Polymerase chain reaction (PCR)-based rapid identification of white spot syndrome virus in shrimp, *Penaeus monodon*

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

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Polymerase chain reaction (PCR)-based rapid identification of white spot syndrome virus in shrimp, *Penaeus monodon*

A Thesis
By

Md. Nurul Islam

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The thesis submitted to the Fisheries and Marine Resource Technology Discipline in partial fulfilment of the requirements for the degree of

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Polymerase chain reaction (PCR)-based rapid identification of white spot syndrome virus in shrimp, *Penaeus monodon*

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DECLARATION

The results submitted in this thesis are entirely of the candidate's own investigations and no part of the results has been accepted for any degree, nor it is being concurrently submitted for any degree

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The Author

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ABSTRACT

The incidence of white spot syndrome virus (WSSV) in *Penaeus monodon* from the coastal region of Bangladesh was determined by a two-step polymerase chain reaction (PCR)-based screening protocol using two pairs of oligonucleotide primers directed towards WSSV DNA. The widespread occurrence of WSSV and the effectiveness of the protocol were further ensured by investigating with horina chingri, *Metapenaeus monoceros* and mud crab, *Scylla serrata.* The experiment was conducted with tissue samples collected from muscle, hepatopancreas and pleopod together with individuals from two different farms; apparently disease infected farm and uninfected farm, situated in the same area. The first-step PCR of total genomic DNA isolated from the tissue samples served as the templates for the second-step PCR, which resulted in DNA amplification of an expected size for each sample. The easiness, cost-effectiveness and high specificity of this method rely on using a single-step DNA preparation without involving hazardous organic solvents as well as two pairs of oligonucleotide primers highly specific for WSSV gene. This is the first scientific report on applying PCR-based WSSV screening protocol in Bangladesh without using patented WSSV detection kits as in elsewhere in the country. The pioneering works in Bangladesh and the results suggest that a PCR screening of WSSV infection for shrimp brood and larval stocks and other potential virus carriers could be an effective tool in fight against the deadly virus that has almost devastated the shrimp industry of the country.

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CHAPTER ONE

INTRODUCTION

CHAPTER ONE

1. INTRODUCTION

1.1 Background

1.1.1 White spot disease (WSD)

The causative agent of WSD is white spot syndrome virus (WSSV) or white spot virus (WSV). WSD of penaeid shrimp is characterised by high and rapid mortality accompanied by gross signs in moribund shrimp of white, initially circular, inclusions or spots in the cuticle, sometimes accompanied by overall red body coloration (Fig.1). Disease progression is characterised by cessation of shrimp feeding followed within a few days by the appearance of moribund shrimp swimming near the surface at the edge of rearing ponds. The cuticular inclusions range from minute spots to discs several millimetres in diameter, which may coalesce into larger plates. These are most easily observed by removing the cuticle over the cephalothorax, scraping away any attached tissue with the thumbnail and holding the cuticle up to the light. However, the appearance of white spots in the cuticle is unreliable even for preliminary diagnosis of WSD as similar inclusions can be produced by high alkalinity and other environmental conditions and bacterial white spot syndrome (Wang et al., 2002). Therefore, preliminary diagnosis must include histopathological examinations. Farms and hatcheries have few defenses against rampaging protozoa, fungi and bacteria, but it is the viral diseases that pose the greatest threat. WSSV is a great threat for cultured *Penaeus monodon*. They have caused major crashes in Taiwan, China, Indonesia, India, Panama, Thailand, Bangladesh, Honduras and Ecuador. Currently the Western Hemisphere fights a virus that arrived from the East (WSSV), and the Eastern

Hemisphere fights a virus that arrived from the West (Taura Syndrome Virus, TSV). There are no medications to treat shrimp viruses, but management techniques have evolved which lessen their impact.

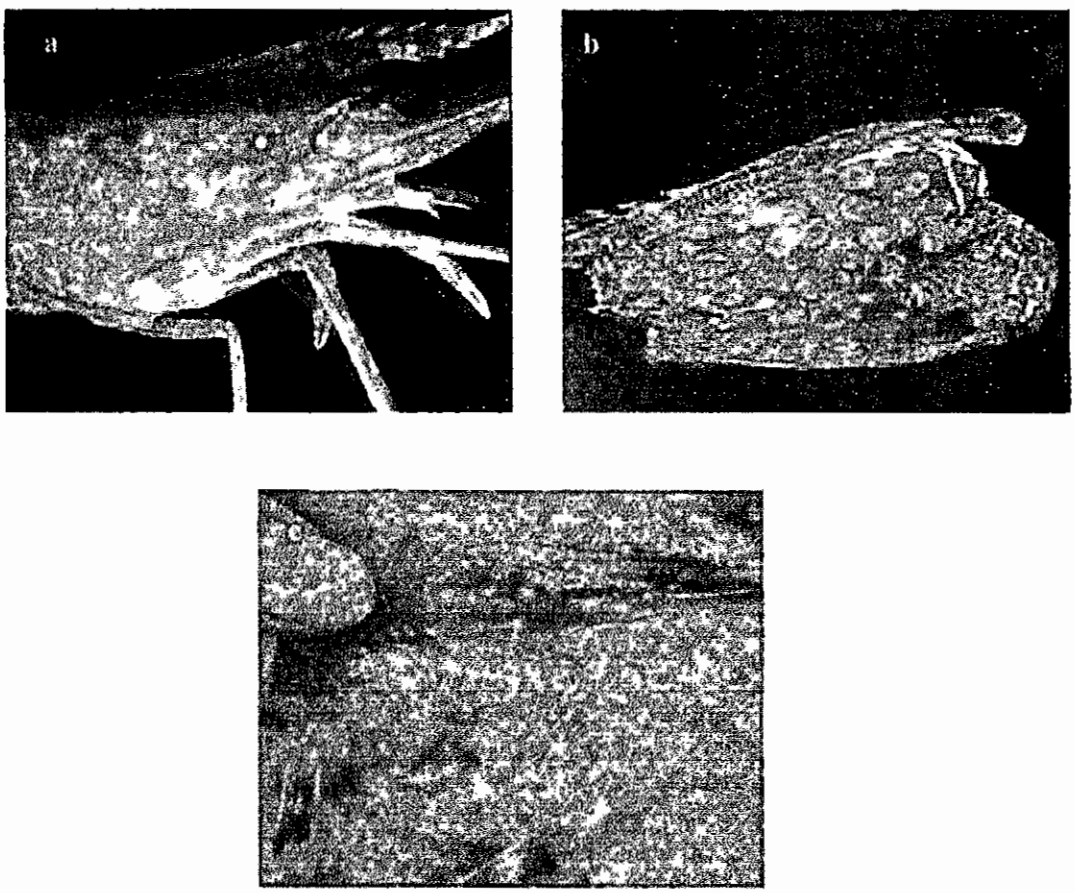

Fig.1: Gross sings of WSSV occurrence in a juvenile *Penaeus monodon* (a) distinctive white spots on head; (b) on the carapace; and (c) throughout the overall external body appendages.

In Latin America, prior to the arrival of the WSSV in 1999, TSV was the biggest killer. Shortly after stocking, it can kill from 40 to 90% of the post-larvae in a shrimp pond. In the Eastern Hemisphere, white spot virus rages on, but in places like Thailand, management techniques have brought it under control. White spot virus

usually strikes when the animals have been in the water for more than sixty days, a critical time for the farmer. In 1996, white spot virus even extensively attacked farms in West Bengal, India, and the Khulna area of Bangladesh (FAO, 1999). Now common in both hemispheres, it is more lethal than TSV, kills many varieties of crustaceans and has many vectors (carriers). Fortunately, WSSV is easier to exclude from a farm than TSV because birds and insects don't appear to be carriers (Vaseeharan et al., 2003).

1.1.2 White spot syndrome virus - viral characterization, transmission pathway and pathology

WSSV, a double-stranded DNA virus of the genus whispovirus within the family nimaviridea (Mayo, 2002a and 2002b) infecting penaeid shrimp (*Penaeus sp.*), is currently the most devastating viral pathogen threatening the shrimp farming industry worldwide. WSSV is also known as baculoviral hypodermal and hematopoietic necrosis virus (Huang et al., 1995), a rod-shaped virus of *P. japonicus* (Inouye et al., 1994), penaeid acute viremia (Takashi et al., 1994), systemic ectodermal and mesodermal baculovirus (Wongteerasupaya et al., 1995), *P. monodon* nonoccluded baculovirus (Wongteerasupaya et al., 1996), white spot syndrome baculovirus (Lo et al., 1996a), Chinese baculovirus (Lu et al., 1997), and white spot virus (Lightner and Redman, 1998). The name WSSV was first suggested by Cesar et al. (1998). Virions are rod-shaped to elliptical with a trilaminar envelope and they are large (80–120 x 250–380 nm) (Durand et al., 1997; Inouye et al., 1994; Inouye et al., 1996; Takahashi et al., 1994; Wang et al., 1995; Wongteerasupaya et al., 1995). Its double-stranded circular DNA genome of 305,107-bp contains 181 open reading frames (ORFs) (Yang et al., 2001). Nine homologous regions containing 47 repeated minifragments that

include direct repeats, atypical inverted repeat sequences, and imperfect palindromes were identified (Yang et al., 2001). Ribonucleotide reductase genes and repeat regions containing genomic segment of white spot syndrome virus of shrimp was analysis by Mariëlle et al. (2000) and the author concluded that the virus is either a member of a new genus (Whispovirus) within the *Baculoviridae* or a member of an entirely new virus family. This is the largest animal virus that has been completely sequenced (Yang et al., 2001). Although WSSV is morphologically similar to insect baculovirus, the two viruses are not detectably related at the amino acid level. Rather, some WSSV genes are more homologous to eukaryotic genes than viral genes. In fact, sequence analysis indicates that WSSV differs from all known viruses, although a few genes display a weak homology to herpesvirus genes. Most of the ORFs encode proteins that bear no homology to any known proteins, either suggesting that WSSV represents a novel class of viruses or perhaps implying a significant evolutionary distance between marine and terrestrial viruses. Molecular epidemiology of white spot syndrome virus within Vietnam was studied by Dieu et al. (2004).

Serious viral outbreaks often cause catastrophic losses in shrimp farming around the globe (Lightner et al., 1996). Infectious hypodermal and hematopoietic necrosis virus and WSSV are the two most important DNA viruses infecting penaeid shrimp in the Western Hemisphere (Lightner, 1996). WSSV was first reported in Eastern Asia during 1992 to 1993 (Inouye et al., 1994). The virus rapidly spread throughout shrimp farming regions of Southeast Asia and North America in the mid-1990s (Flegel, 1997; Jory and Dixon, 1999). WSSV can be spread via-transportation of infected aquatic livestock, birds as transport vectors (fecal contamination) and importation / reprocessing of frozen food products.

The histopathology of WSD is distinctive and can be used for diagnosis with moribund shrimp during outbreaks (Wongteerasupaya et al., 1995; Momoyama et al., 1995 and 1994; Lightner, 1996). Confirmation of WSD requires more detailed analysis by polymerase chain reaction (Kasornchandra et al., 1998; Kim et al., 1998; Kimura et al., 1996; Lo et al., 2001; Nunan and Lightner, 1997; Takahashi et al., 1996), antibody-based assays (Loh et al., 1998; Nadala et al., 1997; Poulos et al., 2001 and Shih et al., 2001), in-situ DNA hybridisation (Durand et al., 1996; Nunan et al., 1998), or transmission electron microscopy (Durand et al., 1997; Takahashi et al., 1994; Wongteerasupaya et al., 1995). Using such techniques, it has been shown that WSSV from captured juvenile and broodstock shrimp or other carriers are identical or closely related (Lo et al., 1996b; Lo et al., 1999; Loh et al., 1998; Takahashi et al.,1996; Wang et al., 1995 and 1997; Wongteerasupaya et al., 1996). These molecular techniques have also been used to confirm infection of more than 40 penaeid and non-penaeid crustacean carriers (Flegel, 1997) and, tentatively, an aquatic insect larval carrier (Lo et al., 1996a). Some of these carriers have been shown to transmit WSSV to shrimp (Kanchanaphum et al., 1998; Supamattaya et al., 1998) in laboratory tests.

1.1.3 Polymerase Chain Reaction

Diagnosis of WSSV is done by bioassay using indicator hosts, monitoring clinical signs, histopathology, dot blot and in situ hybridizations using a virus-specific gene probe, direct dark-field LM, transmission EM, antibody-based assays and PCR (Lightner et al., 1996). PCR is the most effective method. Various scientists have developed various PCR protocol. Kiatpathomchail et al. (2001) develops a protocol for WSSV screening by using 1 sense primer and 2 anti-sense primers. Dhar et al.

(2001) developed a protocol using Real-Time Quantitative PCR and SYBR Green Chemistry.

Polymerase is an enzyme which links individual nucleotides together into a long strand, using another strand as a template. There are two general types of polymerase - DNA polymerases (which synthesize DNA) and RNA polymerase (which makes RNA). Within these two classes, there are numerous sub-types of polymerase, depending on what type of nucleic acid can function as template and what type of nucleic acid is formed. A DNA-dependant DNA polymerase will copy one DNA strand starting from a primer, and the product will be the complementary DNA strand. A DNA-dependant RNA polymerase will use DNA as a template to synthesize an RNA strand. Polymerase chain reaction that uses a thermal cycler, is the invention of Kary Mullis, and has had a tremendous impact on many research areas, including disease diagnosis. PCR is used for replicating a specific piece of DNA *in-vitro* (without using a living organism, such as *E. coli* or yeast), even in the presence of excess non-specific DNA. Primers are added, which initiate the copying of each strand along with nucleotides and *Taq* polymerase (a thermostable DNA polymerase isolated from the bacterium *Thermus aquaticus*).

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1.2 Rational and objectives

The 1980s had witnessed an unprecedented growth in shrimp farming due to the technological breakthroughs (Shang et al., 1998; Leung et al., 2000) in the backdrop of an expanded global demand for high quality sea food. The development of shrimp industries with the rapid expansion and intensification of shrimp farming worldwide has been accompanied by the occurrence of diseases which threaten its total economy. The global shrimp industry has experienced phenomenal growth over the last twenty-four years generating more than 730,000 metric tons of farm cultured product annually. This industry represents a market value exceeding US$3.7-4.5 billion ($5.00-$6.00/kg) ex-farm. In the early 1990's, this rapid growth began to slow. Reasons for declining include lack of knowledge about farming techniques, poor farm management practices, degradation of environment / water quality through industrial pollutions / discharges, and most importantly, shrimp diseases (FAO, 2001). A major outbreak of WSSV infection in 1993 resulted in a 70% reduction in shrimp production in China (Zhan and Wang, 1998; Cen, 1998) and has raised major concerns in aquaculture around the world. The white spot disease was first reported in Eastern Asia during 1992 to 1993 (Inouye et al., 1994; Huang et al.,1999) while by the mid-90s it rapidly spread throughout the shrimp farming regions of Southeast Asia and North America (Flegel, 1997; Jory and Dixon, 1999). As with other Asian countries, the shrimp farms of Bangladesh have also been suffering from heavy economic losses due to the same devastating virus believed to be introduced with imported shrimp fry from Thailand in early 90s.

The disease problem whipped out Thai production in 1996 and 1997 and Ecuadorian production in 1999 (FAO, 2000). Figure 2 shows the occurrence of WSSV in different countries whereas Table-1 shows yearly shrimp production by major producing countries during 1991-2000. Shaded area in the Table shows the sudden fall in shrimp production due to WSSV occurrences in those corresponding countries. The apparent production increases in shrimp farming over the past ten years have been primarily due to tremendous increases in pond area rather than increased output per land area. Worldwide there are approximately 1,251,450 hectares (3 million acres) devoted to shrimp farming with 1,114,050 hectares in the Eastern hemisphere (FAO-UNDP/SCSP, 2003). From 1998 to 1999, Asian countries showed a 75% increase in the number of shrimp farms while North, Central and South America, registered a 39% decrease (FAO-UNDP/SCSP, 2003).

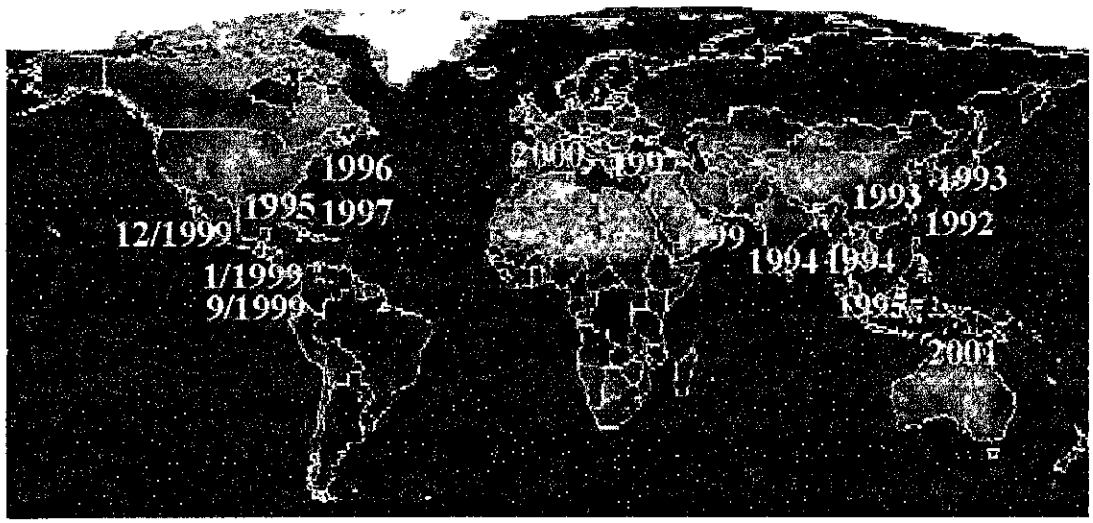

Fig. 2: The spread of white spot virus in different region of the world.

An article by C.G. Lundin(1997) of the World Bank addressed global shrimp disease and concluded that at an average price of US$5.00/kg for heads-on shrimp and total disease-related losses of 540,000 metric tons, the total loss based on 1994 data would be US$3.0 billion. This indicates a very significant problem that has implications for the wellbeing of millions of people in developing countries whose livelihood depends on this industry. Although recovering somewhat, the total losses from disease for all of Asia are estimated to be well over US$1.0 billion in 1997 and over $140 million in the American production. Disease has caused an 11% drop in the yearly harvest total from 1996 and indicates that in all probability the global production of shrimp from aquaculture will not meet expectations for industry growth or consumer demand in the coming years unless resistance to disease is achieved (FAO/WHO, 2001).

Table-1: Yearly shrimp production by major producing countries during 1991-2000 (in 1,000 metric tons; FAO/WHO, 2001).

Country	Year of production									
	1991	1992	1993	1994	1995	1996	1997	1998	1999	2000
China	564.1	574.1	488.7	603.4	665.6	751.8	829.6	970.9	1,222.7	1,241.9
India	300.5	290.4	363.0	446.6	406.1	415.6	366.6	413.1	423.3	405.7
Thailand	289.9	300.6	343.1	385.0	389.3	370.8	350.8	345.4	370.9	398.5
Indonesia	296.8	312.1	300.7	317.1	334.7	343.3	382.2	345.5	384.5	398.4
USA	148.5	156.5	137.9	130.2	140.2	145.0	132.9	128.0	140.1	153.0
Vietnam	81.3	86.2	94.6	111.7	138.1	135.9	147.7	148.4	148.9	151.1
Malaysia	104.7	129.4	109.8	106.4	99.6	108.0	101.0	97.1	102.7	111.9
Mexico	70.6	66.2	79.8	77.3	85.9	78.9	88.5	90.3	95.6	95.1
Greenland	73.1	81.9	76.5	79.8	81.9	72.0	63.9	69.6	79.2	81.5
Philippines	84.9	118.8	130.1	126.6	127.5	113.2	74.5	72.3	73.1	79.4
Norway	49.0	49.1	49.0	38.2	39.8	41.5	42.0	57.1	64.2	66.2
Bangladesh	19.6	21.0	28.5	28.8	34.0	49.3	56.5	66.1	81.1	58.2
Brazil	42.3	44.0	38.4	38.5	43.0	38.9	44.1	42.8	47.7	56.6
Ecuador	118.8	127.0	97.5	98.7	112.1	112.9	137.2	147.4	121.0	51.4
Korea Rep.	55.8	67.1	68.0	58.1	42.5	40.9	41.1	47.6	44.7	37.2
Others	532.7	529.3	542.0	551.7	594.5	622.9	633.7	647.4	599.2	651.7
Total	2,877.3	2,996.8	2,995.0	3,251.3	3,397.4	3,506.6	3,574.4	3,762.1	4,118.9	4,168.4

Among all viral causative agents, the white spot syndrome virus is considered the most serious problem for tiger shrimp (*Penaeus monodon*) aquaculture. The highly virulent nature of WSSV, its wide hosts range as shrimp to marine crustaceans (Hossain, 2001) and its worldwide geographic distribution has overshadowed all other disease agents affecting shrimp as the leading cause of production losses (Flegel, 1997).

The disease is characterized by white spots on the carapace, appendages and cuticle and reddish coloration of the hepatopancreas of the shrimp and also shows signs of lethargy and a rapid reduction in food consumption. Infection of penaeid shrimp by WSSV can result in mortality of up to 90 to 100% within 3 to 7 days of the onset of clinical signs (Zhan and Wang, 1998). WSSV is a member of a new virus family (Van et al., 2001) with high virulence (Yoganandhan et al., 2003). Its vituperative nature contains a wide spreading host range as crayfish, *Penaeus monodon, Macrobrachium rosenbergii, Metapenaeus monoceros*, crabs (eg., *Scylla cerata)*, copepods, lobsters etc. (Lo et al., 1996b; Supamattaya et al., 1998; Cai et al., 1995; Takahashi et al., 1994; Peng et al., 1998; Wongteerasupaya et al., 1995); The virus can transmit through brood to offspring, plankton to shrimp, food prepared from infected animals to cultured shrimp (Wang et al., 1998; Lo et al., 1999; ASCC 1996).

This is the largest animal virus (Yang et al., 2001) and has since been a subject of intensive research as to the molecular basis of disease progression in shrimp. Relating the appearance of white spots to the onset of WSD is unreliable even for preliminary diagnosis as similar inclusions can be produced by bacteria (Wang et al., 2002) as well as high alkalinity and other environmental conditions. The outbreak of WSSV is triggered by a number of different environmental and stress factors (Karunasagar et

al., 1997; Peng et al., 1998; Anonymous, 1995; Flegel et al., 1997; Jory, 2000). However, it is not yet clear as to what factors influence the outbreak of WSSV in shrimp in culture ponds (Hossain, 2001). Besides, the highly virulent nature of WSSV with its wide host range and ability to attack different tissues has made it difficult to identify and to take caution within a short time. There is a strong need for vaccines that can protect against WSSV infections. Some attempts have already been made for the invention of appropriate vaccine. Since complete mortality occurs within a couple of days following the appearance of first clinical sign, an early diagnosis is the only way to manage the infected farm. Hence, require a rapid and sensitive diagnosis protocol that will be able to screen the virus in its various hosts at every life stage.

Histopathological examination of infected tissues provides a preliminary diagnosis, which cannot be performed on a routine basis. The practical application of the use of classical method to diagnose WSSV is sometime difficult and sensitivity is often limited (Durand et al., 1996). In the ongoing effort to control and prevent these diseases, molecular methods are finding increasing application for differential diagnosis, epidemiological investigations and screening of covert infections in hatcheries and on farms. Methods such as the polymerase chain reaction, dot-blot hybridization and *in situ* hybridization have now been developed (Lightner and Redman, 1998; Nadala et al., 1997; Corbel et al., 2001; Tapay et al., 1999). Although technically complex and requiring specialized analytical equipment, these molecular methods have been adopted at a surprisingly rapid rate by diagnosticians, researchers and industry. This is primarily due to their exquisite sensitivity and specificity compared to standard histological procedures. The rapid uptake has also been driven by the lack of other methods that are used commonly in diagnosis of animal viral

infections. Because of the absence of adaptive immunity in invertebrates (Fearon and Locksley, 1996), serology cannot be used to detect existing or prior infections with shrimp viruses. Virus propagation *in vitro* has had very limited application because of the absence of suitable cultured cell lines. Antibody-based methods for viral detection (eg. ELISA, indirect immunofluorescence or immunoperoxidase tests) appear to offer considerable potential but have yet to be explored adequately for most shrimp viruses (Durand et al., 1996; Takahashi et al., 1994; Kim et al., 1998). Among those various technique PCR is the most rapid, sensitive (Lightner and Redman, 1998; Lo et al., 1996b), specific (Tan et al., 2001) and reliable method (Tang and Lightner, 2000). PCR with specific primer sets, have been found useful in diagnosing WSSV infection even with healthy animals showing no signs of disease (Lo et al., 1996a; Takahashi et al., 1996; Maeda et al., 1998). Table-2 summarizes effectiveness of various diagnostic methods.

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Table-2: WSV surveillance, detection and diagnostic methods (GAA, 2003).

Methods	**Screening**				**Presu-mptive**	**Confir-matory**
	Larvae	Post larvae	Juveniles	Adults		
Gross signs	-	-	-	-	+	-
Bioassay	-	-	-	-	+	-
Direct dark-field light microscopy	-	-	-	-	+	+
Histopathology	-	-	-	-	++	++
Transmission electron microscopy	-	-	-	-	+++	+++
Antibody-based assays	?	?	+++	+++	+++	+++
DNA Probes - *in situ*	?	?	+	+	+++	+++
Polymerase chain reaction	+++	+++	+++	+++	+++	+++

-, the method is presently unavailable or unsuitable; ?, The method is available but untested; +, the method has application in some situations, but cost, accuracy, or other factors severely limits its application; ++, the method is a standard method with good diagnostic sensitivity and specificity; and +++, the method is the recommended method for reasons of availability, utility, and diagnostic specificity and sensitivity.

Although molecular genetic methods can provide rapid and accurate information on the infection status of shrimp, there is a considerable risk of misdiagnosis if the various parameters that determine reaction specificity are not carefully monitored and controlled. While the state-of-the-art PCR technology is being routinely applied in the neighbouring countries no such efforts have been made in Bangladesh except some

inconsistent and unpublished attempts by some commercial entrepreneurs. The present study was, therefore, carried out to optimise the screening protocol in Bangladesh context with regard to on-farm preparation of shrimp (*P. monodon*) tissues for rapid extraction of DNA, optimal thermal cycling condition and ingredient compositions for PCR. The simplicity and cost-effectiveness of this protocol rely on using a single-step DNA preparation without involving hazardous organic solvents as well as two pairs of oligonucleotide primers highly specific for WSSV gene. The objective of the study is-

1. To optimize the WSSV screening protocol in Bangladesh context with regard to on-farm preparation of shrimp (*P. monodon*) tissues for rapid extraction of DNA, optimal thermal cycling condition and ingredient compositions for PCR.

CHAPTER TWO

MATERIALS AND METHOD

CHAPTER TWO

2. MATERIALS AND METHOD

2.1 Study area and description of farms

Sample was collected from two different shrimp farms situated in a remote area of Satkhira district of Bangladesh where viral diseases occur frequently. Briefly, farms were situated in the same union Padmapukur under the Shamnagar Thana. Using visual observation farms were categorized as disease infected farm (F1) and uninfected farm (F2). Figure 3 shows the location of farms in the Satkhira district map and the district's position on Bangladesh map.

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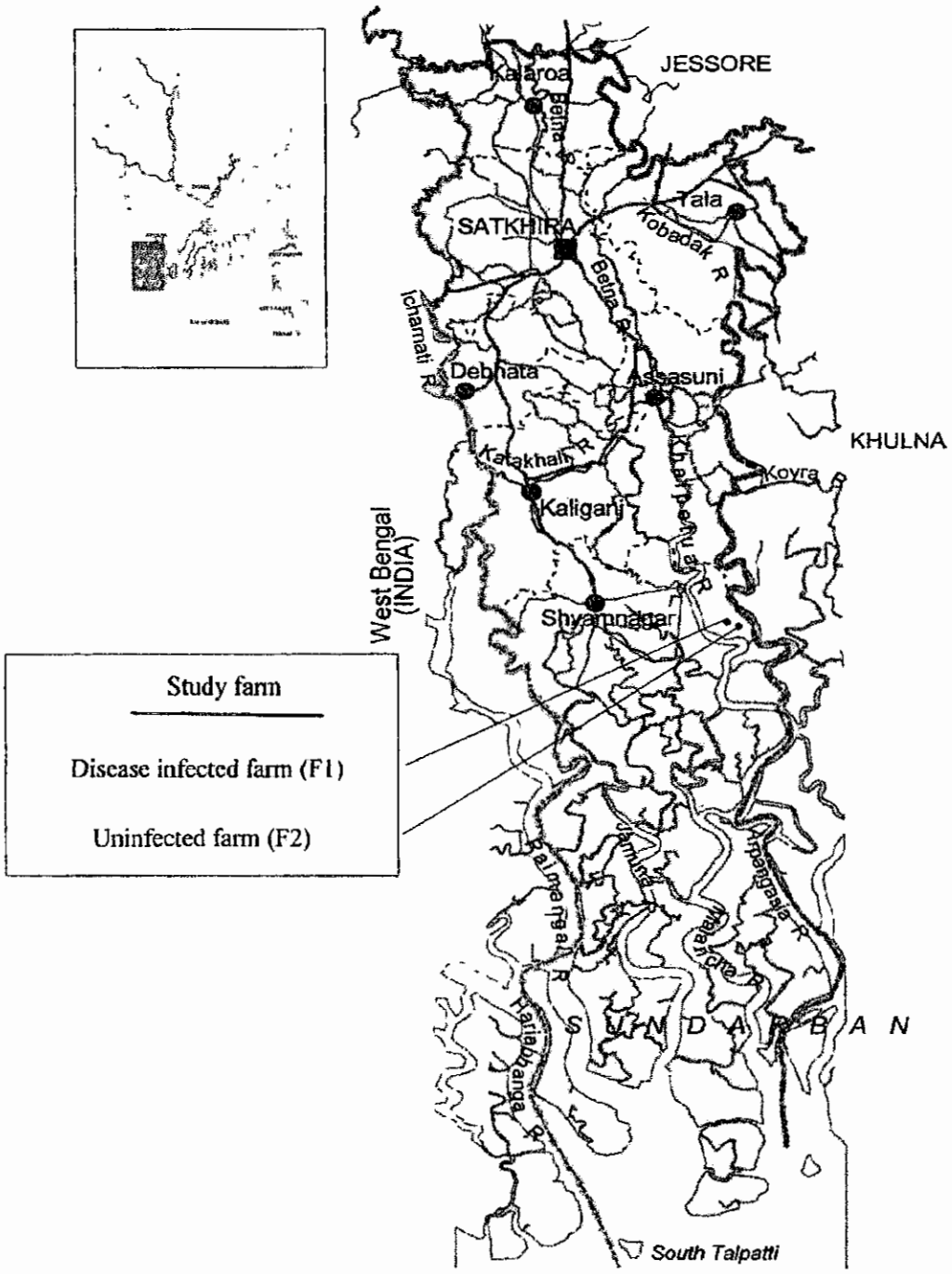

Fig. 3: Spatial distribution of two different shrimp farms from where samples were collected for the present study.

2.2 Experimental design and tissue sample collection

Schematic diagram of sampling procedure was presented in Fig. 4. According to the Fig. 4, eighteen tissue samples were collected from six live individual of three different species viz. Bagda (tiger shrimp; *Penaeus monodon*), horina chingri, *Metapenaeus monoceros* and crab, *Scylla serrata*. Weights of randomly selected individuals were above 30 gm for *Penaeus monodon*, above 10 gm for *Metapenaeus monoceros* and above 60 gm for *Scylla serrata*. Eighteen samples comprised with tissue of muscle, hepatopacrease and pleopod dissected from each individual. Each of the eighteen samples was collected using the same procedure and precaution. Before tissue sample collection, eighteen 1.5-ml tubes were labeled by a marker pen according to numerical order. Labeled tube 1, 2, 3, 4, 5, 6, 7, 8 and 9 were allotted for the tissue sample of muscle, hepatopancrease and pleopod collected from *Penaeus monodon, Metapenaeus monoceros* and *Scylla serrata,* respectively, harvested from farm F1. Next sequenced labeled tube 10, 11, 12, 13, 14, 15, 16, 17 and 18 were allotted for the tissue sample of muscle, hepatopancrease and pleopod collected from *Penaeus monodon, Metapenaeus monoceros* and *Scylla serrata,* respectively, harvested from farm F2. In the second step, 500µl of DNA_{ZOL}, a guanidine isothiocyanate based reagent (Invitrogen, Life Technologies, USA) was dropped in each tube. In the third step, dissection of specimens, individuals was taken on plastic dissection tray covered with thin aluminium sheet (Fig. 5). Using an electric balance (Mettler Toledo, B303-S, Germany) about 100 mg each of muscle, hepatopancreas and pleopod was dissected out from the specimens by sterilized, separate surgical blades to avoid cross contamination. Separate dissection tray was used for each individual. The dissected tissue samples were kept in the correspondingly labeled DNA_{ZOL} containing 1.5-ml tubes and stored in an ice box. Sample containing ice box

was then transported to the Quality Control Laboratory under Fisheries and Marine Resource Technology Discipline, Khulna University, Khulna, for the extraction of total genomic DNA. Approximately 70% ethyl alcohol was used as a disinfectant.

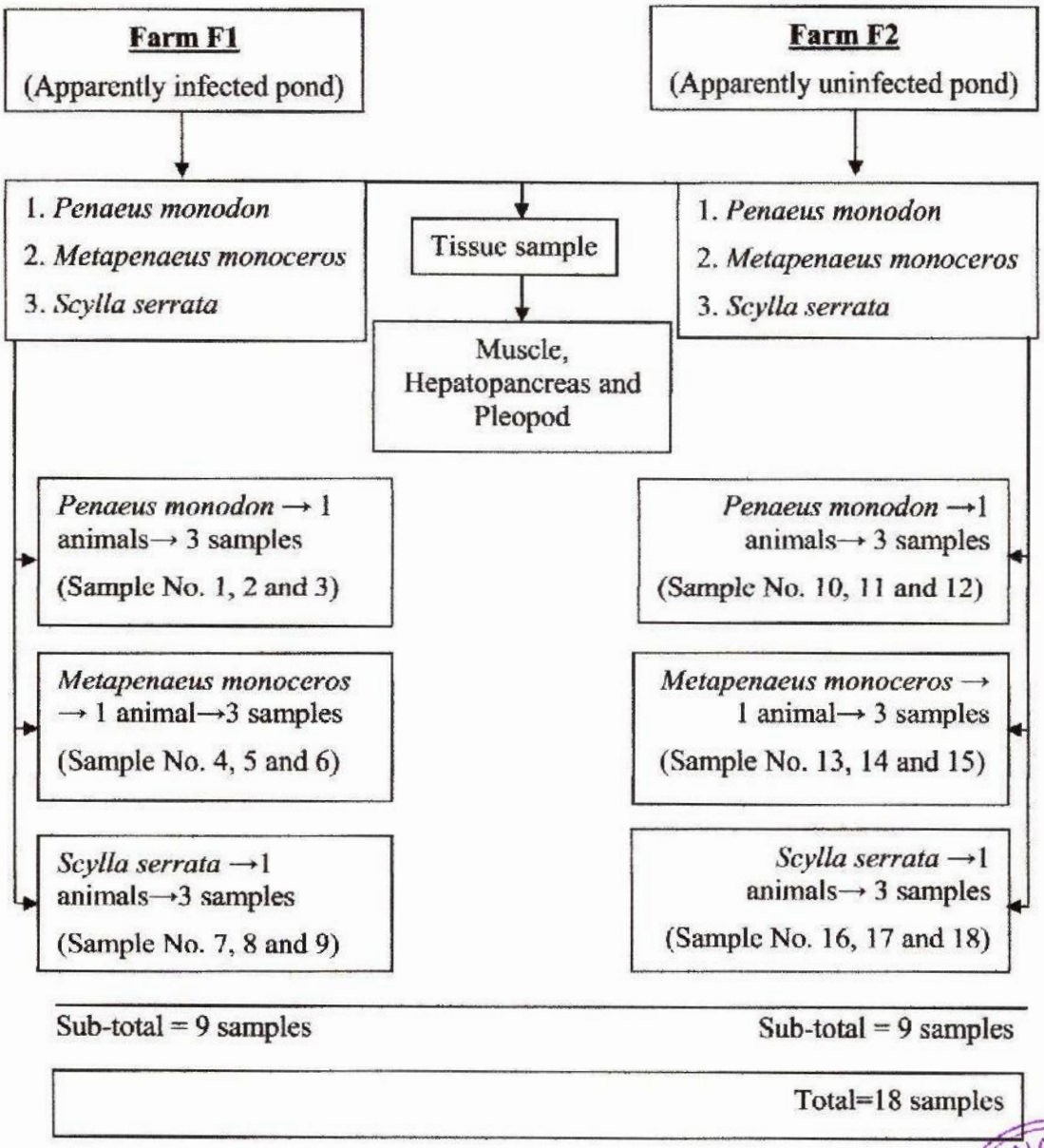

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Fig. 4: Sampling design for WSSV screening of shrimp and other aquatic animals.

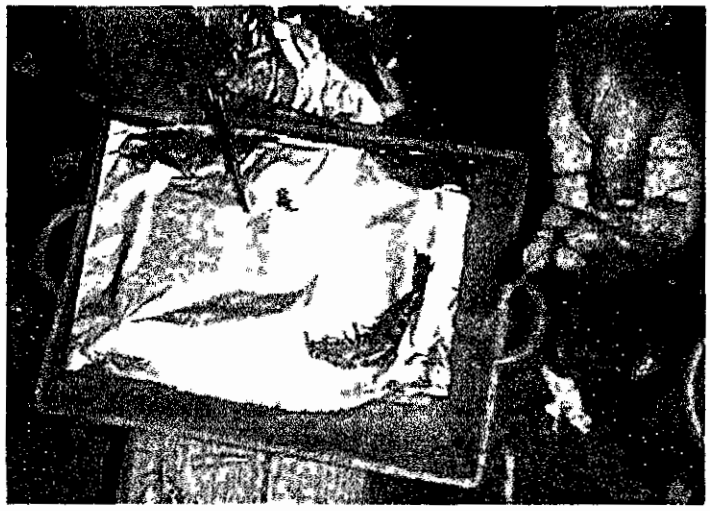

Fig. 5: Dissection of specimen: a plastic dissection tray covered with thin aluminum sheet, effective to avoid cross contamination.

2.3 Extraction of DNA

The tissue samples submerged in DNAzol were homogenized by sterilized tissue grinder and centrifuged at 13,000 rpm for 15 minutes with a tabletop centrifuge machine (Eppendorf, model: 5415D, Germany). For every sample separate sterilized tissue grinder was used. In this time, according to the sample number, eighteen 1.5-ml fresh tubes was labeled and loaded in a tube rack. At the end of centrifugation, two aquas layer appeared in every tube where supernatant layer contains extracted DNA and deposited layer comprised with organic debris. Based on the volume of supernatant layer and to avoid interruption of organic debris in the extracted DNA, three to four hundred micro-liters of supernatant from each tube were transferred to correspondingly labeled 1.5-ml tubes followed by addition of equal volumes of 100% ethanol. The two solutions were mixed well by inverting the capped tubes for a few

times and kept for 10 minutes. The mixture was then subjected to centrifugation at 13,000 rpm for 30 minutes. At this stage, DNA was deposited on the bottom of the tube. The pellet of DNA was obtained by removing supernatant alcohol. Supernatant alcohol was removed by transferring with micro-pipette and then any traces of alcohol were allowed to remove by keeping the tube opened at room temperature for 30 minutes. The DNA pellet thus obtained was washed with 500µl of 70% ethanol, kept for five minutes and centrifuged at 13,000 rpm for another 5 minutes. Finally, the pellet of DNA was obtained at the bottom of tube by removing supernatant alcohol following the previously described alcohol evaporating procedure. Dried DNA pellets were then dissolved in 100µl sterile distilled water in the same 1.5-ml tube and stored at -20°C in a deep refrigerator until subsequent use.

2.4 Quantitative and qualitative determinations of extracted DNA

2.4.1 Spectrophotometric determination of DNA concentration

The concentration of DNA samples were determined from the absorbance at A_{260} (absorbance at 260 nm) using a spectrophotometer (Hitachi U-1100 spectrophotometer, Japan) against distilled water blank. The protocol used in this experiment is designed for a single-beam spectrophotometer. Briefly, the concentration and the purity of DNA samples were determined by the following procedure (Ausubel, 1994):

Materials

1. DNA samples;
2. Single-beam spectrophotometer;

3. Matched quartz semi-micro spectrophotometer cuvette (1-cm-pathlength);
4. Micro-pipette, micro tips and tissue paper; and
5. Distilled water (neutral pH).

Procedure

Following are the procedure performed for one sample and the same procedure was followed for rest of the samples.

1. Spectrophotometer was warmed up before use.
2. Six hundred micro-liter of distilled water was taken in one cuvette;
3. Six hundred micro-liter of the DNA sample (dissolved in the distilled water) was taken in another cuvette;
4. Cuvette, containing distilled water, was loaded in the spectrophotometer;
5. Program was started and reading was taken at 325nm and then the instrument was turned to zero;
6. Blank cuvette was replaced by inserting the cuvette containing DNA sample;
7. Program was started and reading was taken at 260nm and then repeated at 280nm; and
8. The power supply was turned off.
9. Calculation;

(1) Double-stranded DNA concentration (C), µl/ml =Absorbance at A_{260}/0.020;

(2) Purity ($A_{260/280}$) = Absorbance at A_{260} / Absorbance at A_{280}.

sample's serial number, this was done for every sample separately. To avoid cross-contamination, separate micro-tips were used for every sample.

10. Approximately 0.5µl of DNA size marker was mixed with 0.5 µl of loading buffer on a paraflim and were poured into an another sample wells;
11. In this way, if remain, remaining wells of the gel was poured with 6 µl distilled water incorporating with 1.5 µl of loading buffer;
12. The electrophoresis chamber was covered with the lid;
13. The power supply was switched on, only volt was fixed at a constant value of 50, the machine was run until DNA migrated to the two-third of the gel towards the positive electrode (DNA was usually blue colored based on loading buffer);
14. The power supply was switched off and the lid of the electrophoresis chamber was removed;
15. The gel, still in its plastic tray, was removed from the electrophoresis chamber and placed on an ultraviolet (UV) transilluminator (Whatman, Biometra, Transilluminator TI 1, Göttingen, Germany);
16. The plastic tray was left off from the gel, smoothly, gel was placed again on the UV transilluminator;
17. The UV transilluminator was switched on, DNA banding pattern was visible;
18. The resulting banding pattern was captured by using the digital camera; and
19. The photograph was transferred to a desktop computer.

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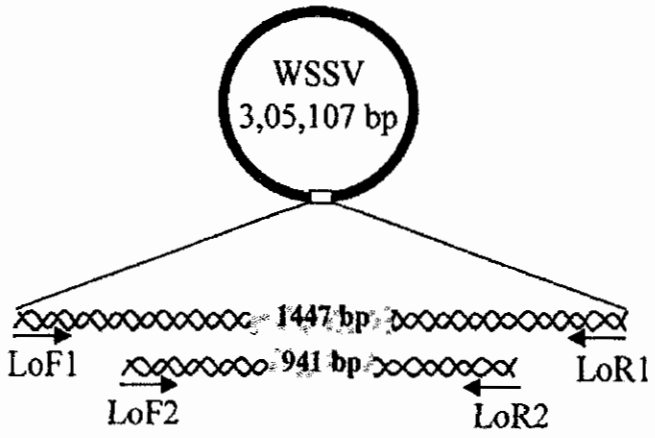
2.5 Description of primers

The DNA fragment to be amplified is determined by selecting primers. Primers are short, artificial DNA strands - not more than fifty (usually 18-25 bp) nucleotides that are complementary to the beginning and end of the DNA fragment to be amplified. They *anneal* (adhere) to the DNA template at these starting and ending points, where the DNA-Polymerase binds and begins the synthesis of the new DNA strand. In this study, two pairs of oligonucleotide primers named as LoF1, LoR1, LoF2 and LoR2 were procured from PROLIGO Primers & Probes, Australia. These primers correspond to a cloned, 1461-bp, *Sal*1-digested WSSV genome fragment as described by Lo et al. (1996a). These primers were designed in a way so that the LoF1 and LoR1 serve as outer primers acting on the WSSV genomic DNA while the remaining primers serve as inner, nested primers that act on amplified DNA resulting from the first pair of primers. The nucleotide sequences of these primers are shown in Fig. 6 with their estimated location and orientation with regard to a hypothetical WSSV genome.



LoF1: 5′-ACTACTAACTTCAGCCTATCTA-3′
LoR1: 5′-TAATGCGGGTGTAATGTTCTTA-3′
LoF2: 5′-GTAACTGCCCCTTCCATCTCC-3′
LoR2: 5′-TACGGCAGCTGCTGCACCTTG-3′

Fig. 6: The nucleotide sequences of two primer sets with their estimated location and orientation with regard to a hypothetical WSSV genome (Lo et al., 1996a).

2.6 Thermal cycler and PCR conditions

Using PCR machine (thermal cycler), by cycling the temperature, the target DNA is repetitively denatured and copied. A single copy of the target DNA, even if mixed in with other undesirable DNA, can be amplified to obtain billions of replicates (First, making two, then four, then eight, then sixteen, and so on, up to millions of copies depending on the number of cycles). Figure 7 shows a PCR machine. PCR takes place in three steps in the same vial: separation of the strands, annealing the primer to the template, and the synthesis of new strands all in less than two minutes. The first part of the process separates the two DNA chains in the double helix by heating the vial to 90-96^0 C for 30 seconds. But the primers cannot bind to the DNA strands at such high temperature, so the vial is cooled to 55-60^0 C. At this temperature, the primers bind or

"anneal" to the ends of the DNA strands, taking about 20 seconds. The final step of the reaction is to make a complete copy of the templates. *Taq* polymerase works best at around 75^0 C (the temperature of the hot springs where the bacterium was found), hence the temperature of the vial is raised. The *Taq* polymerase begins adding nucleotides to the primer and eventually makes a complementary copy of the template (if the template contains an A nucleotide, the enzyme adds on a T nucleotide to the primer, if the template contains a G, it adds a C to the new chain, and so on to the end of the DNA strand). This completes one PCR cycle. This cycle can be repeated 30 or more times. Each newly synthesized DNA piece can act as a new template producing about a million copies of a single piece of DNA within one hour.

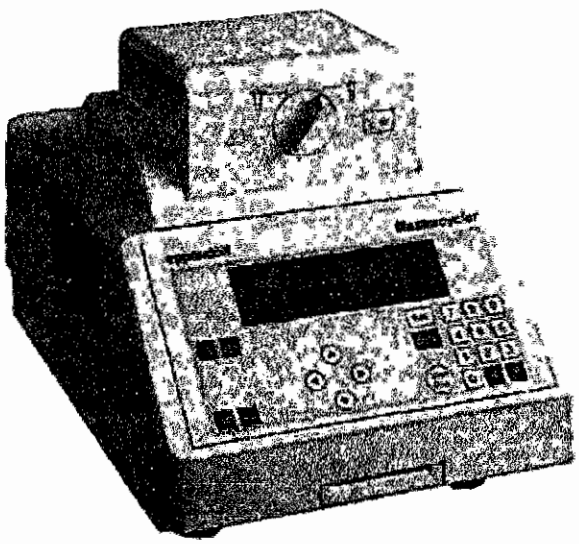


Fig. 7: A thermal cycler for polymerase chain reaction.

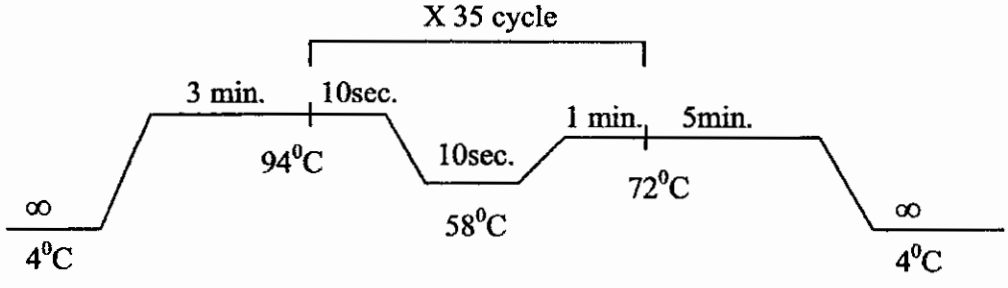
Amplified DNA fragments can then be isolated or separated (using agarose gel electrophoresis) on the basis of size by a process of electrophoresis, in which the fragments are drawn through a thin, flat gel by an electric potential that spans the length of the gel. The gel matrix impedes the larger DNA fragments to a greater degree than it does the smaller ones, and the fragments become distributed on the basis of size. At this point, DNA can be made visible by bathing the gel containing ethidium bromide, making the DNA on the gel intensely fluorescent when irradiated with ultraviolet light.

In the present study, all PCR assays were carried out in 20-μl reaction mixtures with appropriately diluted shrimp tissue DNA or PCR products (for second-step nested PCR) as the templates using 0.2 ml thin-walled PCR tubes. PCR was performed using an automated thermal cycler (Mastercycler Personal, Eppendorf, Hamburg, Germany). Initial denaturation of template DNA was carried out for 3 min. at 94°C, followed by 35 cycles of denaturation for 30 sec. at 94°C, 10 sec. of annealing at 58°C, and 1 min. of extension at 72°C. The last extension step at 72°C was extended for 5 min.

Briefly, following are the structured program performed in the thermal cycler:

	CNTRL		BLOCK	Temperature control for the block
	Lid=105°C			Sets heated lid to 105°C
	NO WAIT		AUTO	NOWAIT= Program is started immediately. AUTO= Lid heating is switched off automatically when temperature (4°C) is maintained for more than 5 minutes.
1	T=94°C		00:03:00	Maintains 94°C for 3 mm. (initial denaturation)
2	T=94°C		00:00:30	Maintains 94°C for 30 sec. (denaturation)
3	T=58°C		00:00:10	Maintains 58°C for 10 sec. (annealing)
4	T=72°C		00:01:00	Maintains 72°C for 1 mm. (elongation)
5	GOTO 2		REP 35	Repeats 35 times from program step 2 onwards. The total number of cycles is 36.
6	T=72°C		00:05:00	Maintains 72°C for 5 mm. (final elongation)
7	HOLD	4°C	ENTER	Cools down to 4°C and maintains temperature.
	End			Program end.

The thermal cycling profile can be schematically shown as follows:



The components of the reaction mixture and their compositions in a 50µl reaction mixture together with final concentration for the first-step PCR (non-nested PCR) was as follows:

Component	Final volume	Final concentration
1. A *Taq* Polymerase (5units / µl; AmpliTaq™ DNA polymerase, Roche Molecular System, USA)	0.2 µl	1 units / 50µl
2. PCR buffer (Pre-combined with 15mM $MgCl_2$; 10XAmpliTaq™ buffer, Roche Molecular System, USA)	5 µl	1X
3. Enhancer * (10XEnhancer, 2.5mM, Roche Molecular System, USA)	3.75 µl	0.75X (1.87mM)
4. dNTP Mix. (dNTP Mix.; 2.5mM, Roche Molecular System, USA)	4µl	0.2mM each dNTP
5. Primers; LoF1 and LoR1 (10-pmole / µl)	1 µl	0.2-pmole/ µl
6. Template DNA	5 µl	<0.5µg/50 µl
7. Nuclease-free water (Invitrogen, Life Technologies, USA)	Upto 50 µl	

* Enhancer was used as an optional component.

In both PCR running, control reactions consisted of: 1) positive control: reaction mixtures with DNA extracted from moribund shrimp sample reported to be highly WSSV positive as diagnosed by a commercial testing agent in Madras, India (personal comm.); 2) negative control, reaction mixtures with DNA extracted using the same procedure from a phylogenetically distinct species, Asian catfish (*Clarias batrachus*); 3) reagent control: reaction mixtures with no template DNA. However, in the present experiment both experimental template (samples of DNA) and three control reactions were assigned as template. A Master Mix of reagents (water, buffer, dNTPs, primers and enzyme) for all templates was prepared first, then aliquoted to individual PCR tubes. Mixture was prepared by sitting materials on ice in a laminar flow. In details, the following procedure was followed to make reaction mixture for a total of 21 templates (18 experimental DNA sample and 3 control reactions).

Materials

1. Automated thermal cycler;
2. Laminar flow;
3. Ice in a box designed to be able to hold PCR tubes and other reagents;
4. Twenty one piece of 0.2-ml PCR tube and one piece 0.5-ml tube;
5. Reagents and template DNA as described above;
6. Approximately 70% ethyl alcohol; and
7. Micro-pipette, micro tips, marker pen and tissue paper;

Procedure

1. Laminar flow was warmed up;
2. All the working area in the laminar flow and hand was disinfected by 70% ethyl alcohol;
3. Ice box was placed in the laminar flow;
4. All reagents was sited on the ice;
5. Twenty one of 0.2-ml thin walled PCR tube was placed on the ice in which eighteen was labeled as a pair for each of the samples / templates and rest three was labeled by the word P, N and R for positive control, negative control and reagents control, respectively;
6. One 0.5-ml tube was labeled as 'Mix' and placed in the ice box. Reaction mixture was prepared in this tube;
7. Approximately 2µl dilution from each template DNA containing tube was transferred to their correspondingly labeled 0.2-ml PCR tube. Again, 2µl distilled water was transferred to the 'R' labeled 0.2-ml PCR tube;
8. A master mix of 378µl was prepared in the "Mix" labeled tube according to the instructions described in the Table-3. (Since, 2µl of template has already been transferred to the 0.2-ml PCR tubes; rest of the amount of the reaction mixture for each template will be 18µl. Therefore, the volume of master mix is 21 X 18=378µl)
9. The automated thermal cycler was switched on;
10. The program was loaded and hold on;
11. The master mix was aliquoted to each of the 21 template containing 0.2-ml PCR tube. Therefore, the final volume of the reaction mixture was 20µl. (Note: After

the addition of primer and enzyme in the reaction mixture it should be transfer to the thermal cycler immediately otherwise unexpected band may produce. Thus program of the thermal cycler was loaded before initiating this step);

Table-3: Compositions for a 378µl master mix.

Components	Amounts (µl)
Water	252.4
Buffer*	42.0
dNTP mix	33.6
Enhancer	31.5
LoF1 primers	8.4
LoR1 primers	8.4
Taq Polymerase (enzyme)	1.71
Total	378.0

* Prior to use, the buffer was completely thawed and thoroughly vortex.

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12. The 0.2-ml thermoblock of the thermal cycler was loaded with the reaction mixture containing PCR tubes (Note that thermoblock is water sensitive therefore any traces of water attached at the outer side of the PCR tube was removed by tissue paper) ;
13. The heated lid of the thermal cycler was closed;
14. Program was run / started by pressing the key;
15. After the completion of 35 cycle; thermal cycler hold on at 4^0C, and then the thermal cycler was switched off;
16. The thermoblock was unloaded and the PCR product was stored at -20°C in a deep refrigerator until subsequent use.

Following the completion of the thermal cycling, a sample of 10 µl from each PCR products was analyzed electrophoretically by running on a 1% agarose gel following the procedure described in the "Agarose gel electrophoresis determination of DNA integrity" section of the "Materials and methods" chapter and the amplified product size was determined by comparison with a DNA size marker (Marker 2: λ/*Hind* III • *Eco*R I double digest; Nippon Gene, Japan).

However, after the completion of the first round of PCR amplification, preparation was undertaken for the second round of amplification (nested PCR). In this case, instead of crude DNA an aliquot of 1µl from the first PCR products was used as the DNA template together with the nested primer pair, LoF2 and LoR2. The rest of the PCR conditions were the same as described above. An aliquots of 1µl of the second step PCR products were analyzed by 1% agarose gel electrophoresis following the same procedure described for first step PCR products, visualized under UV transillumination and the amplified product size was determined by comparison with a DNA size marker (Marker 2: λ/*Hind* III • *Eco*R I double digest; Nippon Gene, Japan)

CHAPTER THREE

RESULTS AND DISCUSSION

CHAPTER THREE

3. RESULTS AND DISCUSSION

Diagnostic screening for the presence of WSSV by PCR starts with extraction of the DNA materials to be amplified. Representative samples are of primary importance in the analysis of pathogens. In the present experiment, the PCR was performed without screening and removing detritus to avoid target loss (Tengs et al., 2000) and to reduce extra time needed assuming that PCR is able to amplify target DNA if present in the sample. In the present experiment, a single-step DNA extraction solution DNAzol was used to extract total genomic DNA from different tissues of *Penaeus monodon*, *Metapenaeus monoceros*, and *Scylla serrate* and the electrophoretic patterns of the extracted DNA samples are shown in Fig. 8 and Fig. 9. As shown in those figures, most of the tissue samples produced a DNA band at about 20-kbp position with varying degree of intensity and smearing pattern. The smearing indicated the presence of numerous smaller size DNAs resulting from the mechanical shearing of the centrifugation process and pipetting involved. The smearing might contain cellular RNAs as well which, however, should not interfere with amplification of DNA. The final yield of purified DNA ranged between 0.87 to 1.32 mg with the highest yield being from hepatopancreas and the lowest from pleopod tissues. The average $A_{260/280}$ ratio of the extracted DNA materials was 1.68 ± 0.22, which was quite enough for semi-preparative diagnostic PCR (Sambrook et al., 1989).

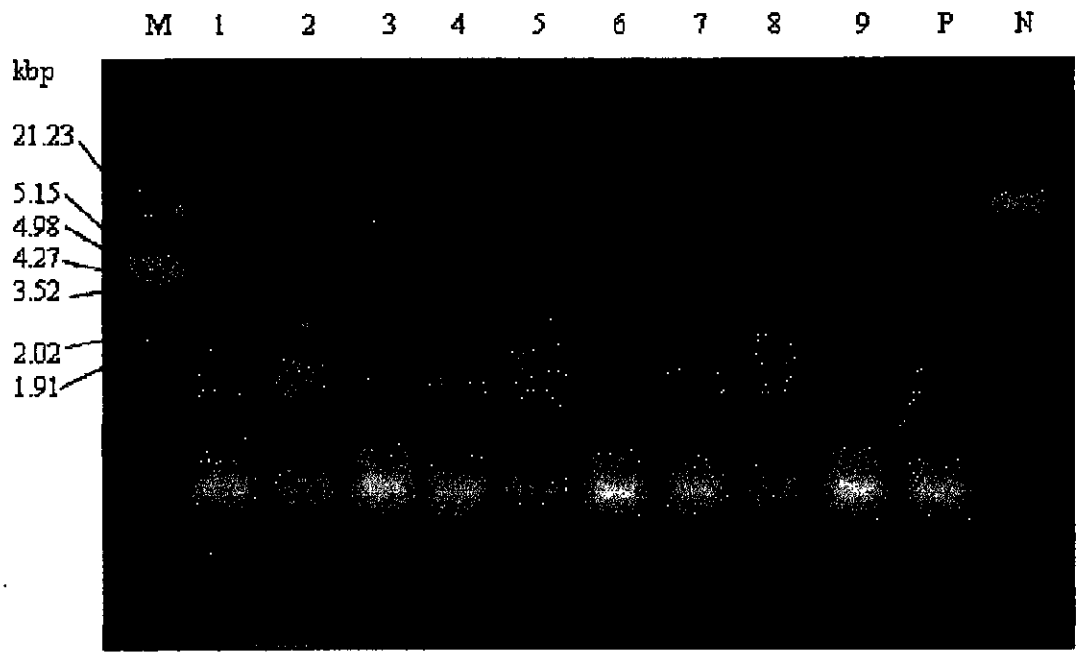

Fig. 8: UV visualization of total genomic DNA extracted from the animals collected from the "farm F1" following electrophoresis in a 0.5% agarose gel. Lanes 1, 2, 3, 4, 5, 6, 7, 8 and 9 are representative of the sample in accordance with the sample number respectively. Lane M, P and N represented 1 kb plus DNA ladder / marker, DNA from positive control and DNA from negative control, respectively.

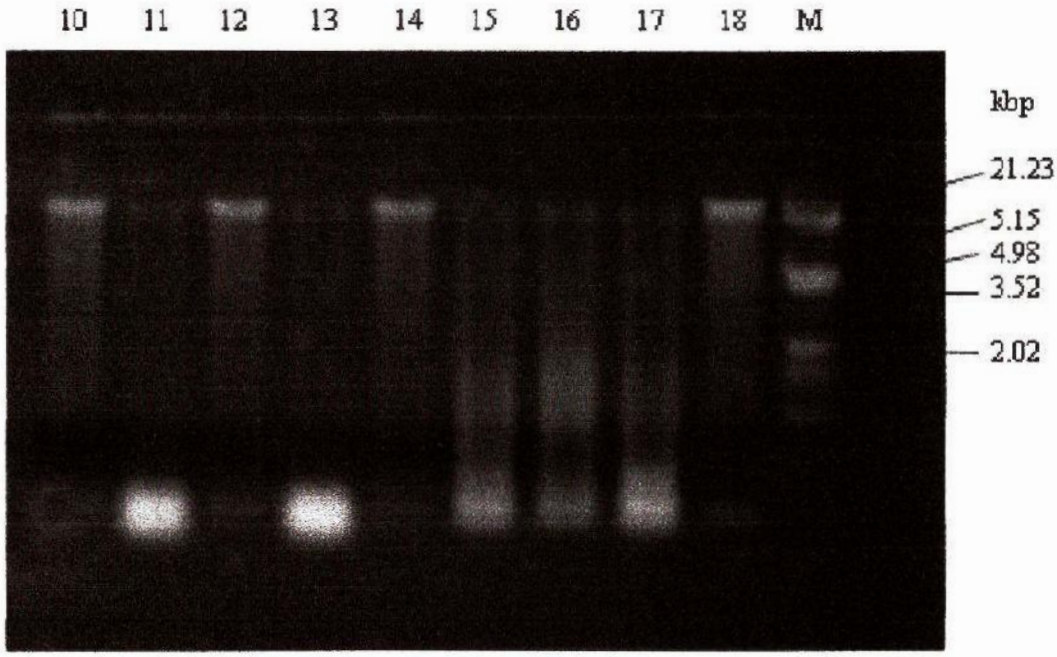

Fig. 9: UV visualization of total genomic DNA extracted from the animals collected from the "farm F2" following electrophoresis in a 0.5% agarose gel. Lanes 10, 11, 12, 13, 14, 15, 16, 17 and 18 are representative of the sample in accordance with the sample number respectively. Lane M, 1 kb plus DNA ladder / marker.

Efficient sample treatment methods are needed to fully exploit the potential of the PCR technique to detect pathogens due to the presence of PCR inhibitors (Rossen et al., 2002). Consequently, several treatments to concentrate DNA for the PCR detection of WSSV in shrimp and to remove PCR inhibitors have been suggested e.g., lysozyme, proteinase K, detergents, centrifugation and filtration (Kroll, 1993), use of proteinase K and organic solvents such as phenol, chloroform and iso-amyl alcohol for deproteinizing cell digests (Lo et al.,1996a; Nonaka et al., 1998).

However, guanidinium isothiocyanate, the main ingredient of DNA_{ZOL} is a strong denaturant of proteins capable of dissolving cytoplasmic and nuclear membranes thus it is talent to solubilize all cellular components yet, allows selective precipitation of DNA in the presence of alcohol as well as improves the release of DNA into solution (Cox, 1968). Therefore, the present method of extraction using DNA_{ZOL} provides an advanced isolation method that combines both reliability and efficiency with simplicity of the isolation protocol as well as it is a fast method and permits isolation of genomic DNA from a large number of samples in less than an hour following tissue grinding compared to previously published or commercially available PCR based methods for the detection of WSSV (Lo et at., 1997). It means that it facilitates the detection of WSSV without a long time requirement and high cost involvement, without a danger of false positivity originating from the contamination during sample preparation. Although beyond the scope and necessity of the present work, the interfering RNA molecules can be easily removed by an additional step of RNAse treatment followed by washing with ethanol. DNA isolated by this method can thus be used for applications such as southern analysis, dot blot hybridization and molecular cloning.

In general, two pairs of oligonucleotide primers are required for a nested PCR amplification. The outer primer pair (LoF1 and LoR1) used in this study amplifies a 1447-bp fragment from the WSSV genome of 305107-bp while the second pair of primers (LoF2 and LoR2) amplifies a 941-bp internal sequence to the first set of primers. The electrophoretic patterns of the first round PCR products using the first primer set are shown in Fig. 10 and 11. From the Fig.10, DNA samples extracted from the animals harvested from the farm F1, a 1447-bp DNA band indicative of the presence of WSSV, observed in every sample and only in the positive control sample suggesting that the tested shrimp specimens were very highly viral infected. However, from the Fig.11, DNA samples extracted from the animals harvested from the farm F2, a 1447-bp DNA band was observed only in the positive control sample suggesting that the tested shrimp specimens were either free from the virus or the infection level was very low. In both case, the appearance of 1447-bp fragment in the positive control sample did not result from PCR artifacts as no such band could also be obtained from the sample containing unrelated, vertebrate DNA as the template or from the sample lacking the template DNA. It also determines the relative accuracy of this PCR-based method.

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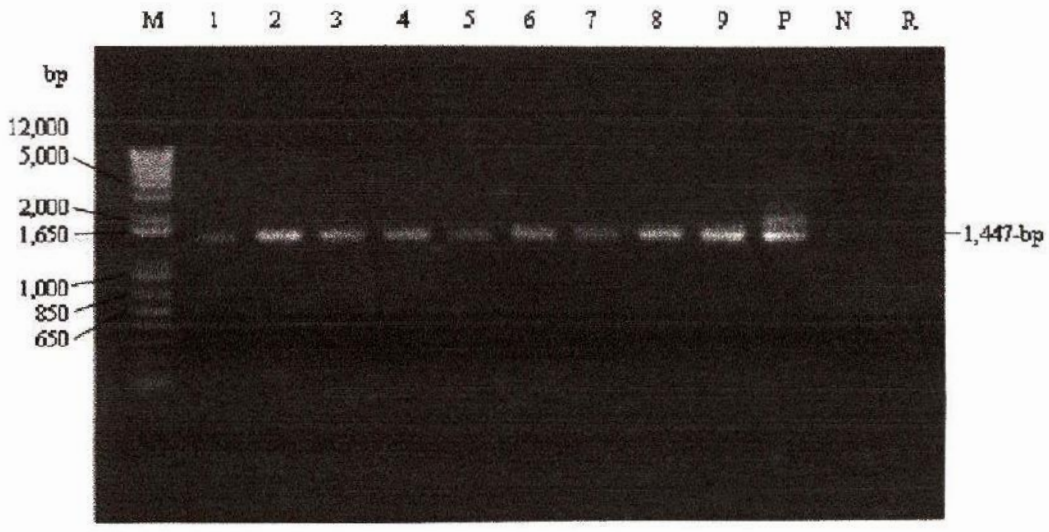

Fig. 10: UV visualization of the first step PCR product following electrophoresis in a 1% agarose gel. Lanes 1 through 9 represent samples as cited in Fig.8 while lane P, N and R correspond to positive control, negative control and reagent control, respectively. Lane M, a DNA size marker, marker 2.

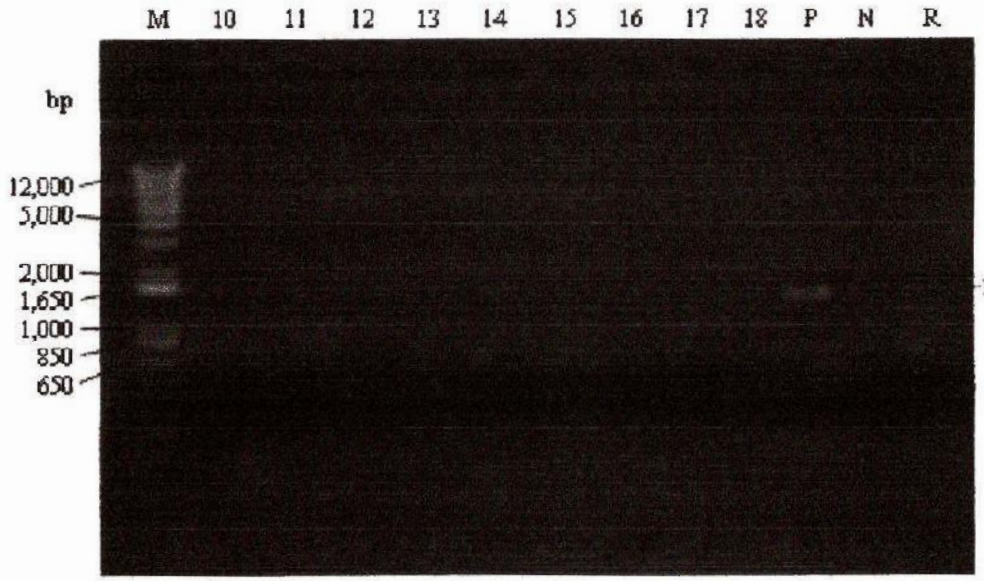

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Fig. 11: UV visualization of the first step PCR product following electrophoresis in a 1% agarose gel. Lanes 10 through 18 represents samples as cited in Fig.9 while lane P, N and R correspond to positive control, negative control and reagent control, respectively. Lane M, a DNA size marker, marker 2.

Following the first round of PCR, a second round of PCR amplification was conducted using the amplified products from the first PCR reaction as template with the nested primer pair, LoF2 and LoR2. Subsequent agarose gel electrophoresis of the nested PCR products demonstrated the presence of WSSV in all the tested samples including the positive control one, as evidenced by an expected DNA fragment of 941-bp with varying degree of intensity (Fig.12 and 13). Visualization of a specific DNA fragment corresponding to the expected 941-bp in shrimp samples including the positive control but not in the negative control and reagent control (no template DNA) confirmed the existence of WSSV in the samples tested from two different farms.

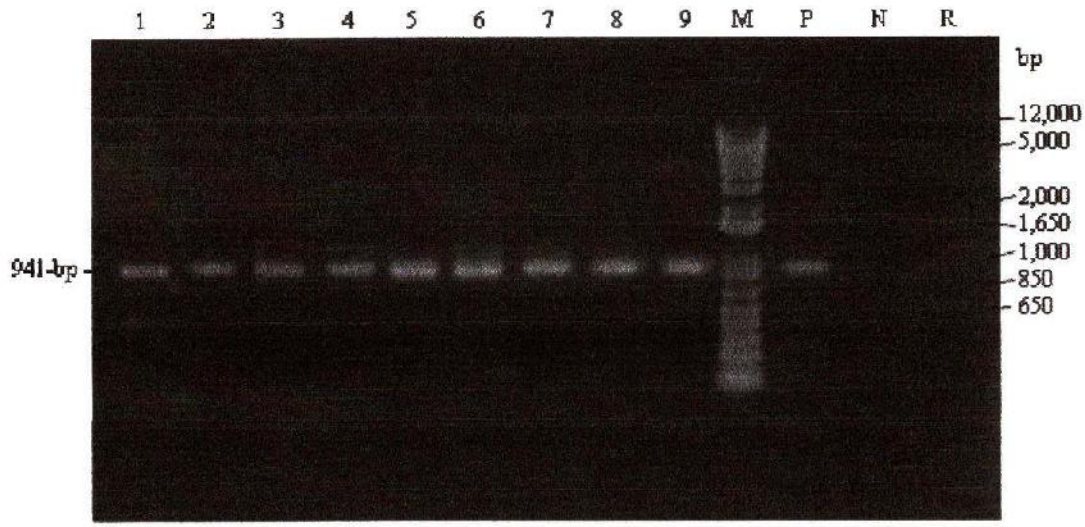

Fig. 12: UV visualization of the second step PCR product (nested PCR) following electrophoresis in a 1% agarose gel. Refer to Fig. 10 for description of the samples in each lane.

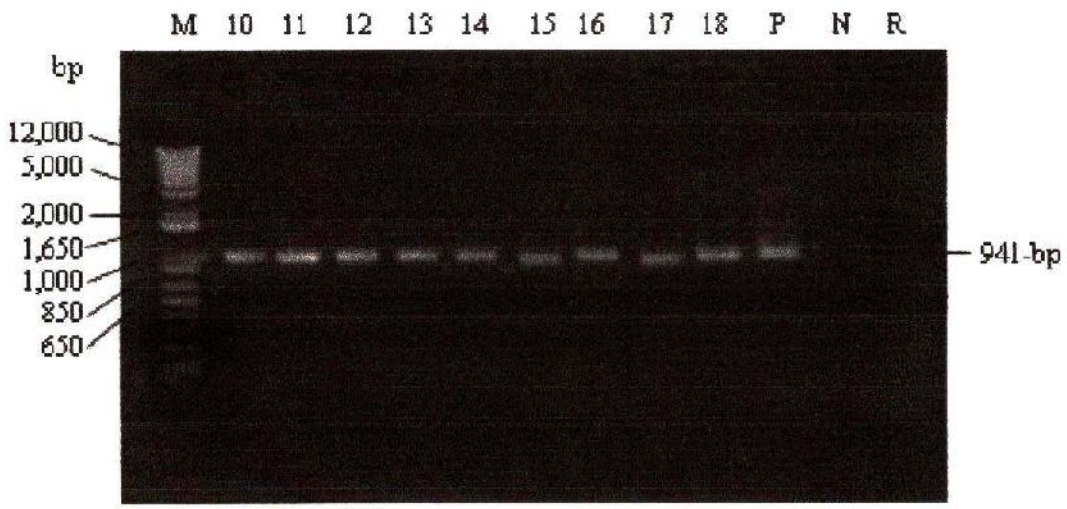

Fig. 13: UV visualization of the second step PCR product (nested PCR) following electrophoresis in a 1% agarose gel. Refer to Fig. 11 for description of the samples in each lane.

The PCR protocol exhibited specificity and high sensitivity towards WSSV since no product was generated when the same primer set was used to amplify negative control sample in both non-nested and nested PCR and the specificity of the protocol was further demonstrated when only the expected PCR product was generated using WSSV-infected sample DNA as template in the nested amplification step. In the present study, for the animals of farm F2, the contamination of the tested shrimp samples with WSSV could only be confirmed by a two-step, nested PCR. This is because the WSSV infection in this stage falls into light or carrier stage characterized by a low viral load of a few hundred viral copies, detectable only by sensitive methods such as nested PCR (Tan et al., 2001).

A single step PCR with moribund shrimp or shrimp having clinical signs of disease is sufficient to amplify enough WSSV-specific DNA to be visualized following electrophoresis (Tan et al., 2001), has been supported by the present study as a DNA band from a single step PCR product was only observed in case of farm F1. However, for healthy shrimp with no clinical syndrome a second round of PCR (nested PCR) is required to confirm the presence of virus as nested PCR has been observed to increase the sensitivity of detection of WSSV by 103 to 104 fold (Lo et al., 1996a). Detecting WSSV in dead or dying shrimp with obvious sign of infection is of no significance as far as viral quarantine is concerned. Since, the present experiment was intended to establish the protocol that could be routinely applied to detect virus in various aquatic inhabitant of shrimp farm in all life stage as brood to offspring, present experiment purposively select both apparently healthy shrimp and apparently infected shrimp. The sampling site has had a consistent and sporadic history of crop destruction due to viral disease. In case of farm F2, at the time of sampling the shrimp appeared healthy

with no signs of macroscopic white spots or abnormalities suggesting an early or latent stage of viral infection whereas results from farm F1 suggesting an mature stage of viral infection. Here the route of infection may be various as because the farmer have no data on the brooders, larvae they used, food, water and plankton.

The nature of WSSV outbreaks and mortality pattern has become most unpredictable and health management effort has become chance. Effective prevention and control methods are urgently needed to control the spread of the WSSV disease. Knowledge on the presence of viruses through diagnostic PCR in post larvae intended for stocking in ponds will greatly help the farmer to understand the risk associated with stocking infected larvae and appropriate management measures for their culture. To this end, the methodological details presented in this thesis for assessment of viral presence by PCR that was not dependent on mortality rate and clinical signs will aid farm managers in decision making in culture management, perhaps allowing proactive steps to be taken to reduce losses when WSSV strikes. The results suggest that a PCR screening of WSSV infection for shrimp brood and larval stocks and other potential virus carriers could be an effective tool in fight against the deadly virus that has almost devastated the shrimp industry of the country.

CHAPTER FOUR

CONCLUSION

CHAPTER FOUR

4. CONCLUSION

The management of viral diseases particularly WSSV in shrimp aquaculture has been a challenge due to the lack of highly sensitive detection methods. Until very recently, viral detection in shrimp aquaculture was primarily based on biological and histological methods, including in situ hybridization using virus-specific gene probes. The detection limits for these methods are low compared to that for PCR, and the process is time-consuming. These challenges have been overcome to some extent over the past few years with the development of screening methods based on conventional PCR technology for WSV screening of broodstock animals in quarantine hatchery facilities and of the postlarvae used in stocking ponds. Traditional PCR sometimes produces false-negative findings, resulting in a putatively negative animal that served as a reservoir for the virus. Although conventional PCR is the most sensitive method, it is unable to detect the presence of a single copy of a viral genome in the infected tissue. This ability is critical for the development of a specific-pathogen-free shrimp-breeding program, for the screening of broodstock held in quarantine facilities, and for the detection of viral pathogens in commodity shrimp imported into or exported from one country to another. In addition, due to the lack of a continuous cell culture system for penaeid shrimp, quantification of viruses infecting shrimp has been severely hampered. To overcome these problems, present protocol based on a two-step nested PCR technique could be an effective way against this fetal virus.

By using the same PCR protocol, two strategies were used in the present experiment viz., 1) sample collection from visually infected and uninfected pond; and 2) application of protocol in two other host species, where muscle, hepatopancrease and

pleopod were collected as tissue sample. In every case positive control shows its positivity accurately on the other hand negative control and other regent control shows the same accuracy. Finally WSSV was identified in every disease sign bearing and nonbearing *Penaeus monodon* even in *Scylla serrata* and *Metapenaeus monoceros* implies that this protocol able to investigate in a wide carrier range. From sampling to UV illumination, protocol consumes 4-5 hrs. Again, simplicity of the protocol: one step DNA extraction, reaction mixture preparation, two step PCR operation, gel electrophoresis, and finally UV illumination is so simple and effective than any microbial and other published PCR protocol in Bangladesh. Moreover, ability of identifying a single piece of target DNA with high specificity, easiness, and cost-effectiveness of this method do not depend on using a patented WSSV detection kits.

Therefore, in context of Bangladesh, this could be an ideal protocol for PCR screening of WSSV infection for shrimp brood and larval stocks and other potential virus carriers.

CHAPTER FIVE

REFERENCES

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