.* (2008) to detect WSSV infection. PCR products were electrophoresed in 1.5% agarose gel stained with ethidium bromide, and visualized under ultraviolet transillumination.

For one-step PCR diagnosis, required amount of reaction mixture were prepared according to sample number. Newly extracted DNA added with reaction mixture in 0.2 ml PCR tube and then taken into thermo cycler with specific thermal profile and observed through agarose gel electrophoresis under UV. For two-step PCR diagnosis, genomic DNA of WSSV negative sample confirmed through one-step PCR and a

WSSV positive PCR product as a control treatment were used and mixed with reaction mixture. Then the PCR tube were taken into thermo cycler with 15 cycle reaction named as first-step PCR. After completing first-step PCR, 10 µl apmlicon from first-step PCR taken as a template and mixed with master mix and other reagents and all the PCR tubes were taken to thermo cycler again with 35 cycle with particular thermal profile which took around 3 hours. After completion of nested PCR 5 µl of 6X loading dye was added to each reaction tube and mixed well. Then sample was ready for electrophoresis and observed under UV transilluminator. PCR confirmed WSSV infected and non-infected (control) shrimp tissues were used for histopathological study.

3.2.3 Histopathological Study

3.2.3.1 Fixation and Preservation of Samples

Histopathological steps were completed by following the handbook of normal penaeid shrimp histology by Bell & Lightner (1988). Shrimp samples from Davidson Fixative solution were taken on the vial containing alcohol 70% and then the samples were transferred into laboratory. The samples were removed from the vial containing alcohol 70% and placed it on a petri dish to dissect and remove the organ to get the target tissues including hepatopancraes and muscle. After cutting organs with a scalpel blade, hepatopancraes and muscle were placed inside the cassette and closed it. Then the cassette was kept inside a beaker containing alcohol 70% for further fixation for 16 hours or overnight. The amount of fixative should be 10 times to bulk of tissue fixed (Humason, 1979; Culling, 1974).

3.2.3.2 Steps of Histopathological Procedure

The cassette were then removed from 70% alcohol and labeled with dark pencil (2B). After that the cassette were taken into a jar and Transfer the cassettes to the cassette holder of the automatic tissue processor (DAIHAN Thermo $Stable^{TM}$ IS-20 Precise Shaking Incubators, BenchTop-type). It was treated with 100% formalin, absolute alcohol and acetone. These treatment together known as dehydration. Dehydration of the organs allows the exchange of the water of the tissue for alcohol. The next step known as clearing with xylene. Clearing helps to replace the dehydration solution with a substance that will be miscible with the inclusion medium called paraffin. For embedding paraffin (protoplast) were used. Alcoholic series of higher concentrations, acetone, xylene and paraffin was (4 series) were used in the processor maintaining at

various time schedules as shown on Table 9. Before use the protoplast is needed to melt at 60℃ in driyer where the melting temperature of protoplast is range from 40-68℃. The tissues are then embedded with melted, steel mold and perforated plastic holder. Proper care should be taken during embedding the hepatopancreas and muscle especially replacement and orientation in the steel mold. The hepatopancreas and muscle should be placed on the bottom of the mold that facilitate the trimming process. At first, the stainless base molds were formulated to receive the organs and placed the stainless base mold below the dispenser and filled it with paraffin. The organs were then eradicate from the cassette and arranged them. Finally let the stainless base mold cool to room temperature for 24 hours and after the paraffin solidifies, unmold the blocks.

Table 9. Time schedule in automatic tissue processor

Process	**Solution**	**Jars**	**Time**
Dehydration	100% formalin and 2g NaCL	1	2 h
	100% Alcohol	2	30 min
	100% Alcohol	3	2 h
	Acetone	4	15 min
		5	45 min
Clearing	Xylene	6	30 min
	Xylene	7	1 h
Infiltration	Molten wax	8	15 min
	Molten wax	9	1h 30 min

The blocks are then kept in the refrigerator (deep fridge) at least half an hour before trimming. Trimming was done from side and surface with the help of scalpel to make

block. The blocks were stored in the refrigerator (deep fridge) at least 15 minutes before cutting on Microtome Machine (HistoCore BIOCUT - Manual Rotary Microtome). With the help of Microtome Machine, the block are placed on the microtome block holder and begin cutting the block to the thickness of 5 micrometers until the paraffin ribbon containing the organs is formed. Take the ribbon into a warm water bath at 45°C to stretched, avoiding overlapping and rupturing points in the organ. Once stretched, the ribbon should be scooped up onto a previously cleaned slide.

Table 10. Staining procedure for hematoxylin and eosin (H & E) stains

Process	**Solution**	**Jar**	**Time**
Clearing	Xylene	1	2 min
		2	2 min
		3	2 min
Rehydration	Alcohol	4	2 min
		5	2 min
		6	2 min
	Distilled water	7	1 min
Staining	Haematoxylin	8	1-2 min
Rehydration	Tape Water	9	1 min
Washing	1% Acid Alcohol	10	Dip
Rehydration	Tape Water	11	1 min
	Scotch Tape Water	12	15-20 sec
Rehydration	Tape Water	13	1 min
Counter staining	1% eosin	14	5-10 sec
Dehydration	Absolute Alcohol	15	Dip

Sample Fixation

Tissue Fixation

Tissue Processing

Tissue Embedding

Tissue Sectioning

Slide Staining

DPX Mounting

Observation

Final Image

Figure 4. Steps of histopathological procedure

After making the slide (in duplicate), let it rest in a drying oven at 30°C. Then the sections are prepared for staining with Hematoxylin and eosin (H & E) stains proceeding through different chemicals shown in Table 10.

In the Hematoxylin-Eosin (H-E) staining process, the slide containing the paraffin ribbon and organs should be transferred. In some cases, the times can vary depending on the tissue; so, it is recommended that run the tests with some duplicate slides before processing all other slides.

After Staining the slides were dried at room temperature. Then the sections were mounted with DPX mountant which is a mixture of distyrene, a plasticizer, and xylene used as a synthetic resin mounting media that replaces xylene-balsam and covered with cover slips. DPX mountant was used to dry quickly and preserves stain and it is suitable for HE stain. The prepared sections were kept over a flat surface for overnight in order to fix the cover slips with the sections permanently. The glass slides were observed under a compound microscope (Nikon ECLIPSE Ts2) to get brilliantly clear images, enabling more efficient cell observation and documentation. Photographs of the stained slides were observed under microscope and saved the photograph for further use.

Chapter IV

4

Results and Discussion

CHAPTER IV

RESULTS AND DISCUSSION

The present study was conducted for molecular detection of white spot syndrome virus infection in *Penaeus monodon* by one-step and two-step PCR and measured the prevalence rate of infection in target areas. PCR detected infection was further analyzed by observing the pathological changes in infected hepatopancreas and muscle tissues through histopathological study. Results obtained from the present study were presented below under the following headings.

4.1 Confirmation of WSSV infection in the studied shrimp by one-step and two-step PCR assay

A total of 36 samples were collected to detect the prevalence of WSSV in shrimp (*P. monodon*) from three different areas of each district of Satkhira and Bagerhat of Bangladesh. As mentioned above three sets of primers were used to detect WSSV of which one set of primers is used to check the DNA quality.

From the 18 samples of Satkhira district, fifteen (15) samples were found to be WSSV infected and the rest three (3) samples were negative by one-step PCR. It has been shown in the PCR product of Figure 5. From the 18 samples of the Bagerhat district, ten (10) samples were found to be WSSV infected and the rest eight (8) samples were negative by one-step PCR. It has been shown in the PCR product of Figure 6. Among 36 samples, Thirty-three (33) samples were found WSSV positive and only 3 samples were found to be WSSV negative confirmed by two-step PCR. In nother 18 samples of the Bagerhat district, ten (10) samples were found to be WSSV infected and the other eight (8) samples were negative by one-step PCR.

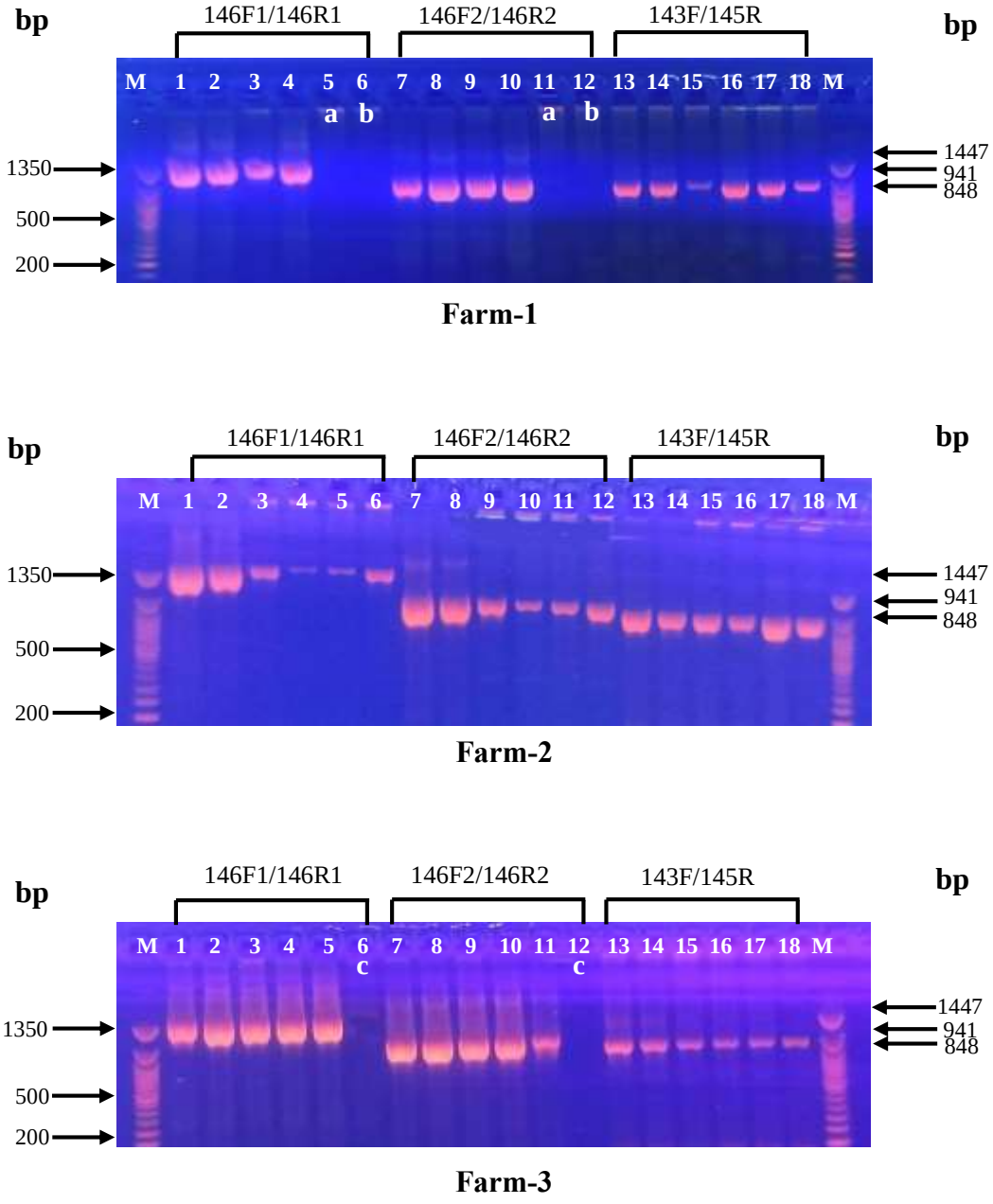


Figure 5. Detection of White Spot Syndrome Virus in *Penaeus monodon* collected from three farms of Satkhira district (6 samples from each farm) by one-step PCR with primer pairs 146F1/146R1 (lanes: 1, 2, 3, 4, 5, 6) and 146F2/146R2 (lanes: 7, 8, 9, 10, 11, 12). The 143F/145R primer pair was used as positive control to check the quality of the DNA (lanes: 13, 14, 15, 16, 17, 18). The letters a, b and c was used for indicating WSSV negative samples. Lanes M represent 50 bp DNA ladder.

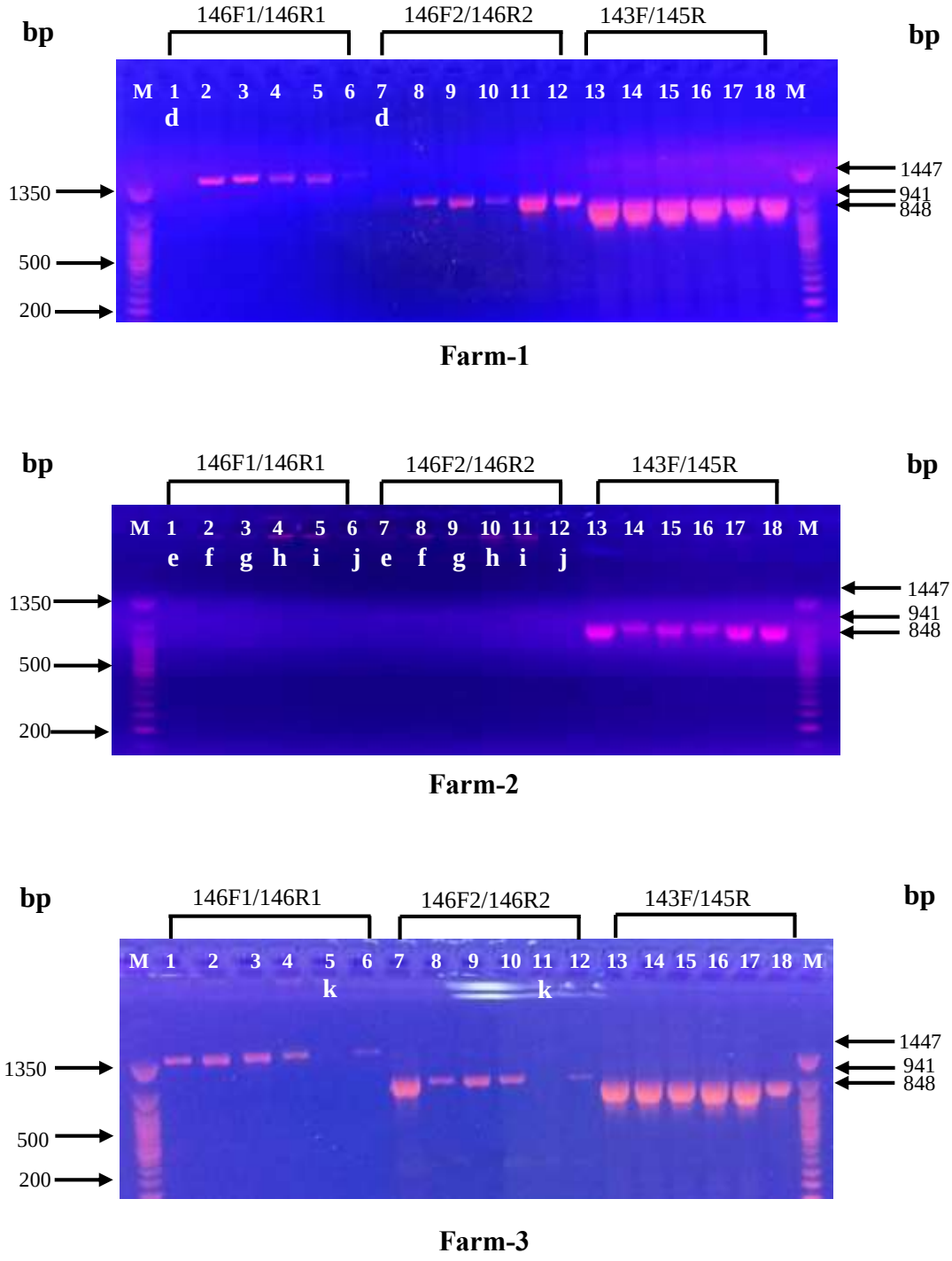


Figure 6. Detection of White Spot Syndrome Virus in *Penaeus monodon* collected from three farms of Bagerhat district (6 samples from each farm) by one-step PCR with primer pairs 146F1/146R1 (lanes: 1, 2, 3, 4, 5, 6) and 146F2/146R2 (lanes: 7, 8, 9, 10, 11, 12). The 143F/145R primer pair was used as positive control to check the quality of the DNA (lanes: 13, 14, 15, 16, 17, 18). The letters, d-k were used for indicating WSSV negative samples. Lanes M represent 50 bp DNA ladder.

A total of eleven (11) negative samples were confirmed by one-step PCR is further prepared for two-step PCR. The result of two-step PCR was that three (3) samples of Satkhira dostrict was remained WSSV negative and the other 8 samples of Bagerhat district was WSSV infected shown in Figure 7.

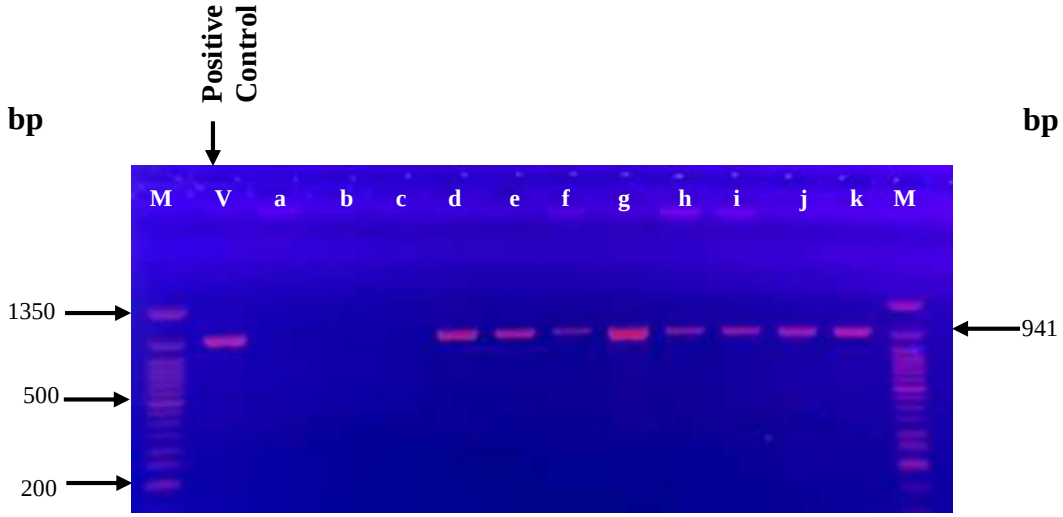


Figure 7. Detection of White spot syndrome virus (WSSV) in *Penaeus monodon* by two-step PCR with inner primer pair 146F2/146R2 using WSSV negative samples obtained by one-step PCR (lanes: a, b, c, d, e, f, g, h, i, j, k). Lane V denotes WSSV positive control. Lanes M represent 50 bp DNA ladder.

Based on the one-step PCR result, the prevalence rate of WSSV infection in collected shrimp was 83.33% and 55.56% in Satkhira and Bagerhat districts, respectively. So the overall prevalence rate of WSSV-infected shrimp by one-step PCR was 69.44% in these two study areas of Bangladesh. On the other hand, in the case of two-step PCR of 11 samples, the prevalence rate of WSSV infection in collected shrimp was 83.33% and 100% in Satkhira and Bagerhat districts, respectively and the overall prevalence rate of WSSV-infected shrimp in both one-step and two-step PCR was 91.67%.

Table 11. The overall PCR result of all collected sample after one-step and two-step PCR

Districts	**Farm No.**	**No. of samples**	**One-step PCR Redults**		**Two-step Nested PCR Results**		
			WSSV +(ve)	WSSV -(ve)	Sample for Nested PCR	WSSV +(ve)	WSSV -(ve)
Satkhira	Farm-1	6	4	2	3	-	2
	Farm-2	6	6	-		-	-
	Farm-3	6	5	1		-	1
Bagerhat	Farm-1	6	5	1	8	1	-
	Farm-2	6	-	6		6	-
	Farm-3	6	5	1		1	-

Table 12. Prevalence rate of all collected samples after one-step and two-step PCR

Districts	**No. of samples**	**Prevalence rate after one-step PCR**	**Overall Prevalence rate**	**Samples for Two Step PCR**	**Prevalence rate after two-step PCR**	**Overall Prevalence rate**
Satkhira	18	83.33%	69.44%	11	83.33%	91.67%
Bagerhat	18	55.56%			100%	

White spot syndrome virus (WSSV) is now a most pathogenic and devastating threat to the commercial shrimp farming industry all over the world. WSSV is highly virulent in shrimp farms and causes 100% mortality within a few days. So detection and diagnosis of WSSV are necessary to take some remedial measures to prevent the virus in valuable commercial shrimp industry all over the world (Hakami, 2018). According to the OIE Manual (OIE 2003), PCR is the best method to diagnose and detect WSSV infection in shrimp. Due to higher sensitivity limits than classical diagnostic methods, PCR has

become the preferred as the best method for the diagnosis of most shrimp viruses (Tang and Lightner, 2000).

This study was conducted to detect WSSV in juveniles and sub-adults or adult samples of shrimp. According to the OIE Manual (OIE, 2003), juveniles and sub-adult shrimp samples provide the best samples for WSSV detection without any false negatives. But in the case of post-larvae, an entire and higher number of samples must be analyzed (Cavalli *et al.*, 2008). Thakur *et al*. (2002) recommended the analysis of at least 300 post-larvae shrimp to significantly reduce the probability of false negatives. A basic survey carried out by the Central Institute of Brackishwater Aquaculture, CIBA, Chennai in 2004 and found the prevalence of WSSV in brood stock of Tiger Shrimp, *P.monodon* was 25% whereas other crustaceans showed a low prevalence of WSSV at 13% was found in Andaman water. During sampling, brood stock tiger Shrimp, *P.monodon* should be taken to get accurate prevalence. For competitive PCR, I used pleopods and muscle tissue to get high quantities of the WSSV genome. Tang and Lightner (2000) reported that the quantities of WSSV genomes in hemolymph were generally much lower than that in tissues of pleopods and muscle due to the higher cell mass in the tissues relative to the hemolymph. In the present study primer pair 143F/145R is a highly conserved region of 18SrRNA sequence of decapods and was used as a positive control to check the quality of the DNA extracted from shrimp samples and the PCR conditions used because DNA extracted from either hemolymph or tissues decreased during the later stages of infection. These 18S rRNA were designed by Lo *et al*. (1996b). Tang and Lightner (2000) stated that for assessing the preparation of WSSV DNA, the use of shrimp 18s rRNA DNA for a quality and quantity control may be restricted to the early stages of infection. Therefore, 18S rRNA were used in the later stages of WSSV infection when DNA is degraded due to the disintegration of infected cells.

This study was conducted by using the primer sets, 146F1/146R1 and 146F2/ 146R2 developed by Lo *et al.* (1996b) in Taiwan for the detection of WSSV isolates from Bangladesh. Natividad *et al.* (2006) reported that these primer pairs were only able to amplify the isolates from Japan but not the isolates from the Philippines while working on Philippine and Japanese WSSV isolates. So, the present study will reveal whether the effectiveness of the primer sets can also detect WSSV samples from Bangladesh. The present study revealed that both primer pairs were effective to amplify expected

size fragments from Bangladesh isolates of WSSV successfully. Ayub *et al.* (2008) also found that both primer pairs were able to amplify expected size fragments from Bangladesh isolates of WSSV successfully.

In the present study, the prevalence rate of WSSV infection in collected shrimp was 83.33% and 55.56% in Satkhira and Bagerhat districts, respectively. The highest occurrence was found in the Satkhira district confirmed by one-step PCR. From July 2013 to April 2014, Hossain *et al.* (2014) found a similar result of the high prevalence of WSSV in Satkhira (79%) and Cox's Bazar (25%) of Bangladesh. A recent report provided by Siddique *et al.* (2018) stated that the prevalence rate of WSSV was nearly 78% in Satkhira from 2014 to 2017. A very recent study conducted by Talukder *et al.* (2021) reported similar results that the highest prevalence (79%) of WSSV was found in the Satkhira region, and the lowest prevalence was detected within samples of Cox's Bazar region whereas the WSSV prevalence rate for the samples collected from Khulna and Bagerhat regions was 50% and 38% respectively. The higher prevalence found in these studies indicated an outbreak of WSSV in Bagerhat which may be commenced by the stocking of WSSV-infected PL. The infected shrimp PL transmits the WSSV horizontally to the other non-infected shrimp PL through water, sediment or other carrier organisms in the shrimp culture ponds/ghers which may the possible reason of the higher WSSV prevalence in cultured shrimp of Bagerhat. Durand and Lightner (2002) had done a significant study that confirmed a minimum dose of 10^5 copies of WSSV is needed for WSD transmission which is similar to the results which were observed by Meng *et al.*, 2010. Talukder *et al.* (2021) and Debnath *et al.* (2012) found similar results where they observed the highest prevalence in the pre- monsoon season, a decreasing trend of WSSV during the monsoon season and again an increase in the month of August. Mallik *et al.* (2016) reported that infected shrimp in Kolkata, India, faced the lowest WSSV prevalence in the time of pre-monsoon season, while the highest WSSV infection was in the post-monsoon season. The WSSV concentration in shrimp samples may varied with seasonal changes and these viral loads directly declined from the pre-monsoon to post-monsoon seasons in Bangladesh. I collected shrimp samples in the mid of May which was early pre-monsoon in Bangladesh and the result was similar to Talukder *et al.* (2021) and Debnath *et al.* (2012).

On the other hand, in the case of two-step nested PCR of 11 samples, the prevalence rate of WSSV infection in collected shrimp was 83.33% and 100% in Satkhira and

Bagerhat districts, respectively. The prevalence rate of WSSV-infected shrimp by one-step PCR was 69.44% and after two-step PCR the prevalence rate of WSSV-infected shrimp was 91.67%. Otta *et al.* (1999) conducted a study in India along the west coast only 5% were positive by one-step PCR, whereas 48% were positive by two-step PCR with a sample size of 20 to 30 PL per hatchery. Another study carried out by Thakur *et al.* (2002) and found 49% of the PL were positive for WSSV by two-step PCR, of which only 4% were positive by one-step PCR. These results indicated that those samples that were positive by one-step PCR were highly infected with a higher viral load by WSSV and those that were positive only by nested PCR indicated a lower level of infection with a lower viral load by WSSV. However, shrimps with minor spots or apparently healthy animals were positive by either one-step PCR with primers yielding smaller amplicons or by two-step PCR. Hence, it is clear that the sensitivity of detection and number of positivity increases as the amplicons size of the primers used decreases (Hossain *et al.*, 2004). Furthermore, Hossain *et al.* (2001) found that when the viral load in shrimp is low, the chances of getting positive results by nested PCR are better with primers yielding smaller amplicons. Two-step PCR has the advantage of increasing level of sensitivity over single-step PCR. Only a shrimp shows clinical signs of WSSV, it can easily be detected by single-step PCR stated by Bir *et al.* (2017). It has been also mentioned by Lo *et al.* (1996b) that two-step nested PCR was 10^3 to 10^4 times more sensitive than the one-step PCR alone. This supports the results of nested PCR sensitivity for WSSV detection as reported by Saravanan, (2017) & Otta *et al.* (2002). This statement recommended that samples will be one-step PCR-positive if they have at least 10^3 to 10^4 times more viral DNA than samples detected positive only by two-step PCR. A similar result was also found by Saravanan (2017) & Otta *et al.* (2003).

Although one-step PCR positive brooders spawned successfully (Lo *et al.*, 1997), it may possible to rear such eggs to post larvae that are two-step PCR negative or at most two-step PCR positive for WSSV. Lo *et al.* (1998) reported that ponds stocked with postlarvae that are two-step PCR positive can be successfully harvested under low-stress conditions and monitored to reduce the risk of WSSV outbreak (Hsu *et al.*, 1999). So, it has been recommended that the broods with light infection of WSSV, which could not be detected by one-step PCR, should not be used as a good spawner in the commercial shrimp farming industry because when it lay eggs that PL will be highly infected by WSSV and the broods could not lay the higher amount of eggs.

Among different PCR techniques, nested PCR is now widely applied for the detection of viruses including hepatitis C virus (Newman *et al*. 1996), bovine herpesvirus (Ashbaugh *et al.,* 1997) and fish rhabdoviruses (Miller *et al.,* 1998). This two-step PCR are also used widely for the detection of different viruses in penaeid shrimp including WSSV (Lo *et al.,* 1996b), monodon baculovirus (Belcher and Young, 1998), gill-associated virus and lymphoid organ virus (Cowley *et al.,* 2000). However, two-step PCR methods use conventional steps where a portion of the PCR amplicon from the first step is transferred to a second PCR reaction tube for the two-step amplification. But a high contamination risk is associated with the transfer step. To overcome these problems Kiatpathomchai *et al.* (2001) suggested a semi-quantitative, non-stop, semi-nested PCR detection method in penaeid shrimp. Bastien *et al.* (2008) designed a rapid, dynamic, precise and contamination-proof quantitative real-time PCR (qPCR) technology with excellent sensitivity, efficacy and precision, extra sensitivity over conventional PCR for confirmation of WSSV DNA in shrimp. A similar statement established by Espy *et al.* (2006) stated that real-time PCR technology, which facilitates the detection of viral DNA contains only 1 to 2000 copies of viral DNA in every reaction. Furthermore, the method requires a low concentration of template DNA and therefore can be used even with a low virus load in the sample. Hossain *et al.,* 2014 reported that the percentile value of the specificity of this assay was 100% and sensitive enough to detect WSSV-specific gene from 0.035 ng of total sample DNA which was similar to the findings of Durand and Lightner (2002); Khadijah *et al.* (2003) & Dutta *et al.* (2015). But Guevara & Meyer (2006) reported that Real-time PCR is a relatively expensive and sophisticated technique, nested PCR represents the best alternative to determine the status of the virus in the shrimp population, whereas single-step PCR is not sensitive enough.

4.2 Histopathological Assay of WSSV Infected Shrimp

A number of four samples were tested of which two samples were WSSV infected confirmed by visual inspection and another one sample was collected from Bagerhat Shrimp Research Institute, BFRI where shrimp were cultured under very controlled condition. However, these three samples were confirmed by both one-step PCR with primer pair 146F1/146R1 and 146F2/146R2 and two-step PCR with primer pair 146F2/146R2 and then processed for histopathological observation. Results of the electrophoresis of the PCR products of positive and negative controls are shown in Figure 8.

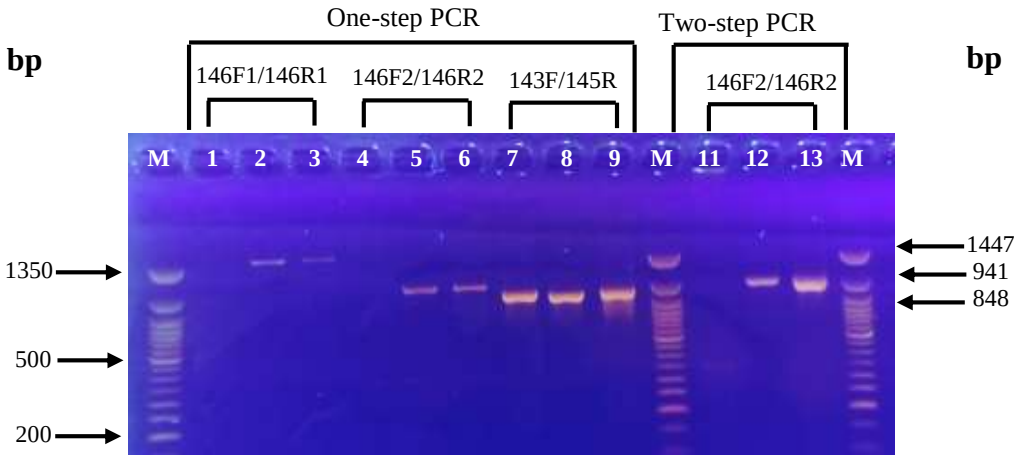


Figure 8. PCR confirmation of WSSV infection for histopathological study by one-step PCR and two-step PCR using primar pairs 146F1/146R1 and 146F2/146R2. Lanes 1 & 4 represent WSSV negative sample (control) confirmed by one-step PCR which was further confirmed by two-step PCR (lane 11). Lanes M represent 50 bp DNA ladder.

For histopathological studies of WSSV in *P. monodon*, two samples were detected WSSV positive by using both one-step and two-step PCR and only one sample was used as a representative of WSSV positive samples. Similarly, one sample was found WSSV negative by one-step and two-step PCR and used as a representative of WSSV negative samples. Then WSSV-induced pathological changes were observed in the subcuticular epithelial cells of the hepatopancreas and muscle in these clinically and PCR-confirmed non-infected shrimp and infected shrimp.

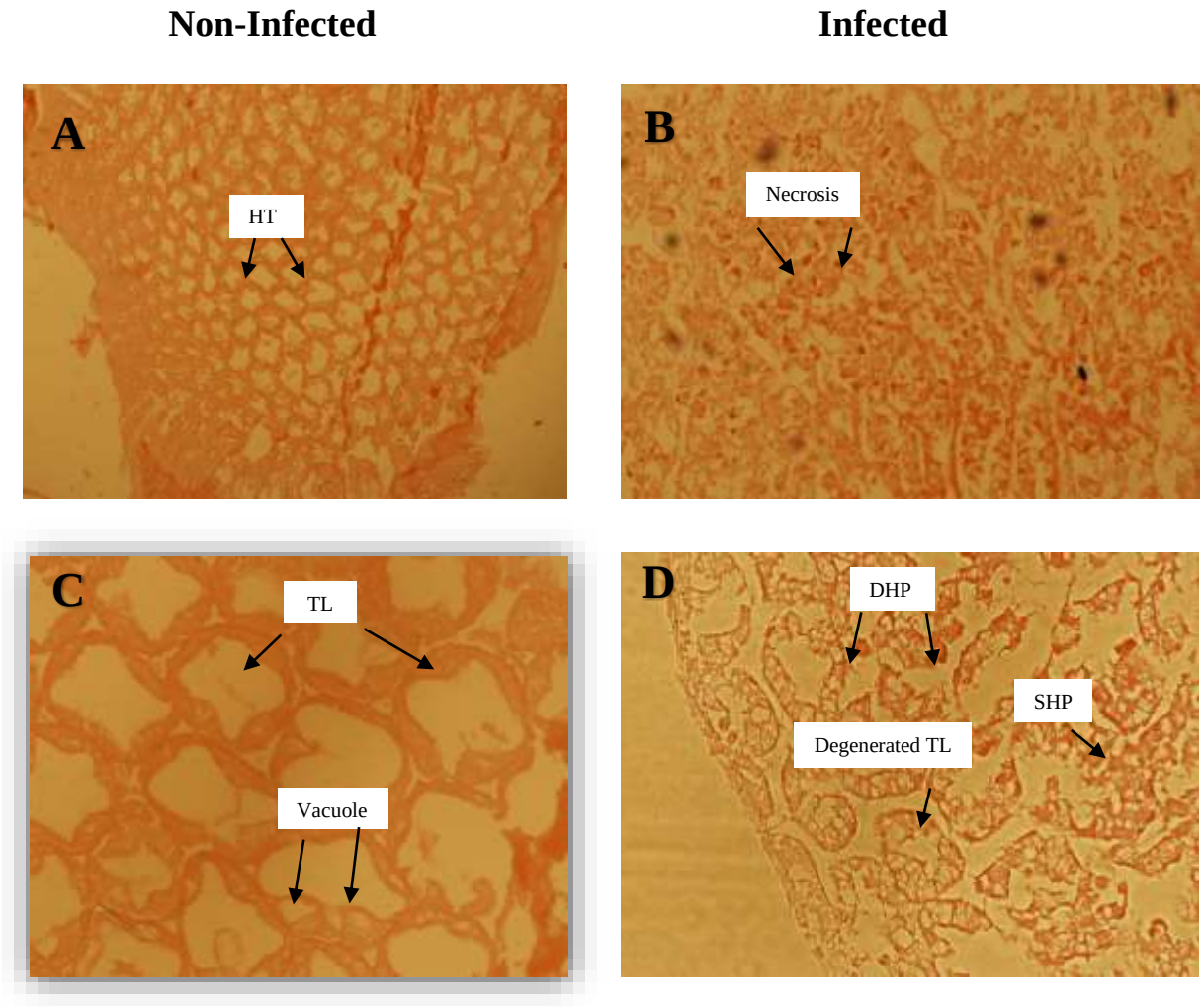


Figure 9. Histopathological (H & E) stained WSSV infected and non-infected hepatopancraeas of *Penaeus monodon*. Histopathological study of HP for non-infected shrimps with (A&C) 10X and 40X magnification showed healthy tubule (HT), TL (Tubule lumen), and vacuole. No inclusion or necrosis was observed. Infected tissue was observed with DHP (Degenerated HP), SHP (Sloughing HP), necrosis, vacuolization of cell (B & D) at 10X and 40X magnification.

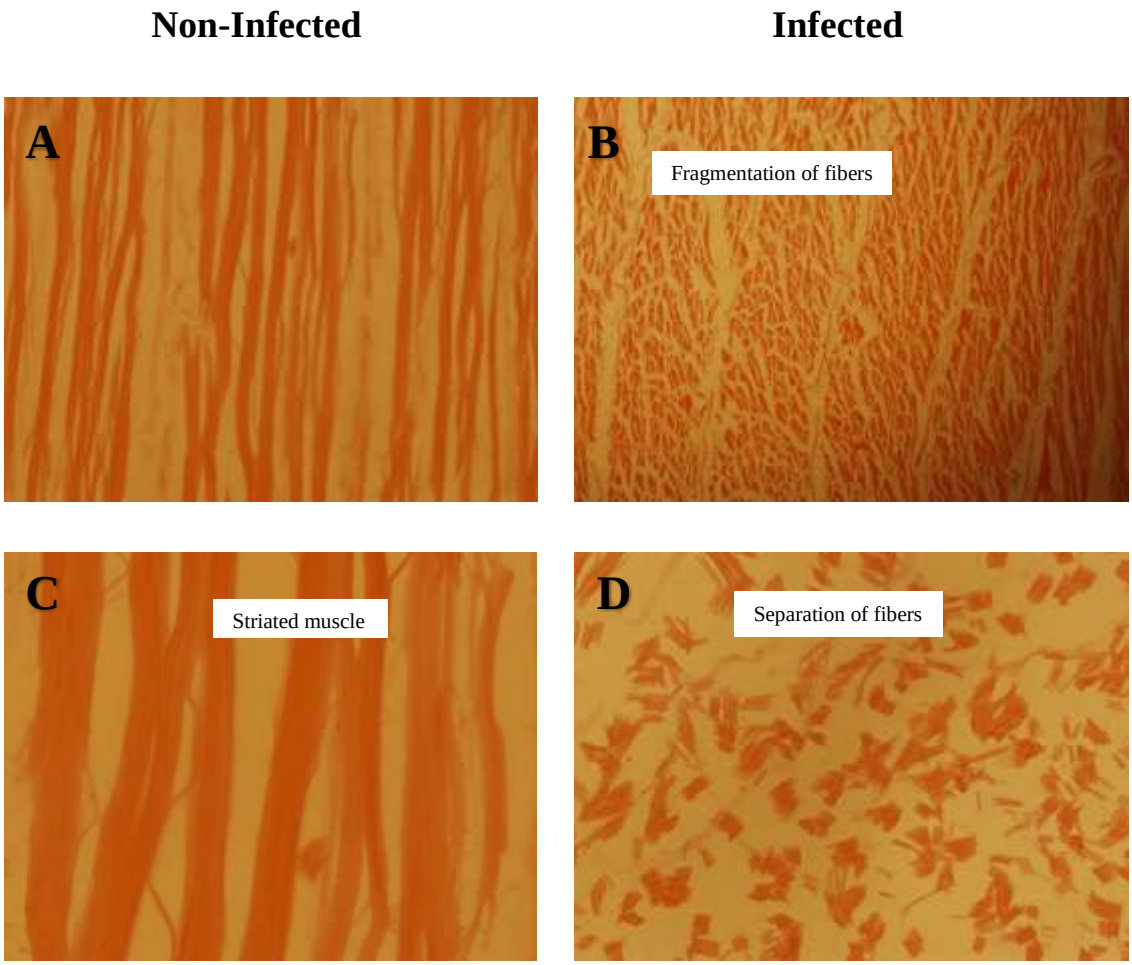


Figure 10. Histopathological (H & E) stained WSSV infected and non-infected muscle of *Penaeus monodon*. Histopathological study of muscle for non-infected shrimps (A & C) with 10X and 40X magnification showed striated fibers without any damage. Infected tissue observed DHP (Degenerated HP), loss of myofibrils and muscle striation and fragmentation and separation of muscle fibres (B & D) with 10X and 40X magnification.

Using PCR, WSSV infection could be diagnosed by pleopod tissues collected from shrimp. The negative control group was PCR-negative. At this time point, the control tissues showed normal cell architecture including healthy tubule, tubule lumen and vacuole. The appearances of white spots over the exoskeleton, pinkish-red body color, lethargy and anorexia are the common clinical signs associated with the WSSV that is clinically confirmed by visual inspection in the infected shrimp.

Tissues of hepatopancreas and muscle from both infected and non-infected shrimp were processed and stained by haematoxylin and eosin. (H & E) stained histopathological study indicates the destruction or degeneration of HP tissue observed due to the WSSV-affected the healthy shrimp. Histopathology of HP is characterized by widespread cellular degeneration, degenerated tubule lumen, degenerated and sloughing hepatopancreas with necrosis. These studies showed the degeneration of the epithelial tissue, the collapse of the epithelial tubules of HP, massive sloughing of central HP tubule epithelial cells and massive inter-tubular hemocytic aggregation followed by secondary bacterial infections causes necrosis. WSSV-infected hepatopancreas tissues have degenerated and necrosis was observed. But large eosinophilic intranuclear inclusion bodies Cowdry type A was absent in the hepatopancreas tissue.

In the case of infected muscle tissue loss of myofibrils, muscle striation, fragmentation and separation of muscle fibers were observed as shown in Figure 10.

This study also observed histopathological evidence of WSSV infection in *P. monodon* after confirmation by both one-step and two-step PCR. However, samples are confirmed by both one-step and two-step nested PCR and then processed for histopathological observation to ensure the PCR result was further supported by histopathological investigation. The destruction of HP tissue observed may be due to the disease affecting the healthy shrimp. Histopathology of HP is characterized by widespread cellular degeneration, degenerated tubule lumen, degenerated and sloughing hepatopancreas with necrosis, degeneration of the epithelial tissue, the collapse of the epithelial tubules of HP, massive sloughing of central HP tubule epithelial cells followed by secondary bacterial infections causes necrosis. I also found that hepatopancreas of WSSV-infected shrimp showed vacuolization of the entire tissue which was reported by Wang *et al.* (1997) & Wang *et al.* (1999). Tang and Lightner (2000) examined that the sample did not show any signs of disease and PCR was positive only in nested reactions indicating low viral load suggesting large Cowdry type

A eosinophilic intranuclear inclusion bodies. But our study showed that there was no Cowdry type A eosinophilic intranuclear inclusion bodies due to low viral load on hepatopancreas. Mannan *et al.* (2015) conducted a study to observe the WSSV infected tissue of hepatopancreas of *P. vannamei* and found that histopathological results showed signs of infected conditions including degeneration of Tubules Lumen(TL), slough hepatopancreas tubules cells (SHP), enlarged of the hepatopancreas nuclei cell and lack of B, F and R epithelial cells in the hepatopancreas tubules. They also commented that HP conditions might be a good indicator to determine the conditions of the shrimp health status either infected or not. Pazir *et al.* (2011) observed basophilic Cowdry type A inclusion bodies in all tissues such as gills, haematopoietic tissue, cuticle epithelium, lymphoid organ and connective tissue. It also has been observed that the histopathological change of the striated muscle abnormality showed severe necrosis. In case of infected muscle tissue loss of myofibrils and muscle striation was observed through our study. Hepatopancreatic epithelial cells showed no viral infection, but haemocytes in the hepatopancreas (HP) are heavily infected. HECs are highly vacuolated, resulting in diminution of the tubule lumens. B-cells (B) are rarely seen reported by Wang *et al.* (1999).

Histopathological changes of the post larvae, sub adults and broodstocks infected tissues collected from hatcheries and shrimp farms were characterized by widespread cellular degeneration, severe nuclear hypertrophy and margination of chromatin in tissues, especially in the hepatopancreas, cuticular epithelium, gills, connective tissues, hematopoietic tissues and digestive epithelium reported by Pazir *et al.* (2011). Histopathology on shrimp HP and muscle sections has been a routine practice in diagnostics. Finally, the PCR-based detection of WSSV in shrimp provides a sensitive tool to know the prevalence and the changes in the tisssues level due to WSSV infection as confirmed by histopathological changes in examined species of shrimp.

5 Chapter V
Summary and Conclusion

CHAPTER V
SUMMARY AND CONCLUSION

Shrimp farming has rapidly expanded all over the world due to the high demand for shrimp in the international markets. In Bangladesh, shrimp are culturedin the semi-intensive system, in gher and ponds by the local farmers in the Southeast region exclusively coastal area. Geographically, Bangladesh is situated near the Bay of Bengal and shrimp cultures require enough saline water. That's why the interest of shrimp culture in Bangladesh is increasing day by day. But the shrimp culture industry is facing severe problems due to the attack of different bacterial and viral pathogens. Among different viral pathogens, white spot syndrome virus is a great threat to shrimp culture that resulted in 100% mortality with 3 to 5 days of infection and 60% production loss as well. There is no treatment to stop these viruses but an early, accurate and timely diagnosis and detection of the virus makes good disease management to prevent production loss. To reduce the incidence of WSSV in shrimp, early diagnosis of WSSV is necessary in brood stock, PL, juvenile and sub-adult. There is various method for disease diagnosis and detection in shrimp. Polymerase chain reaction known as PCR is a sensitive, reliable and powerful tool for early-stage disease diagnosis. The present study has been conducted to detect WSSV by PCR techniques including one-step and two-step PCR which was further confirmed by histopathological observation.

To conduct this study, 36 samples were randomly collected from the Bagerhat and Satkhira districts of Bangladesh for PCR analysis and 4 samples were collected for histopathological analysis. Through one-step and two-step PCR techniques, all samples were diagnosed with specific three sets of primer and detected WSSV positive and negative samples. Among 36 samples, Thirty-three (33) samples were found WSSV positive and only 3 samples were found to be WSSV negative combined by one-Step and two-step PCR. From the 18 samples of Satkhira district, 15 samples were found to be WSSV infected and the rest 3 samples were negative by one-step PCR. In another 18 samples of the Bagerhat district, 10 samples were found to be WSSV infected and the other 8 samples were negative by one-step PCR. A total of 11 samples were WSSV negative confirmed by one-step PCR was further prepared for two-step PCR. The result of two-step PCR was resulted that 3 samples of Satkhira remained WSSV negative and the other 8 samples of Bagerhat was WSSV infected. On the basis of the one-step PCR

result, the prevalence rate of WSSV infection in collected shrimp was 83.33% and 55.56% in Satkhira and Bagerhat districts, respectively. So the overall prevalence rate of WSSV-infected shrimp by one-step PCR was 69.44% in these two study areas of Bangladesh. On the other hand, in case of two-step PCR of 11 samples, the prevalence rate of WSSV infection in collected shrimp was 83.33% and 100% in Satkhira and Bagerhat districts, respectively and the overall prevalence rate of WSSV infected shrimp by two-step PCR was 91.67%. These results indicated that those samples that were positive by one-step PCR were highly infected with higher viral load by WSSV and those that were positive only by two-step PCR indicated a lower level of infection with lower viral load by WSSV. After confirmation of the WSSV positive and WSSV negative samples, two representative samples were taken for histopathological study to observe the change of tissue due to WSSV and compared with non-infected samples. Haematoxylin and eosin (H & E) stained histopathological study indicates the destruction or degeneration of HP tissue observed due to the WSSV-affected the healthy shrimp. Histopathology of HP is characterized by widespread cellular degeneration, and severe nuclear hypertrophy hepatopancreas. This study showed the degeneration of the epithelial tissue, the collapse of the epithelial tubules of HP, massive sloughing of central HP tubule epithelial cells and massive inter-tubular hemocytic aggregation followed by secondary bacterial infections causes necrosis. In case of infected muscle tissue loss of myofibrils and muscle striation, fragmentation and separation of muscle fibers were observed.

Knowledge of the presence of viruses and viral load in brood stock, post larvae, juvenile and adult in ponds or ghers will greatly help the shrimp farmer to understand the risk associated with stocking infected spawners and larvae and appropriate disease management for shrimp culture. Recently, the prevalence and the WSSV entry along with spawners and PL batches have been suggested to play a crucial role in WSSV outbreaks from PL to PL and gher to gher. Therefore, testing of spawners and the nauplii/PL for the presence of WSSV and the removal of WSSV in infected PL or spawner of shrimp before stocking may be helpful for shrimp farmers. It may be a good remedial measurement to avoid severe loss in shrimp production in the coastal areas of Bangladesh.

6 Chapter VI
Recommendations

CHAPTER VI

RECOMMENDATIONS

The results obtained from the present study on molecular detection and histopathological studies of white spot syndrome virus infections provides some useful implications for shrimp farmers of Bangladesh to reduce significance loss due to white spot disease. But some limitations were occured during these study. Due to Covid-19, enough sample couldn't be collected within that time. Even entry of outsider was limited in the hatcheries. Therefore, for further studies following points might be considered to study WSSV detection.

- Study area should be covered more than two districts to understand the better prevalence rate.
- Seasonal sampling should be maintained as WSSV infection varies with season.
- Two-step PCR must be conducted with care due to the chance of cross contaminations.
- It is suggested that more molecular-based studies about WSSV is required for disease management and to increase shrimp production in Bangladesh.

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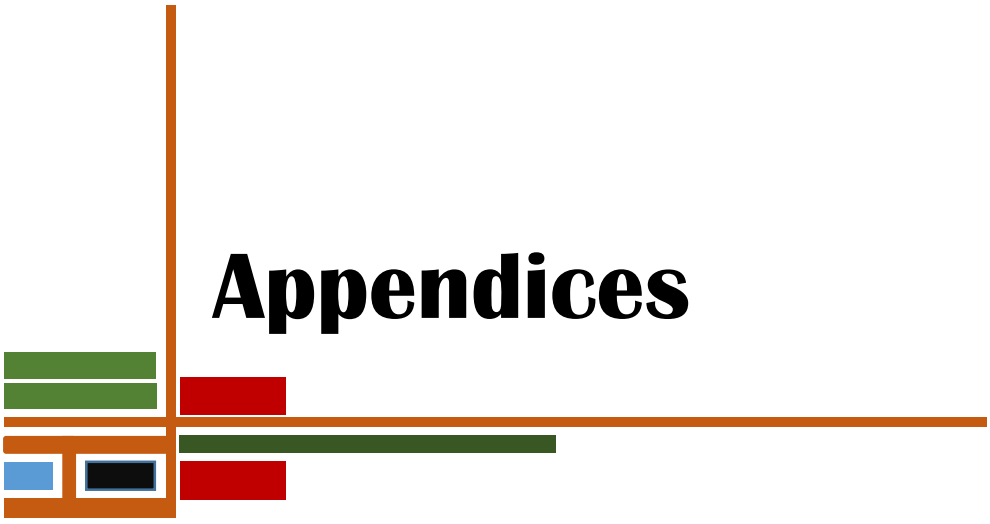
Appendices



APPENDICES

Appendix 1: Calculation to prepare lysis buffer for DNA isolation

Given the following stock solutions: 1 M Tris-HCl, 0.5 M EDTA, 5 M NaCl, and 10% SDS to make 1 L of a working solution containing 200 mM Tris-HCl, 25 mM EDTA, 250 mM NaCl and 0.5% SDS requires the necessary calculations are as follows.

Following formula is used to calcultae the required volume:

$$V_1S_1=V_2S_2$$

Here,

V_1= initial volume

S_1 = initial concentration

V_2 = final volume

S_2= final concentration

For Tris-HCl:

(1000 mM) (X ml) = (200 mM) (1000 ml)

X ml = (200 mM) (1000 ml)/ 1000 mM

X ml = 200 ml of 1 M Tris

For EDTA:

(500 mM) (X ml) = (25 mM) (1000 ml)

X ml = (25 mM) (1000 ml)/ 500 mM

X ml = 50 ml of 0.5 M EDTA

For NaCl:

(5000 mM) (X ml) = (250 mM) (1000 ml)

X ml = (250 mM) (1000 ml)/ 5000 mM

X ml = 50 ml of 5 M NaCl

For SDS:

(10 %) (X ml) = (0.5 %) (1000 ml)

X ml = (0.5 %) (1000 ml)/ 10 %

X ml = 50 ml of 0.5% SDS

650ml ddH_2O was added to make the volume 1L and then autoclaved before use.

Appendix 2: Preparation of 10x TBE buffer for gel electrophoresis

- Dissolve 108 g Tris and 55 g Boric acid in 900 ml distilled water.
- Add 40 ml 0.5 M Na_2EDTA (pH 8.0)
- Adjust volume to 1 Liter.
- Store at room temperature.

Appendix 3: Calculation to prepare 1X TBE buffer from 10X stock solution

Following formula is used to calcultae the required volume:

$$V_1S_1=V_2S_2$$

Here,

V_1= initial volume

S_1 = initial concentration

V_2 = final volume

S_2= final concentration

For 100 ml:

(10X) (X ml) = (1X) (100 ml)

X ml = (1X) (100 ml)/ (10X)

X ml = 10 ml of a 10X

90 ml ddH_2O was added to make 100 ml working buffer solution.

Appendix 4: Equalization of different values of DNA concentration to prepare working solution

Following formula is used to calcultae the required volume:

$$V_1S_1=V_2S_2$$

Here,

V_1= initial volume

S_1 = initial concentration

V_2 = final volume

S_2= final concentration

Then to calculate how much solvent need for dilution the samples:

Required volume of solvent = V_2-V_1

Followings are the examples of DNA concentration equalization with calculations:

Samples (Farm-3, Satkhira)	**Initial concentration (S_1) ng/µL**	**Final concentration (S_2) ng/µL**	**Final volume (V_2) µL**	**Initial volume (V_1) µL**	**Volume of solvent (V_2-V_1)µL**
1	72.54	50	50	34.46	15.54
2	100	50	50	25	25
3	164.82	50	50	15.17	34.83
4	142.56	50	50	17.54	32.46
5	74.19	50	50	33.7	16.3
6	75.72	50	50	33.01	16.99