Multiplex PCR Protocol Development for Rapid Screening of White Spot Syndrome Virus (WSSV) in Shrimp

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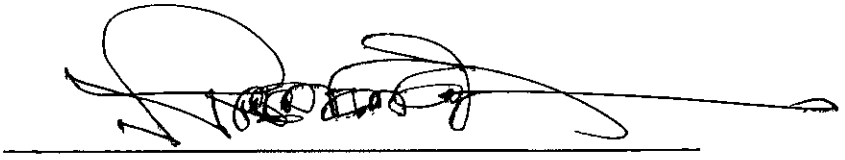

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Declaration

Multiplex PCR Protocol Development for Rapid Screening of White Spot Syndrome Virus (WSSV) in Shrimp

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

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Dedicated to

My Beloved Parents

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Abstract

One of the most serious problems confronted by the shrimp farming industry is the disease caused by white spot syndrome virus (WSSV). For definite diagnosis and certification of the virus, nested polymerase chain reaction (PCR) technology based imported kit has been used that are expensive, time consuming and vulnerable for cross-contamination. Therefore, this study aimed to develop a non-stop, faster,single tube, single step multiplex PCR protocol using locally available reagents for the simultaneous detection of WSSV with its host(shrimp) as internal positive control.In the multiplex PCR procedure, two sets of WSSV specific primer and one set of shrimp specific primerwere tested. A total of eight (08) primer combinations based on the WSSV specific primer (Lo F1R1, Lo F2R2, Lo F1R2, Lo F2R1, Lo F1R1F2, Lo F1R1R2, Lo F2R2F1, Lo F2R2R1)being incorporated with one (01) pair of the shrimp specific primer (16S rRNA) were evaluated and optimized in a single tube to find out the most suitable protocol. All the primer combinations yielded their expected amplicon sizes with stronger band resolution intensity. Any one of the developed multiplex PCR protocols with its reagent composition and thermal cycling conditions could be applicable for routine diagnosis of the virus with simultaneous presence of the host. Among the protocols, it was suggested to follow the protocol based on 16S rRNA and Lo F2R2 considering time, cost, effort, sensitivity and specificity. If sensitivity is not the major problem, this study recommended the multiplex protocol with the primer combination of 16S rRNA and Lo F1R1. The study also recommended one of the semi nested multiplex PCR protocols with the primer combination of 16S rRNA and Lo F1R1R2 (combination-6) that would detect both of the target DNA as well as the severity of the infection.The developed multiplex protocols could be used for saving time and effort, eliminating chance for cross-contamination and it ensured thirty five (35) times reduced cost in comparison with a renowned imported kit named 'IQ2000 WSSV detection System' certified by Office International des Epizootics (OIE).

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List of Abbreviations

Abbreviation	Meaning
bp	Base pair
PCR	Polymerase Chain Reaction
WSSV	White Spot Syndrome Virus
DNA	Deoxyribonucleic acid
dNTPS	Deoxyribonucleotide triphosphates
OIE	Office International des Epizootics
FAO	Food and Agricultural Organization
FRSS	Fisheries Resource Statistical Survey
USAID	United States Agency for International Development
rRNA	Ribosomal Ribonucleic Acid
mtDNA	Mitochondrial DNA
IHHNV	Infectious Hypodermal And Hematopoietic Necrosis Virus
MBV	Monodon Baculovirus
YHV	Yellow head Virus
GAV	Gill-associated Virus
TSV	Taura Syndrome Virus
WSD	White Spot Disease

Chapter One

INTRODUCTION

1. Introduction

1.1 Background of the study

Aquaculture is one of the fastest growing sectors in the world. It is mainly contributing to the increase in export earning, income, employment generation and food and nutritional security for the developing countries. In the last fifty years the production of aquatic animals increased with an average rate of 8.8% while the world wide population grows at the rate of 1.7% per year (FAO, 2012). World aquaculture production attained another all-time high of 90.4 million tonnes (live weight equivalent) in 2012 (US$144.4 billion), including 66.6 million tonnes of food fish (US$137.7 billion) which rose by 5.8 percent to 70.5 million tonnes in 2013 (FAO, 2014). Bangladesh is one of the resourceful countries with its wide range of aquatic bio-diversities and has achieved remarkable progress in the fisheries sector since its independence in 1971.

Fish play an important role among the population in Bangladesh as indicated by the proverb *machte bhate Bangali* (fish and rice make a Bengali). Situated in the delta of the Brahmaputra, Meghna, and Ganges rivers, the climate, water, and soil conditions of Bangladesh are favorable for inland fisheries and aquaculture. Fisheries sector contributes 4.20% to the national GDP and almost one-fourth (22.23%) to the agricultural GDP by attaining a total production of 32.62 MT in the last financial year 2012-13 (FRSS, 2014). Bangladesh is widely recognized as one of the most suitable countries in the world for prawn, *Macrobrachium rosenbergii* and shrimp, *Penaeus monodon* farming because of its vast resources of shallow water bodies which provide a unique opportunity for production.

Shrimp is the second largest export item in Bangladesh after readymade garments. At present, the area under tiger shrimp production is 188,046 hector and it alone adds to the economy of Bangladesh more than 70% of the total export earnings from all the agro-based products and contributes 2.73% of total export earnings (FRSS, 2014). In the last fiscal year (2012-13), export earnings from the fish and fish products were nearly US$ 620 million, of which shrimp alone contributed about 80% (US$ 500) of the total value (FRSS, 2014). Shrimp sector not only contributes to earn valuable foreign exchange, but the sector alone directly employs in excess of 600,000 people (Karim *et al.*, 2011; USAID, 2006). The rapid expansion of shrimp farming over last decade and its contribution to foreign earnings has been quite remarkable.

Despite of all glorious achievements, the aquaculture industry still battles with serious constraints. The sustainability of this important sector largely depends on addressing a number of challenges, most notably the occurrence of disease that is severely undermining the productivity of the sector and leading to severe economic loses. Bacterial and viral diseases along with some fungal and parasitic diseases are major problems in the shrimp aquaculture industry which cause a significant drop in shrimp production resulting in tremendous losses among shrimp farmers.

Viruses are considered to be the most important pathogens in shrimp and viral diseases signify a permanent threat to the shrimp aquaculture which has contributed to massive economic losses in the aquaculture industry. Almost more than 20 viruses have been reported to infect shrimp (Bonami, 2008) within which nine are responsible for substantial economic losses. These include white spot syndrome virus (WSSV), infectious hypodermal and hematopoietic necrosis virus (IHHNV), monodon baculovirus (MBV), hepatopancreatic parvovirus (HPV), yellowhead virus (YHV), gill-associated virus (GAV), Taura syndrome virus (TSV), infectious myonecrosis virus (IMNV), and Mourilyan virus (MoV).

The white spot syndrome virus (WSSV) that causes white spot disease (WSD) has been considered the most crucial one. The virus is by far the most devastating pathogen threatening the shrimp industry worldwide. The first reported epidemic due to WSSV was found from shrimp farms in Taiwan in 1992 (Chou *et al.*, 1995). In Bangladesh, WSD or WSSV is now endemic and is generally regarded as one of the most important constraints to the industry's sustainability and further expansion of the industry (Karim *et al.*, 2011). White spot syndrome was first recorded in Cox's Bazar in 1994 (Larkins, 1995) and since then the shrimp farming industry in Bangladesh has been seriously affected by WSSV resulting in substantial loss.

WSSV is an enveloped, rod-shaped virus, containing double-stranded DNA (Wang *et al.*, 1995; Wongteerasupaya *et al.*, 1995) belonging to the family Nimaviridae, genus Whispovirus (Mayo *et al.*, 2002). It is large, the virions are 70-138 nm x 240-340 nm in diameter and it contains a rod shaped nucleocapsid of 70-90 x 200-350 nm (Kasornchandra *et al.*, 1998; Wang *et al.*, 2000a). The genome of different WSSV isolates ranges from 292 kbp to 307 kbp in size (van Hulten *et al.*, 2001a; Yang *et al.*, 2001; Chen *et al.*, 2002). It is extremely virulent and presently overshadows all other disease agents as the leading cause of production losses in Asia (Flegel,

1997; Flegel *et al.*, 1997). Although WSSV has a very wide host range, it can remain in a virion state for a considerably long period of time, thereby increasing the chances of further infection (Durand *et al.*, 1997; Yang *et al.*, 2001) and thus posing a major threat to the shrimp farming industry.

WSSV is capable of causing 100% mortality within a few days of the onset of clinical signs (Ayub *et al.*, 2008; Sanchez- Martinez *et al.*, 2007; Vaseeharan *et al.*, 2003). One important reason why white spot syndrome (WSS) is so hard to control is that it is capable of transmitting both vertically and horizontally. A major route of introduction is vertical transmission, occurs when WSSV is transmitted from parent shrimps to post-larvae in hatchery ponds before the post-larvae (PL) are transferred to culture ponds. The clinical signs of the syndrome include lethargy, anorexia, the presence of white spots on the cuticle and, often, generalized reddish to pink discoloration (Durand *et al.*, 1997). Since complete mortality of shrimp occurs within a couple of days following the appearance of first clinical sign, stocking of virus free healthy PL, as the preventive measure of vertical transmission, is the only available alternative to hand for minimizing the WSSV propagation.

At present, no treatments are available to control the disease and mortality, so finding ways to minimize the incidence of WSD could have significant implications for the future success of the industry. For this a rapid and efficient diagnosis of diseases prior to the onset of heavy infection is a major concern for successful shrimp farming operations. In an effort to minimize the spread of viruses in shrimp farms and hatcheries, various types of histological and molecular diagnostic methods have been developed for detection of WSSV. These include histopathological techniques (Wongteerasupaya *et al.*, 1995; Supamattaya and Boonyaratpalin, 1996) in situ hybridization using gene probes (Durand *et al.*, 1996; Wongteerasupaya *et al.*, 1996) immunological methods such as Nitrocellulose-enzyme immunoblot (Nadala and Loh, 2000) and western blot techniques (Nadala *et al.*, 1997; Hameed *et al.*, 1998) and more recently polymerase chain reaction based methods (Lo *et al.*, 1996a; Kim *et al.*, 1998). Among them PCR is the most reliable and important tool for detection of viral pathogens in shrimp aquaculture. PCR based methods have been successfully used to detect and eliminate WSSV from infected brood stock and PL before they enter the production system (Momoyama *et al.*, 1995; Lo *et al.*, 1996a, b; Withyachumnarnkul, 1999).

The polymerase chain reaction (PCR) is a scientific technique in molecular biology to amplify a single or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence developed by Kary Mullis in the 1980s. The PCR reaction is carried out in a thermal cycler with a small tube containing the DNA to be amplified, free nucleotides (dNTP mixture), one pair of single-stranded DNA's usually about 20 nucleotides in length that are complementary to a short region on either side of the template DNA (primer), and thermo-stable polymerase enzyme (Usually Taq polymerase enzyme). It is quick, easy, and automated. In this regard, the state-of-the-art polymerase chain reaction (PCR) can be conveniently used for rapid screening for WSSV presence in shrimp brood, PL and growing juveniles as it provides a high degree of sensitivity and specificity in detection of WSSV (Lightner, 1996).

Apparently, there is no shortage of policies or initiatives both by the GoB and the major development partners towards application of PCR technology in addressing the virus issue. Accordingly, PCR testing facilities have been introduced by some private hatcheries, Department of Fisheries (DoF) and other development agencies particularly, USAID-funded projects managed by WorldFish. Notably, WorldFish has been successfully using this technology to provide screened PL to selected farmers through various projects. However, costing for a single sample remains substantially high even though the costs for technical personnel and kits/chemicals are being borne by WorldFish.

The high cost for WSSV testing can be attributed to the fact that all PCR testing laboratories in Bangladesh including the government ones employ the use of patented kits imported from countries like Thailand, Taiwan etc. The open-access database together with bioinformatics tools can be conveniently used to access and design primers from WSSV genome that can be tested with easily obtainable chemicals from local suppliers to develop a cost-effective protocol for WSSV screening. Apart from being highly cost-prohibitive the methodology employed with the imported kit poses the risk of contamination because of two-step PCR. Opening the PCR tube after first 15 cylces, as required by the imported kit protocol, to add second-step PCR reagents greatly increase the chance of cross contamination and increasing the risk of generating false positive or negative results thereby.

To overcome the drawbacks, it is necessary to design a non-stop, single-tube multiplex PCR method, thereby greatly reducing the overall reaction time and increasing the sensitivity. Multiplex polymerase chain reaction (PCR) is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction by using multiple primer sets. The primer design for all primers pairs has to be optimized so that all primer pairs can work at the same annealing temperature during PCR. Multiplex-PCR was first described in 1988 as a method to detect deletions in the dystrophin gene (Chamberlain *et al.*, 1988). This technology, comparing with PCR assay in separate reaction tubes, reducing the cost and shortens the time required for screening and ensures reduced use of targeted samples as well as reagents makes it reliable to screen WSSV virus in shrimp. In Bangladesh context, unfortunately, little attention has been paid and no initiative for the development of multiplex PCR protocol for the screening of WSSV in shrimp yet to be undertaken.

Thus, the research problem in Bangladesh context has two dimensions: one is to develop a protocol for PCR testing using non-patented bulk available reagents and the other one is to develop a protocol that rely on multiplexing the PCR. Recently, an optimized PCR-based screening protocol for WSSV without employing imported kits has been developed by FMRT Discipline with funding support from IDRS-BFRI project of Shrimp Research Station. However, development of a multiplex PCR system targeting both WSSV- and shrimp-specific DNA in a single tube, as proposed herein under, were beyond the scope of the IDRS-BFRI study. However, these components are expected to contribute substantially to our knowledge base that would ultimately pave the way for developing a multiplex WSSV kit of our own.

Multiplex-PCR consists of multiple primer sets within a single PCR mixture to produce amplicons of varying sizes that are specific to different DNA sequences. By simultaneously amplifying more than one locus in the same reaction in a single PCR tube, multiplex PCR is becoming a rapid and convenient screening assay in both the clinical and the research laboratory. Thus considering the simplicity and cost- effectiveness, this study was carried out to optimize a multiplex PCR protocol for detection of both WSSV and shrimp DNA as the internal positive control in a single tube thereby reducing the time and chance of carry over contamination.

1.2 Objective of the study

- To adapt, optimize and validate a multiplex PCR system targeting both WSSV and shrimp DNA in a single tube without employing costly imported kit;
- To analyse the cost-effectiveness for the optimized protocol in comparison to an existing imported kit protocol that is currently followed by the laboratory technicians.

Chapter Two

REVIEW OF LITERATURE

2. Review of Literature

Shrimp is one of the most popular seafood in the world. Given that shrimp aquaculture is faced with the constant threat of bacterial and viral pathogens, among them viruses are ubiquitous and extremely abundant in the natural environment. One of such viruses, the White Spot Syndrome Virus (WSSV) has emerged globally as one of the most contagious, widespread and lethal pathogen of cultured shrimp that causes mass mortality, leading to huge economic loss to the shrimp industry. However, at present there is no such existing treatment procedure to restrict the uncontrolled occurrence and rapid spread of the disease in the wild, for this an early detection technique is necessary. Recent advancement in the field of molecular biology has made it possible to obtain information on the factors, mechanisms and strategies used by this virus to infect and replicate in susceptible host cells.

Diagnosis of disease is performing using transmission electron microscopy (Lei *et al.*, 2008), DNA hybridization (Dhar *et al.*, 2003), histopathology by H and E (Duangsuwan *et al.*, 2008), monoclonal antibody (Wang *et al.*, 2008). However, these methods are time consuming and labor intensive. To date, various molecular techniques have been developed to detect pathogens directly targeting the genetic material. Literally various DNA based detection methods for the most important viral pathogens have been reported and a few methods are commercially available in the shrimp culture industry. The use of DNA-based techniques enables the detection of pathogenic microbes exposed to stress conditions with greater sensitivity and reliability than conventional culture methods (Mandal *et al.*, 2011). The first probe was developed by Lightner for detecting the shrimp virus IHHNV (Lightner *et al.*, 1992, Mari *et al.*, 1993). DNA based probes for WSSV have been developed by several laboratories (Rajan *et al.*, 2000, Zhan *et al.*, 2000).

At present molecular assays, such as DNA probes and PCR methods have been used for rapid and sensitive detection of these viruses. Development and application of these methodologies in the diagnosis of shrimp disease is very recent. PCR based diagnostic methods are being widely used, because of its high sensitivity. Development of PCR based modified methods have been published for rapid, sensitive detection of food-borne pathogens in many previous studies.

Different variations of PCR methods include one step PCR, semi nested, two step PCR, quantitative competitive PCR, real time PCR, reverse transcription polymerase chain reaction (RT-PCR), PCR–ELISA, Multiplex PCRs for different pathogens along with WSSV and for different isolates, are the most popular and widely used methods, that has improved sensitivity, versatility and flexibility for detection of WSSV. One step PCR detects WSSV in shrimp containing a substantial concentration of viral DNA. Two step PCR can detect light infections in brood stock, nauplii, postlarvae and juveniles and quantitative PCR can be used for the quantification of the viral load. Recently a sensitive, specific and rapid method was developed, called multiplex PCR that would allow detection of multiple pathogens simultaneously from foods will be useful for food processors and regulatory bodies (Bai *et al.*, 2010).

Dieu *et al.* (2004) studied molecular epidemiology of WSSV and suggested that there are different types of virus. Vaseeharan *et al.* (2003) were detected WSSV by PCR in cultured and captured crustaceans in India. They concluded that WSSV is widespread in cultured and captured shrimp in India.

In India, Manivannan *et al.* (2002) reported that *P. monodon* post-larvae were simultaneously infected by three different viruses including MBV, hepatopancreatic parvovirus (HPV) and WSSV as detected by histology and non-nested PCR. Jian *et al.* (2005) compared in situ PCR with other methods for detection of WSSV in *Panaeus vannamei*.

Hameed *et al.* (2005) developed a non-stop, single tube and semi-nested polymerase chain reaction (PCR) technique with simple procedure for simultaneous detection and grading the level of white spot syndrome infection in penaeid shrimp, (*Penaeus monodon*). In this study PCR was done by using three sense primers and one antisense primer with uniform annealing temperature of 55°C and these primers amplify three PCR products (500, 300 and 200 base pairs) depending upon the severity of infection. This PCR technique was assessed for early detection of WSSV in shrimp.

Kiatpathomchai *et al.* (2001) established a non-stop, single-tube, semi-nested PCR technique for grading the severity of white spot syndrome virus infections in *Penaeus monodon* by using 1 sense primer and 3 antisense primers that produce up to 3 PCR products (1100, 526 and 250 base

pairs) depending upon the severity of infection and was able to detect a low quantity of viral target as little as 5 fg WSSV DNA.

In a study Park *et al.* (2013) developed a highly sensitive single-tube nested PCR protocol directed toward the sequence of virion envelope proteins for detection of white spot syndrome virus (WSSV) infection. In this study they developed an improved diagnostic PCR method for WSSV by designing primers directed toward the nucleotide sequence encoding envelope proteins of WSSV, VP19 and VP28.

A nested PCR protocol for white spot syndrome virus (WSSV) screening for major crustaceans inhabiting shrimp farms in Bangladesh was developed by Islam *et al.* (2007). In this study a two-step polymerase chain reactions (PCR)-based screening protocol were applied by using two pairs of oligonucleotide primers of 22 bp and 21 bp named as LoF1, LoR1 and LoF2, LoR2 specific for WSSV DNA sequences to identify the prevalence of WSSV. The outer primer pair (LoF1 and LoR1) amplifies a 1,447 bp fragment while the second pair of primers (LoF2 and LoR2) amplifies a 941 bp internal sequence to the first set of primers. The experiment was highly specific, easy, and cost-effective and is the first scientific report on applying PCR-based WSSV screening protocol in Bangladesh.

Sritunyalucksana *et al.* (2006) made a comparison of PCR testing methods for white spot syndrome virus (WSSV) infections in penaeid shrimp and shown that real time PCR could detect with certainty at dilutions of approximately 5 copies per reaction, whereas 1000 copies were needed for common one step PCR and 50 for a common single tube nested PCR (1N-PCR) and of 2 two-tube nested PCR protocols tested, one required 100 and the other 1000 copies. Overall, the results confirmed the validity of PCR-based methods in Thailand for detection of WSSV.

A rapid PCR assay for detection of white spot syndrome baculovirus (WSBV) in cultured and captured shrimp, crabs and other arthropods was developed by Lo *et al.* (1996) based on the nested PCR procedure which was outlined as the recommended PCR diagnostic assay in the Manual of Diagnostic Tests for Aquatic Animals published by the Office of International Epizootics (OIE, 2009). The detection of WSBV had been achieved by means of 1-step PCR and 2-step amplification with nested primers on the sensitivity of WSBV diagnostic PCR. In this

study the DNA sample yielded a visible band of 1447 bp in first of step amplification and 2 visible bands of 1447 and 941 bp in second step of amplification and showed that the sensitivity of the 2-step amplification was 10^3 to 10^4 times higher than that of l-step amplification.

A real-time PCR method was developed by Yan *et al.* (2009) to detect monodon baculovirus (MBV) in penaeid shrimp by developing a pair of MBV primers to amplify a 135 bp DNA fragment.

Dhar *et al.* (2002) developed real-time RT-PCR for measuring TSV and YHV load in shrimp. The method is highly robust and high throughput assays which are useful for diagnostic, epidemiological and genetic studies in shrimp.

Esparza-Leal *et al.* (2009) detected white spot syndrome virus in filtered shrimp-farm water fractions and experimental evaluation of its infectivity in *Penaeus vannamei.* WSSV detection was performed by nested PCR. First, a one-step PCR was done with primers that produced a 982 bp amplicon from the WSSV genome. Nested PCR was done that amplified a genome fragment of 570 bp.

Natividad *et al.* (2008) developed a specific and highly sensitive 2-step nested PCR protocol for the detection of WSSV DNA in pond soil. They used previously described primers for the first round of amplification and designed an inner primer based on the sequenced amplicon for the 2nd round of amplification. They used plasmid DNA containing the 211 bp target WSSV sequence and showed that the 2-step nested PCR protocol was able to detect down to 0.015 fg of the plasmid DNA.

Otta *et al.* (2003) investigated the simultaneous presence of monodon baculovirus (MBV) and white spot syndrome virus (WSSV) in apparently healthy postlarvae of *Penaeus monodon* from different hatcheries in India was studied by nested polymerase chain reaction (PCR) and showed simultaneous presence of WSSV and MBV in many samples at various degrees of infection.

Hossain *et al.* (2001) studied the presence of white spot syndrome virus (WSSV) of shrimp in various marine crustaceans by using polymerase chain reaction (PCR). They stated that detection of carrier animals required two-step nested PCR.

Nunan *et al.* (1998) worked on the detection of White Spot Syndrome Virus (WSSV) and Yellow Head Virus (YHV) in shrimp. A nested PCR amplification procedure was used for detection of WSSV by using two sets of oligonucleotide primers. The first set primers amplified a 750 bp sequence of the WSSV genome. The second set of primers amplified a 500 bp internal sequence to the first set of primers.

Saberi *et al.* (2008) designed a diagnostic kit for white spot syndrome virus. They designed 2 series of primers for diagnosis of viral VP24 gene and also primers as internal controls which amplify part of genome in both positive and negative samples and tested successfully.

Ayub *et al.* (2008) detected prevalence of white spot syndrome virus infection by one-step and nested PCR in tiger shrimp (*Penaeus monodon*). The study was conducted by using two pairs of primers, namely, 146F1/146R1 and 146F2/146R2, amplifying the 1447 bp and 941 bp fragments, respectively, for both one-step and nested PCR. They concluded that among the two primer-pairs, the inner pair amplifying the 941 bp fragments was more sensitive than the outer primer pair amplifying the 1447 bp fragment.

Nunan and Lightner (2011) optimized PCR assay for detection of white spot syndrome virus WSSV). They developed one-step PCR method for this study based on the nested primers developed by Lo *et al.* (1996) that generates a 942 bp amplicon. This method takes approximately 1 h 30 min to run 40 cycles at an annealing temperature of 62 °C.

Tang and Lightner (2000) worked on quantification of white spot syndrome virus DNA through a competitive polymerase chain reaction. In this study they used a pair of WSSV primers, designated WSSV341F/R, to amplify a 341-bp DNA fragment from the WSSV genome and 18S primer for shrimp to amplify 441-bp from shrimp 18S rRNA DNA for multiplex PCR detection.

Hossain *et al.* (2004) carried out a study on detection of WSSV in cultured shrimps (*Penaeus monodon*), brooders, shrimp postlarvae and water samples in Bangladesh by Polymerase chain reaction (PCR). They applied both non-nested PCR reaction and nested PCR reaction by using five different pairs of primers designated as KF 1–2 (2310 bp), Lo 1– 2 (1447 bp), Lo 5–6 (775

bp), IK 1–2 (486 bp) and IK 3–4 (310 bp) and concluded that use of primers yielding smaller amplicons would give more accurate results.

Tapay *et al.* (1999) developed a PCR protocol to detect at least four geographic isolates of WSV from both experimentally- and naturally-infected shrimp. In addition to high specificity, the one-step and two-step PCR protocols were determined to have sensitivities of 10-100 pg and 100 femtograms respectively and two-step PCR protocol is recommended as a very sensitive and specific protocol.

A duplex polymerase chain reaction (PCR) protocol was developed by Natividad *et al.* (2006) for the simultaneous detection of two penaeid shrimp viruses, namely, White spot syndrome virus (WSSV) and Monodon baculovirus (MBV) infecting *Penaeus monodon*. The method was designed for screening postlarval samples with dual infections of MBV and WSSV in a single reaction.

Miniarray system and single-step multiplex PCR for simultaneous diagnosis of white spot syndrome virus and infectious hypodermal and hematopoietic necrosis viruses were introduced by Quere *et al.* (2002).

Tsai *et al.* (2002) designed a multiplex reverse transcription-polymerase chain reaction (RT-PCR) method for detection of WSSV and Taura Syndrome Virus (TSV) in penaeus shrimp in Taiwan. It was an assay using a single-tube, one-step multiplex RT-PCR for the simultaneous detection of WSSV and TSV. In this study three primer sets, 9195 F/9992 R, 94 F2/R2, and ITS F/28S R, were mixed at a ratio of 3:1:1 to amplify specific fragments of the TSV, WSSV, and *Penaeus vannamei* genome, respectively, in the RT-PCR reaction.

Yang *et al.* (2006) developed a single-step multiplex PCR for simultaneous detection of white spot syndrome virus and infectious hypodermal and haematopoietic necrosis virus in penaeid shrimp. They believed that WSSV is important pathogen of cultured shrimp that effect on cultured industry in China.

Xie *et al.* (2007) developed and optimized a multiplex reverse transcription polymerase chain reaction (mRT-PCR) for simultaneous detection of three viral pathogens of shrimp. Three sets of

specific oligonucleotide primers for Taura syndrome virus (TSV), white spot syndrome virus (WSSV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) were used in the assay yielding 231 bp for TSV, 593 bp for WSSV and 356 bp PCR product for IHHNV.

Xie *et al.* (2008) developed and optimized a real-time multiplex polymerase chain reaction (rtm-PCR) assay to simultaneously detect three viral pathogens of shrimp in one reaction by using three sets of specific oligonucleotide primers for white spot syndrome virus (WSSV), infectious hypodermal and haematopoietic necrosis virus (IHHNV) and Taura syndrome virus (TSV).

Xie *et al.* (2009) developed a multiplex real-time PCR assay for detection of White spot syndrome virus (WSSV) and Infectious hypodermal and haematopoietic necrosis virus (IHHNV) simultaneously and massively by using 2 pairs of primers.

Labreuche *et al.* (2012) developed a quantitative multiplex PCR method with an internal amplification control for simultaneous detection of *Vibrio penaecida* and *Vibrio nigripulchritudo* from shrimp samples with high specificity and sensitivity.

Sibonga *et al.* (2013) optimized multiplex PCR assays for simultaneous detection of viruses infecting hatchery-reared shrimp, *Penaeus monodon*. Multiplex PCR conditions were developed for simultaneous detection of white spot syndrome virus (WSSV), *Penaeus monodon*-type baculovirus (MBV) and infectious hypodermal and hematopoietic necrosis virus (IHHNV) in shrimp. Reactions were performed by using three pairs of primer for the detection of MBV, WSSV and IHHNV resulting in the amplification of a 193 bp, of 824 bp and 703 bp and the best results found at annealing temperature of 58^0C.

Kawasaki *et al.* (2009) developed another multiplex PCR method for detection of *Salmonella* spp., *Listeria monocytogenes* and *E. coli* O157:H7 from various types of artificially inoculated food samples including shrimp.

Liang *et al.* (2010) establish a multiplex PCR method for simultaneous and rapid detection of *Spiroplasma eriocheiris* and white spot syndrome virus (WSSV) in *Procambarus clarkii* by using three primer sets mixed at a ratio of 1: 3: 1 to amplify specific fragments of the *S.*

eriocheiris, WSSV, *P. clarkii* crayfish (control organism) genomes, at an optimal temperature of 57^0C respectively and thereby increasing the efficiency of pathogen detection.

Panichareon *et al.* (2011) developed a multiplex real-time PCR to detect simultaneously three of the major viruses of penaeid shrimp including white spot syndrome virus (WSSV), yellow-head virus (YHV), and *Penaeus monodon* densovirus (PmDNV) by using three sets of viral specific primers.

Fakruddin *et al.* (2013) carried out a study to evaluate overall performance (sensitivity and specificity) of the multiplex PCR assay for detection of *Vibrio cholerae*, *Vibrio parahaemolyticus, Salmonella* sp. and *Escherichia coli* O157:H7 from spiked shrimp samples by using four different pairs of primer. Twenty different uniplex and multiplex PCR assay were performed and band sizes for multiplex PCR showed 470, 250, 588 and 889 bp, for *Salmonella, V. parahaemolyticus, V. cholerae* and *E. coli* O157:H7 respectively.

In a previous study (Fakruddin *et al.*, 2012), multiplex PCR method for simultaneous detection of *Salmonella* spp. and *Vibrio parahaemolyticus* from enrichment cultures and spiked shrimp samples were developed by using two pairs of primer and showed 470 and 250 bp of amplified PCR product respectively for *Salmonella* and *V. parahaemolyticus.*

Galaviz-silva *et al.* (2004) established a new multiplex PCR method for detection of White spot syndrome virus genetic variants in Mexico. In this study they used a set of five primer pairs in a multiplex PCR reaction and WSSV-positive shrimp from Thailand yielded five expected amplicons of 1447, 941, 775, 365, and 306 bp.

Kim *et al.* (2007) has developed a multiplex PCR assay for simultaneous detection of five foodborne pathogen such as *E. coli* O157:H7, *Salmonella, Staphylococcus aureus, Listeria monocytogenes* and *Vibrio parahaemolyticus*.

Recently a multiplex reverse transcription-polymerase chain reaction (mRT-PCR) was developed for simultaneously detecting six major shrimp viruses, including Yellow Head Virus (YHV), White Spot Syndrome Virus (WSSV), Taura Syndrome Virus (TSV), Hepatopancreatic Parvovirus (HPV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV) and

Monodon Baculovirus (MBV) by Khawsak *et al.* (2008) by using six primer sets that could amplify viral nucleic acids resulting in different sized PCR products.

In Bangladesh the coastal aquaculture mainly shrimps constitute major export sector and is increasingly shaped by international trade conditions and by national responses to those stringent quality and safety standards. PCR based validated methods for detection of major bacterial and viral pathogens in shrimp might be very useful tool for ensuring quality and safety standards of exportable shrimps. PCR is the method of choice for detection of microorganisms in food (Sherfi *et al.*, 2006; Seidavi *et al.*, 2008). Simplicity, rapidity, reliability, reproducibility, sensitivity and specificity of PCR make it most competitive (Cheng *et al.*, 2012). Uniplex PCR method amplify single gene at a time while multiplex PCR method simultaneously amplify more than one target sequence (Seidavi *et al.*, 2008). Multiplex PCR method saved considerable time and effort in detection of pathogens. It is now proved that multiplex PCR method have compatible or even superior sensitivity over conventional cultural detection methods (Kawasaki *et al.*, 2005; Yasmin *et al.*, 2007).

Chapter Three

MATERIALS & METHODS

3. Materials and Methods

The experiment was conducted at biochemistry and molecular genetics (BMG) laboratory of Fisheries and Marine Resource Technology (FMRT) Discipline, Khulna University.

3.1 Study period

The study was conducted over a period of twelve (12) months during July 2014 to June 2015.

3.2 Sample collection

Juvenile/adult tiger shrimp, bagda (*Penaeus monodon*) from diseased (animals containing grossly external disease symptoms) pond and apparently disease free pond (having no external disease symptoms) were collected from ashashuni under shatkhira district, a remote area of coastal region of Bangladesh devoted to viral diseases endlessly. Some post larvae (PL) being WSSV positive were also collected from WorldFish (WF) by personal communication. After collecting, the samples were transported to the laboratory preserving in 95% ethyl alcohol and finally preserved in -80^{0}C for long time preservation to keep the quality before further analysis.

3.3 Sample preparation

Taking each adult shrimp, about 25-30 mg tissue from pleopods were collected separately by dissecting with sterilized, separate surgical blades to avoid cross contamination. The tissue samples were placed into a 1.5 ml sterile microcentrifuge tube.

3.4 Total genomic DNA extraction

Sodium dodecyl sulfate (SDS) based DNA extraction protocol was followed for the extraction of total genomic DNA from the shrimp tissue samples. One percent (1%) SDS buffer (Table-01) was treated to the samples for lysis which followed 100% ethanol precipitation, 75% ethanol wash and solubilization with TE buffer (Table-01). This method of DNA extraction was previously developed by FMRT Discipline and found quite satisfactory result. The process followed for the shrimp DNA extraction is described below:

Table-01: Composition of the reagents for DNA extraction buffer

Solution	Composition
DNA Extraction buffer (DEB)	10 mM Tris-HCl (pH 8), 25 mM EDTA, 100 mM Nacl, 1% SDS
TE buffer (pH 8)	10 mM Tris-HCl (pH 8), 1 mM EDTA
75% ethanol	75% of 100% ethanol+25% ddH_2O

*Detail preparations of the reagents are given in annex II

3.4.1 Homogenization and lysis

Lysis of the samples was done by adding 500 µl of DNA extraction buffer in the tube containing 25-30mg tissues sample. Then, it was homogenized with a disposable grinder/homogenizer. At first, homogenization was done applying 100-200 µl of the solution and gradually rest of the solution was added for complete dispersion of tissue. After homogenization, incubation was done at 100°C in a heat block for 10 minutes.

3.4.2 Centrifugation

The lysate was centrifuged at maximum speed, 12000 rpm, for 5 minutes at room temperature. Then the clear supernatant (~350 µl) was transferred carefully to a new, sterile 1.5 ml microcentrifuge tube.

3.4.3 DNA precipitation

DNA precipitation was done adding 700-µl (two volumes of the supernatant) of 100% ethanol. Samples were mixed by inversion slowly and kept it at room temperature for 5 minutes. After centrifugation at 10000 rpm for 5 min, the ethanol was decanted carefully.

3.4.4 DNA wash

About 500 µl 75% ethanol was added into the tubes for washing the DNA. At 8000 rpm, the tubes were centrifuged for 2 minutes. Finally, the ethanol was removed by pipetting/decanting

carefully. The DNA pellet into the tubes were air dried immediately by keeping tubes open at room temperature for 10~15 min.

3.4.5 DNA solubilization

The extracted DNA inside the tube was solubilized with 100 µl TE buffer by slowly passing the pellet through a pipette tip.

3.5 DNA quantity and purity determination

DNA quality and quantity was measured spectrophotometrically. The quantity or concentration (C) was determined according to Aquadro *et al.* (1998) as given below:

$C = A_{260}$ (observed) × (50 µg/ml)* × Dilution factor for measured sample

*Where, A_{260} **(observed)** = Concentration of DNA sample determined by a spectrophotometer at the absorbance of 260 nm.*

***Dilution factor** = (Amount of solvent for dilution/Amount of original sample)*

**Given that a 50 µg/ml solution of double stranded DNA has an absorbance of 1.0 at 260 nm.*

Again, the DNA purity was determined by using the following formula:

Purity = A_{260}/A_{280}

Where, A_{260} = Concentration of DNA sample determined by a spectrophotometer at the absorbance of 260 nm

A_{280} = Concentration of DNA sample determined by a spectrophotometer at the absorbance of 280 nm.

3.6 Design of PCR primers

A total of three (3) pairs of oligonucleotide primer were evaluated for the development of multiplex PCR protocol. Among the three primer sets, two pairs were WSSV specific and one pair was shrimp specific as the internal positive control. Each of the WSSV specific primer had

the length size of 22 bp and 21 bp named as Lo F1R1 and Lo F2R2 respectively. The WSSV specific primers correspond to 1 cloned, 1461-bp *Sal*I-digested WSSV genome fragment as described by Lo *et al.* (1996). Here, Lo F1R1 acts as the outer primer and Lo F2R2 acts as inner primer (Fig. 01). The rest of the primer pair specific for shrimp ribosomal RNA (rRNA gene) DNA described by Dieu *et al.* (2004). After procuring the primers, these were diluted properly to get a final concentration of 10 pmole/µl (Annex II). All the primer names, nucleotide sequences, melting temperatures, lengths and their amplicon sizes are shown in table 02.

Table 02: Name, sequence, melting temperature, length, type and amplicon size of the primer

Primer Name	**Nucleotide Sequence (5'-3')**	**Length (bp)**	**Type**	**Melting temperature (°C)**	**Amplicon size**	**Reference**
LoF1	ACTACTAACTTCA GCCTATCTA	22	WSSV-specific	49.6	1447	Lo *et al.* (1996)
LoR1	TAATGCGGGTGTA ATGTTCTTA	22	WSSV specific	51.9		
LoF2	GTAACTGCCCCTT CCATCTCC	21	WSSV specific	57.1	941	Lo *et al.* (1996)
LoR2	TACGGCAGCTGCT GCACCTTG	21	WSSV specific	62.8		
16S rRNA-FW	GTGCGAAGGTAGC ATAATC	19	Shrimp specific	52	414	Dieu *et al.* (2004)
16S-rRNA RV	CTGCTGCAACATA AGGATAC	20	Shrimp specific	52		

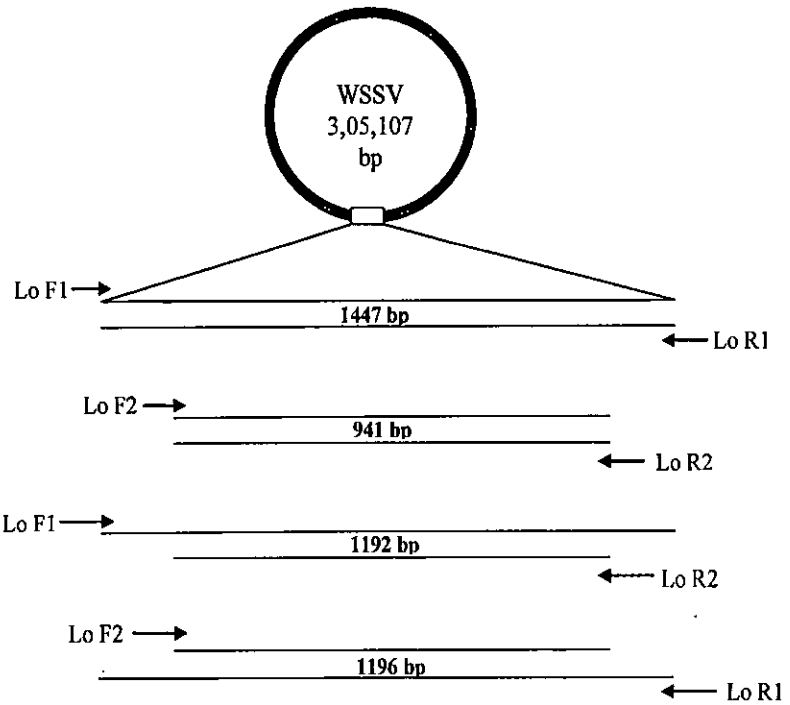

Fig. 01: *The nucleotide sequences of two selected primer sets and probable two more primer combinations with their estimated location and orientation with regard to a hypothetical WSSV genome.*

3.7 Development of a multiplex PCR protocol

The present study of multiplex PCR protocol development will ensure simultaneous detection of both WSSV and shrimp DNA in a single tube. Prior to the development of multiplex PCR protocol, all the three primer pairs were tested in separate PCR tube reactions. An additional two step (nested) PCR using the WSSV specific primers (outer and inner) was also tested. After testing the primers separately, both shrimp specific and WSSV specific primer pairs were evaluated in employing single step PCR. In addition, semi-nested single tube PCR was evaluated to find out the most suitable protocol for the simultaneous detection of both shrimp DNA and WSSV DNA in a single tube. The procedures followed in the study are described below:

3.7.1 Optimization of PCR protocol in separate tube reactions

For separate tube reactions, 10 µl reaction mixtures for each specific tube was prepared with a combination of appropriately diluted approximately 10-25 ng sample tissue DNA as the templates with 2 µM of each primer and 5 µl of premix (TAKARA BIO INC.) in 0.2 ml thin-

walled PCR tubes. The reactions were performed using an automated thermal cycler (BioRed C1000, Singapur). The premix was the mixture of double concentrated 1X PCR buffer, 2 mM $MgCl_2$, 200 µM dNTP's and 0.25 unit Taq polymerase enzyme.

PCR thermal cycling conditions were empirically determined based on previous studies (Islam *et al.*, 2007). To optimize the PCR using the separate primer, several combinations of thermal cycling conditions were applied for getting the most suitable one. Thermal cycling conditions that were tested ranged 25-30 sec at 95°C for the first round followed by 30 cycles of denaturation for 30-40 sec at 94°C, 25-30 sec of annealing at 54-62°C, and 40-45 sec of extension at 72°C. The last extension step at 72°C was extended for 5 min. For nested PCR reaction, 1st step PCR was performed only for 15 cycles and another more 30 cycles were done using nested primer and 10 times diluted 1 µl of 1st step PCR product.

3.7.2 Optimization of multiplex PCR protocol

After optimizing thermal cycling conditions for separate tube reaction having separate primers, the multiplex PCR protocol was developed by manipulating the primer and template concentration, thermal cycling conditions (temperature and time), buffer, dNTP, MgCl2, Taq polymerase concentration. For the development of multiplex PCR protocol in a single tube, both WSSV DNA and shrimp DNA were amplified using their respective primers.

All the PCR assays for the multiplex protocol were done with a 10 µl PCR cocktail in a single tube using the thermo cycler (BioRed C1000). Four combinations of primers for single step multiplex PCR and four combinations of primers for semi nested single step multiplex PCR were theoretically selected for the evaluation. PCR thermal cycling conditions were empirically determined based on the optimized conditions found for each of the separate primers.

The optimum thermal cycling conditions, reagents compositions, cycling numbers of the separate primer were taken under consideration for the development of the multiplex protocol. If all the conditions were found to be optimum for the protocol, only annealing temperatures were empirically altered to obtain the best banding pattern with stronger intensity. Appropriate control reactions (reagent control and negative control) were run in parallel to rule out the possibility erroneous amplification or no amplification at all. The PCR mixture being all the reagents

without primers was considered as reagent control and the PCR mixture being all the reagents with DNA of tilapia species replacing shrimp DNA was considered as negative control.

3.8 Detection of the amplified products

Gel electrophoresis

1. Agarose gel prepared (Annex II) earlier was placed into electrophoresis tank that was filled with TAE buffer at appropriated level

2. An amount of 5 µl sample (PCR amplified product) was mixed with loading dye and it was loaded slowly and carefully into the gel well

4. Meanwhile 2 µl of 100 bp DNA ladder marker was loaded in one of the wells independently.

5. Electrophoresis tank was connected to power supply, making sure the anode and cathode were correctly connected to ensure proper direction of current flow. The current was adjusted at 100 amperes. Migration of the bands was monitored by visualizing bromophenol blue.

3.9 Visualization under UV trans-illuminator

The gel was carefully exposed to UV trans-illuminator. The bands were analyzed by taking picture in a desktop computer.

3.10 Cost effectiveness analysis

All the reagents and chemicals used for the multiplex protocol development were procured from local suppliers. One of the objectives for developing such type of the multiplex protocol was to replace costly imported kit. As a result, after the development of the protocol, all the reagents and their amounts required for the DNA extraction, PCR operation, gel electrophoresis and gel documentation were calculated and an approximate cost was estimated based on the price quotation of a local supplier called City Scientific Store, 31, Ahsan Ahmed Road, Khulna. In addition, a comparative analysis was also done for the multiplex PCR protocol. For this, cost required for a 200 runs of multiplex PCR protocol was compared with the cost required for purchasing a WSSV detection imported kit named 'IQ2000 WSSV detection system' which had facility for 200 reactions per kit.

The cost for the following reagents was taken under calculation (Annex-II) for both protocols:

- Reagents required for DNA extraction buffer
- Primers required for operating protocols (maximum amount)
- Premix- (Included *Taq* polymerase, buffer, dNTP's etc)
- Molecular weight marker
- Gel loading dye.

The costs for following categories, for both groups, were deducted from the calculation:

- Reagents/chemicals category not supplied with the kit e.g. alcohol, TE buffer etc
- All the laboratory supplies and consumables
- Labor cost

4. Result

In this study, a multiplex PCR protocol was optimized and validated for the screening of WSSV in shrimp by single tube non-stop PCR without employing costly imported kit. The reagents for DNA extraction and PCR amplification were prepared with their proper concentration and the optimization was done empirically. Using the optimized multiplex PCR protocol, some samples from field were tested. The presence of WSSV and shrimp DNA were simultaneously detected by the optimized multiplex PCR, amplifying of published primers described by Lo *et al.* (1996) and Dieu *et al.* (2004).

4.1 Optimization of PCR protocol using the primers in separate tube

4.1.1 Single primer in separate tube with single step PCR

Prior to the development of multiplex PCR protocol, the three primer pairs (Table 02) were tested separately in separate tube employing single step PCR. The testing of the primers in separate single tube reaction was done for two reasons. One reason was to test efficiency of the primers which revealed the effectiveness of the primer pairs (Table 02) developed by Lo *et all.* (1996) and Dieu *et al.* (2004) can also amplify the WSSV DNA and the shrimp DNA. It was found that both the WSSV specific primer and the shrimp specific primer yielded expected size fragments (Table 02 and Fig. 02). Another reason for testing the primers separately was to optimize the thermal cycling conditions for each single primer as well as to find out the most suitable annealing temperature with the best band resolution intensity. This optimization for each single primer made it easier to manipulate the thermal cycling condition optimum for the multiplex PCR.

Primer name: 16S rRNA, LoF1R1 and Lo F2R2

Each of the primer was tested in separate single tube employing single step PCR and each of them yielded the expected size band fragment (Table 02, Fig. 02). The three primer pairs of the study annealed at 56-60°C temperature with good band resolution intensity. The 16S rRNA primer pair specific for shrimp DNA annealed at 58°C temperatures for 25 sec (Fig. 02A). The WSSV specific outer primer Lo F1R1 pair annealed at 56°C for 30 sec (Fig. 02B) whereas the

another inner primer Lo F2R2 for WSSV detection annealed at 60°C temperature for 30 sec (Fig. 02C).

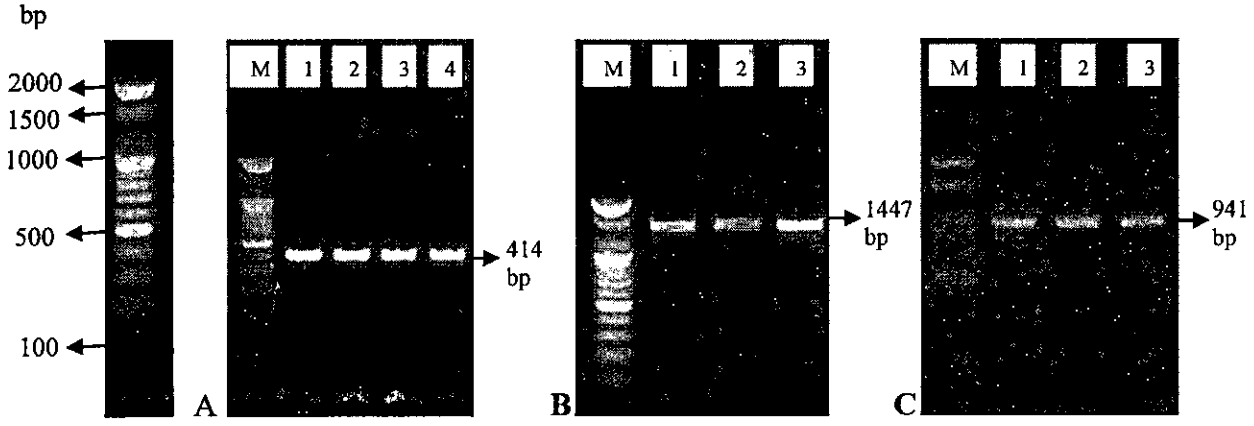

Fig. 02: *Electrophoretic patterns of single step PCR products using 16S rRNA, LoF1R1 and LoF2R2 primers.* ***A.*** *Electrophoretic pattern of 16S rRNA primers; Lane 1-4: shrimp DNA amplification using 16S rRNA primer pair at an annealing temperature of 58°C where amplicon size: 414 bp.* ***B.*** *Electrophoretic pattern of Lo F1R1 primers; Lane 1-3: WSSV DNA amplification using Lo F1R1 primer pairs at an annealing temperature of 56°C where amplicon size: 1447 bp.* ***C.*** *Electrophoretic pattern of Lo F2R2 primers; Lane 1-3: WSSV DNA amplification using Lo F2R2 primer pairs at an annealing temperature of 60°C where amplicon size: 941 bp. Lane M: 100 bp DNA marker;*

Table 03: Reaction components for single set primer pair (16s rRNA, Lo F1R1 and lo F2R2)	
Reagents	***Amount***
DW	01 µl
Primer-F	01 µl
Primer-R	01 µl
Template*	02 µl
Taq premix	05 µl
Total	***10 µl***
*20 times diluted	

Besides, the annealing temperature, the thermal cycling conditions were found to be as initial denaturation at 95°C for 45 sec, denaturation at 95°C for 25 sec, elongation or extension at 72°C for 40 sec and final extension at 72°C for 5 min.

4.1.2 Nested PCR

In addition, a nested PCR was tested using the two pairs of WSSV specific primer. In nested PCR, the amplified products of the first step PCR served as the template DNA for the second step of amplification. In this case, the first-step amplification was carried out by the outer primer pair Lo F1R1 with the following cycle parameters: initial denaturation at 95°C for 01 min, followed by 15 cycles of 95°C for 45 sec, annealing at 56°C for 30 sec and elongation at 72°C for 40 sec and a final extension at 72°C for 5 min. After the completion of the first step, ten times diluted 1 µl of the reaction mixture of 1st step PCR product was used as the template and it was added to make another 10 µl of standard PCR cocktail containing the inner primer pair. The second step PCR was found to be optimum at an annealing temperature of 60°C for 30 sec with 30 cycles amplification. Thus, at least total 45 cycles of PCR amplification was required for the nested PCR to run.

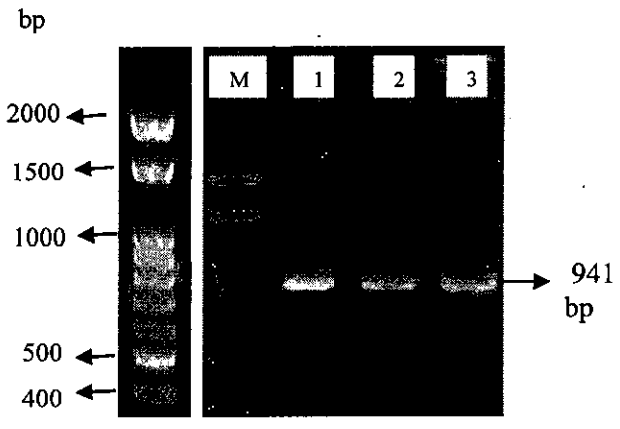

Table 04: Reaction components for nested PCR using Lo F1R1 and Lo F2R2 primers

Reagents	***1st step***	***2nd step***
DW	01 µl	01 µl
Primer F1	01 µl	
Primer R1	01 µl	
Primer F2		1.5 µl
Primer R2		1.5 µl
Template	02 µl*	01 µl**
Taq premix	05 µl	05 µl
Total	***10 µl***	***10 µl***

**20 times diluted from extracted DNA **10 times diluted from the 1st PCR product*

Fig. 03: *Electrophoretic pattern of the nested PCR product using Lo F1R1 primes for the 1st 15 cycles and Lo F2R2 primer for the nested step. The optimized annealing temperatures for the thermal cycling were 56°C for 1st step and 60°C for nested step. Lane M: 100 bp DNA marker; Lane 1-3: detection of WSSV using the nested primer pairs, amplicon size of the products-941 bp.*

4.2 Optimization and development of the multiplex PCR protocol

After testing the three primer pairs separately, a multiplex PCR system was developed for simultaneous detection of WSSV DNA with shrimp DNA as the internal positive control of WSSV. To do so, two pairs of the primer comprising one pair of shrimp specific primer and one pair of WSSV specific primer were amplified in a single tube employing a single step PCR. Four (04) different types multiplex PCR protocol were optimized based on the four (04) different theoretical combinations of the WSSV specific primer (Lo F1R1 and Lo F2R2). The theoretical combinations and their expected band sizes were:

	Primer combination	Expected size (bp)
1.	16S rRNA+Lo F1R1	414, 1447
2.	16S rRNA+Lo F2R2	414, 941
3.	16S rRNA+Lo F1R2	414, 1192
4.	16S rRNA+Lo F2R1	414, 1196

The thermal cycling conditions found to be optimum for separate primers (section 4.1.1) were used for developing the multiplex PCR protocol. Only annealing temperature and reagents composition were altered empirically and the different combinations of the primer were evaluated for optimizing each of the different multiplex PCR protocol.

Combination 1: 16S rRNA + Lo F1R1

Annealing temperatures of 56°C and 58°C for 30 sec with at least 30 cycles were found to be optimum for the primer combination of shrimp specific primer (16S rRNA) and WSSV specific outer primer (Lo F1R1). But the annealing temperature of 56°C showed stronger band intensity of the expected amplicon sizes (Fig. 04). The amplicon sizes viz. 414 bp and 1447 bp were visualized after gel electrophoresis. The presence of shrimp DNA was confirmed through the amplification of 414 bp PCR products (amplicon) and the amplification of 1447 bp (amplicon) confirmed the presence of WSSV DNA (Fig. 04). Time required for thi single step PCR was estimated approximately 1 h and 18 min.

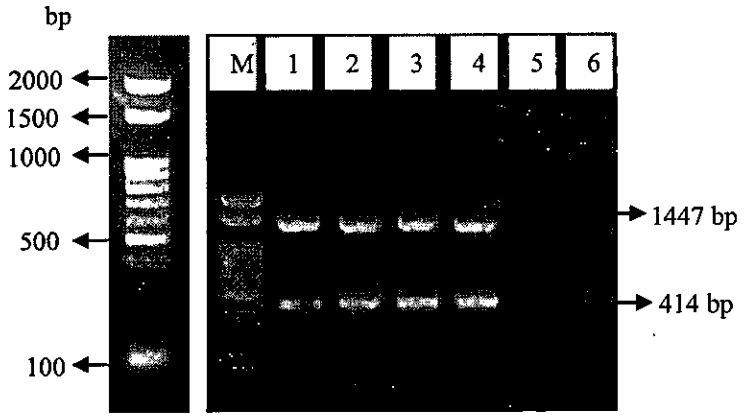

Table 05: Reaction components for the PCR using 16S rRNA and Lo F1R1 primers	
Reagents	***Amount***
Primer 16S rRNA F	0.75 µl
Primer 16S rRNA R	0.75 µl
Lo F1	01 µl
Lo R1	01 µl
Template*	1.5 µl
Taq premix	05 µl
Total	***10 µl***
*20 times diluted DNA	

Fig. 04: *Electrophoretic pattern of single step multiplex PCR products for the simultaneous detection of WSSV DNA and shrimp DNA in a single tube using Lo F1R1 and 16S rRNA primer sets. The optimized annealing temperature for the thermal cycling was 56°C. Lane M: 100 bp DNA marker; Lane 1-4: simultaneous detection of WSSV and shrimp DNA, WSSV amplicon size-1447 bp and shrimp amplicon size-414 bp; Lane 5: negative control; Lane 6: reagent control.*

The other thermal cycling conditions viz, intial denaturation, denaturation, elongation and final extension were found to be optimum at 95°C for 45 sec, 95°C for 25 sec, 72°C for 40 sec and 72°C for 5 min respectively. Again, approximately ~15 ng concentration of the template (genomic DNA) DNA was found to be optimum with a total of 3.5 µM primer concentration and 1.25 unit taq polymerase enzyme to make a total of 10 µl PCR mix for carrying out the single tube single step reaction. The electrophoretic pattern of the PCR product is shown in Fig. 04 and the reaction composition for the reaction combination is summarized in table 05.

Combination 2: 16S rRNA + Lo F2R2

For the combination of shrimp specific (16S rRNA) and WSSV specific inner primer (Lo F2R2) pairs, some of the amplification conditions viz. thermal cycling conditions and cycling numbers were found to be different from the combination 1. The PCR amplifications were carried out annealing temperatures ranging 56°C-60°C for 30 sec, although good resolution with stronger intensity of the bands were obtained at an annealing temperature of 58°C with at least 32 cycles of PCR (Fig. 05).

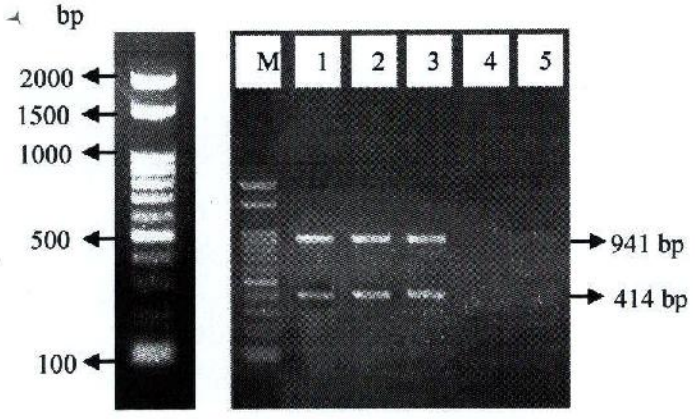

Table 06: Reaction components for the PCR using 16S rRNA and Lo F2R2 primers	
Reagents	***Amount***
DW	0.5 µl
Primer 16S rRNA F	0.75 µl
Primer 16S rRNA R	0.75 µl
Lo F2	0.75 µl
Lo R2	0.75 µl
Template(20 times diluted)	1.5 µl
Taq premix	05 µl
Total	***10 µl***

Fig. 05: *Electrophoretic pattern of single step multiplex PCR products for the simultaneous detection of WSSV DNA and shrimp DNA in a single tube using Lo F2R2 and 16S rRNA primer sets. The optimized annealing temperature for the thermal cycling was 58°C. Lane M: 100 bp DNA marker; Lane 1-3: simultaneous detection of WSSV and shrimp DNA, WSSV amplicon size-941 bp and shrimp amplicon size-414 bp; Lane 4: negative control; Lane 5: reagent control.*

Concentration of the reaction components for this combination was found to be similar that of combination 1 except primer concentration. A total of 3 µM primer concentration was found to be optimum for the total 10 µl PCR mix (Table 06). Time required for the combination was estimated approximately 1 h and 40 min.

Combination 3 and 4: 16S rRNA + Lo F1R2 and 16S rRNA + Lo F2R1

Using the primer combination 3 and 4, two amplicon sizes for WSSV detection namely 1192 bp and 1196 bp were visualized respectively after gel electrophoresis. No size difference was observed for the WSSV specific amplicon size because of only 4 bp size difference between the two combinations. The 414 bp amplicon size confirmed the presence of internal positive control (shrimp DNA) in each the above two combinations.

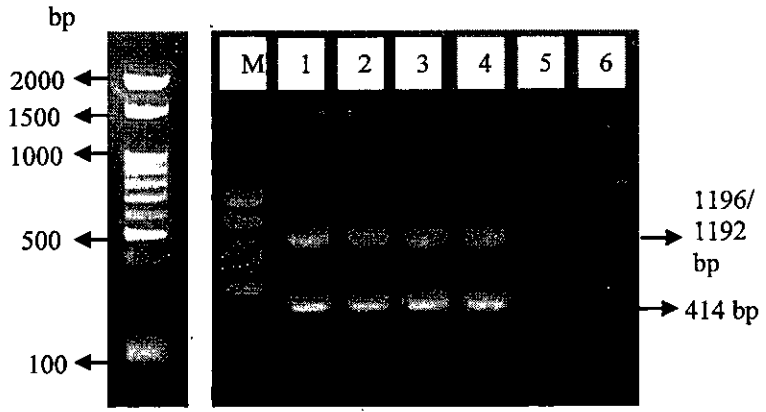

Table 07: Reaction components for the PCR using 16S rRNA and Lo F1R2/F2R1	
Reagents	***Amount***
Primer 16S rRNA F	0.75 µl
Primer 16S rRNA R	0.75 µl
Lo F1/F2	01 µl
Lo R2/R1	01 µl
Template (20 times diluted)	1.5 µl
Taq premix	05 µl
Total	***10 µl***

Fig. 06: *Electrophoretic pattern of single step multiplex PCR products for the simultaneous detection of WSSV DNA and shrimp DNA in a single tube using Lo F1R2, Lo F2R1 and 16S rRNA primer sets. The optimized annealing temperature for the thermal cycling was 58°C. Lane M: 100 bp DNA marker; Lane 1-2: simultaneous detection of WSSV and shrimp DNA using 16S rRNA and Lo F1R2 primers, WSSV amplicon size-1192 bp and shrimp amplicon size-414 bp; Lane 3-4: simultaneous detection of WSSV and shrimp DNA using 16S rRNA and Lo F2R1 primers, WSSV amplicon size-1196 bp and shrimp amplicon size-414 bp; Lane 5: negative control; Lane 6: reagent control.*

Concentration of the PCR mix and the thermal cycling conditions were found to be similar optimized for the primer combination 1. Annealing temperatures of 56-58°C for 30 sec yielded the expected amplicon sizes with at at least 32 cycling amplification though the 58°C annealing temperature for 30 sec with 32 cycles yielded the best banding pattern with stronger intensity.

4.2.1 Semi-nested multiplex PCR protocol with single step PCR

Another type of optimization was also done for semi nested multiplex PCR protocol employing single step PCR. To do the optimization, forward or reverse primer of the nested primer pair was introduced to the outer primer combination (16S rRNA + Lo F1R1) and forward or reverse

primer of the outer primer pair was introduced to the inner primer combination (16S rRNA + Lo F2R2). The primer combinations for the semi nested single step PCR and their expected band sizes were as follows:

Primer combination	**Expected size (bp)**
5. 16S rRNA + Lo F1R1/F2	414, 1447, 1196
6. 16S rRNA + Lo F1R1/R2	414, 1447, 1192
7. 16S rRNA + Lo F2R2/F1	414, 941, 1192
8. 16S rRNA + Lo F2R2/R1	414, 941, 1196

Each of the above combinations annealed at 58°C for 30 sec with its expected amplicon sizes, though one of the amlicon sizes of each combination was not amplified might be due to lower infection of the sample. The other thermal conditions; initial denaturation, denaturation, elongation and final extension were found to be optimum at 95°C for 01 min, 95°C for 40 sec, 72°C for 45 sec and 72°C for 5 min respectively. Time required for each of the multiplex combination was estimated approximately 2h and 10 min.

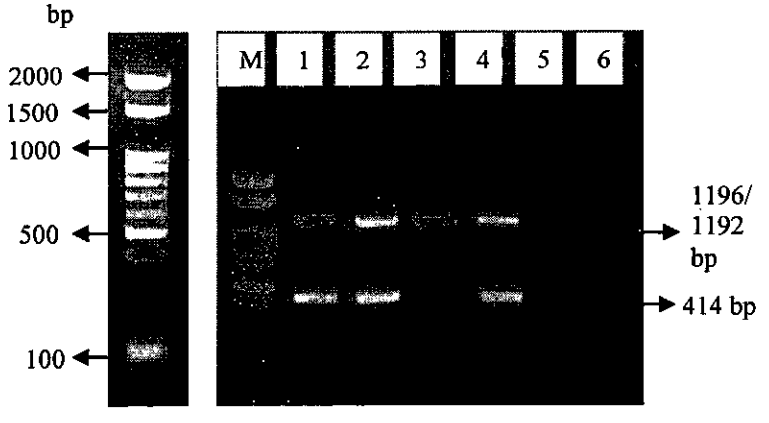

Table 08: Reaction components for the PCR using semi-nested combinations

Reagents	***Amount***
DW	0.5 µl
Primer 16S rRNA F	0.5 µl
Primer 16S rRNA R	0.5 µl
Two F or R (Lo)	01µl (each 0.5)
Single F or R (Lo)	01 µl
Template (20 times diluted)	1.5 µl
Taq premix	05 µl
Total	***10 µl***

Fig. 07: *Electrophoretic pattern of single step multiplex PCR products for the simultaneous detection of WSSV DNA and shrimp DNA in a single tube using semi-nested and 16S rRNA (F/R) primer sets. The optimized annealing temperature for the thermal cycling was 58°C. Lane M: 100 bp DNA marker; Lane 1-4: simultaneous detection of WSSV and shrimp DNA using 16S rRNA and Lo F1R1/F2 primers, WSSV amplicon size-1196 bp (Lane 1); Lo F1R1/R2 primers, WSSV amplicon size-1192 bp (Lane 2); Lo*

F2R2/F1 primers, WSSV amplicon size-1192 bp (Lane 3) and Lo F2R2/R1 primers, WSSV amplicon size-1196 bp (Lane 4). The shrimp amplicon size-414 bp; Lane 5: negative control; Lane 6: reagent control.

Based on the banding intensity and clarity of the products, the primer combination of 16S rRNA and Lo F1R1/R2 showed best results among the four combination of semi nested multiplex PCR system.

4.2.2 Specificity by testing samples from the field

Two types of field testing were performed to show the specificity of the multiplex protocol. Of the two, one was the shrimp samples of three different life stages (PL, Juvenile and Adult). The DNA's were extracted from PL, juvenile and adult collecting the grossly and apparently WSSV infected shrimp from a firm of satkhira district, Khulna. Samples from the three (03) different stages of shrimp were evaluated using two of the optimized multiplex protocols viz. the 16S rRNA and Lo F1R1; and 16S rRNA and Lo F2R2 primer combination (combination 1 and 2).

All the samples were found to be virus positive with the presence of its host using the two mentioned multiplex PCR protocol with their respective DNA extraction procedures, primer combinations, thermal cycling conditions and other reagent compositions optimized in the section 4.2.

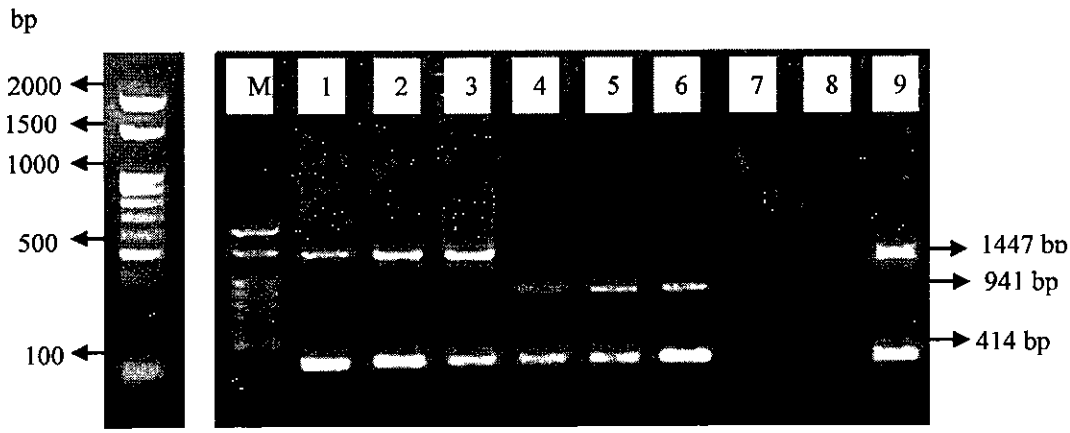

Fig. 08: *Multiplex PCR detection of WSSV DNA and shrimp DNA using the primer combination of 16S rRNA, Lo F1R1 and 16S rRNA, Lo F2R2 primers combinations. Aliquots of PCR product were analyzed on 1.5% agarose gel. Templates used in the reactions were the DNAs extracted from three different stages affected shrimps. Lane M: 100 bp DNA marker; Lane 1-3: Multiplex PCR amplification using 16S rRNA, Lo F1R1 primer combination where templates were extracted from shrimp PL, juvenile and adult*

respectively; Lane 4-6: Multiplex PCR amplification using 16S rRNA, Lo F2R2 primer combination where templates were extracted from shrimp PL, juvenile and adult respectively; Lane 7: negative control; Lane 8: reagent control; Lane 9: known positive sample.

Another type of field testing was done by collecting random samples from satkhira district, Khulna. Using one of the above mentioned two multiplex protocol, 16S rRNA and Lo F1R1, a total of nine random sample was screened for WSSV virus detection and it was found that of the nine samples five (05) were found to be virus positive (Fig. 09).

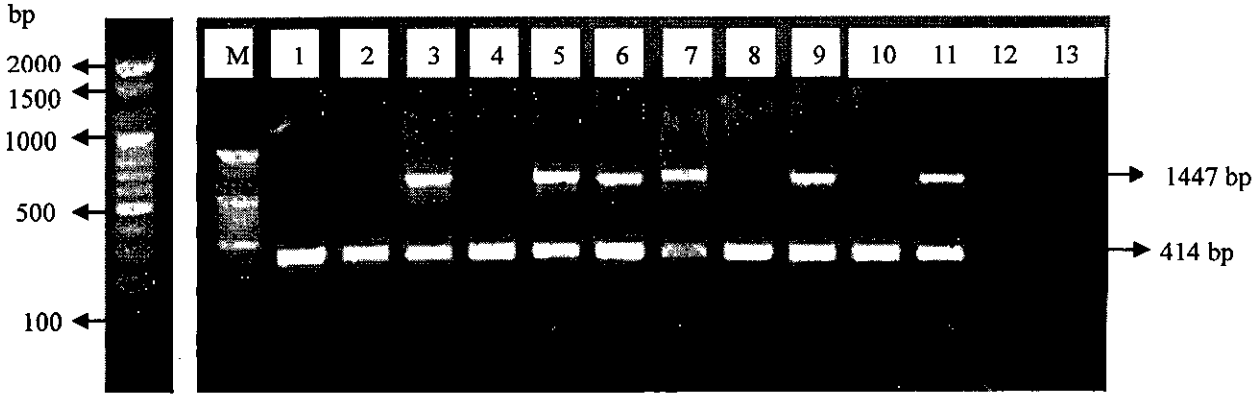

Fig. 09: *Multiplex PCR detection of WSSV DNA and shrimp DNA using the primer combination of 16S rRNA and Lo F1R1. Aliquots of PCR product were analyzed on 1.5% agarose gel. Templates used in the reactions were the DNAs extracted from nine different shrimp samples collected randomly from nine different shrimp farms. Lane M: 100 bp DNA marker; Lane 1-9: Nine different shrimp random samples; Lane 10: WSSV negative sample; Lane 11: known positive sample; Lane 12: negative control; Lane 13: reagent control.*

4.3 Cost benefit analysis and comparative cost of the multiplex protocol

This study had optimized eight (08) different multiplex PCR systems by using eight (o8) primer combinations. Each of the multiplex protocol represented the individual screening procedure with its primer combination. The reagents composition, reagents amount, thermal cycling conditions were showed in the 4.2 and 4.2.1 section.

Estimation of the expenditure was done by calculating the cost of each reagents and chemicals required for the developed protocol. For a total of two hundred (200) reactions, maximum an amount of four thousand five hundred and eighty nine (4,589 BDT) BDT was estimated for any of the multiplex protocol. Only twenty three (23 BDT) BDT was required for per reaction (Table 09).

Again, the study showed that about one lac and sixty thousands (160,000) BDT was claimed by the local suppliers for the procurement of 'IQ2000 WSSV kit' as an all-in-one detection kit imported from Taiwan (Table-09). Two hundred reactions (200) could be performed using one imported kit. About eight hundred (800) BDT was required for per reaction/run which was 35 times higher than the cost required for the developed protocol per reaction/run.

The following table (Table-09) showed a comparative study in terms of cost required for procuring reagents and chemicals for the compared protocols. All the costs were calculated here for 200 reactions. Detailed calculations were figured out in annex-II.

Table-09: Cost effectiveness analysis of the multiplex protocol comparing with a imported kit

Name of reagents	**Multiplex protocol**			**IQ2000 WSSV kit**
	Concentration for each reaction	**Required amount (200 reactions)**	**Cost (BDT) (200 reactions)**	**Cost (BDT) (200 reactions)**
Reagents required for genomic DNA extraction				
DNA extraction buffer				
Tris-HCl	10 mM	0.16 g	4.8	-
EDTA	25 mM	0.73 g	5.84	-
NaCl	100 mM	0.59 g	1	-
SDS	1% (W/V)	1 g	6	-
Subtotal			***17.64***	-
Reagents required for PCR amplification				
Primers	-	-	-	-
Lo F1/R1	1 µM of each (F/R)	200 µl (F+R)	294	-
Lo F2/R2	1 µM of each (F/R)	200 µl (F+R)	280	-
16s rRNA (F+R)	1 µM of each (F/R)	400 µl (F+R)	267	-
Premix	-	1000 µl	1,380	-
Taq polymerase	1.25 U	-	-	-
PCR reaction buffer	1 X	-	-	-
MgCl2	2 mM	-	-	-
dNTP's	200 µM	-	-	-
Subtotal			***2,221***	-
Reagents required for gel electrophoresis				
DNA ladder	N/A	25 µl	1,900	-
Loading dye	N/A	50 µl	450	-
Subtotal			***2,350***	
				160,000
Total Cost for 200 reactions (BDT)			**4,589**	**160,000**
Cost per reaction (BDT)			**23**	**800**

Chapter Five

DISCUSSION

5. Discussion

Shrimp is considered as one of the most important commercially cultured species in Bangladesh. It is the most susceptible host of WSSV (Wongteerasupaya *et al.*, 1995). This species has been badly affected by WSSV in all of the shrimp-producing countries in Asia, including Bangladesh (Larkins 1995; Wongteerasupaya *et al.*, 1995; Karunasagar *et al.*, 1997). For definitive diagnosis and certification of the WSSV infection status of broodstock and fry, PCR technology has been recommended (OIE 2003). Molecular-based detection methods for shrimp viruses include normal PCR using degenerate primers (Chang *et al.*, 1993) and nested PCR (Lo *et al.*, 1996b; Belcher and Young 1998), among others.

Due to higher sensitivity limits than most classical diagnostic methods, PCR has become the preferred method for the diagnosis of most shrimp viruses (Tang and Lightner 2000). Multiplex PCR is a variant of PCR which enabling simultaneous amplification of more than one template in a mixture by addition of more than one set of oligonucleotide primers and thus saves considerable time and effort. Multiplex PCR is becoming a rapid and convenient screening assay in both the clinical and the research laboratory. This assay technology can also be used for the rapid and routine screening of WSSV in shrimp. In this study, a non-stop, faster multiplex PCR protocol was developed to diagnosis the WSSV infection with an internal positive control simultaneously.

The primers that were used for the study had been developed by Lo *et al.* (1996) for WSSV DNA and Dieu *et al.* (2004) for shrimp as the internal positive control. Four (04) different combinations were theoretically selected based on the WSSV specific primer (Lo F1R1 and Lo F2R2). Using the primer combinations, four different single step multiplex PCR protocol were optimized for the detection of WSSV pathogen with the host sample simultaneously. Each of the optimization was carried out preparing a 10 µl reaction mixture with appropriately diluted (20 times diluted having ~15 ng concentration) shrimp tissue DNA as the template employing a single step PCR in a single tube (0.2 ml thin-walled PCR tube). Result of the work on the multiplex PCR protocol optimization viz. thermal cycling conditions, reagents combinations and banding patterns are shown in fig. 04-06 and table 05-07. Initial denaturation at 95°C for 45 sec, denaturation at 95°C for 25 sec, extension at 72°C for 40 sec and a final extension at 72°C for 05

min were found to be optimum for each of the multiplex combinations. Based on the intensity and clarity of the products (bands), annealing temperature at 58°C for 30 sec yielded the best results for all the assays, except 16S rRNA and Lo F1R1 combination (combination-1). An annealing temperature at 56°C for 30 sec yielded the best results for the assay when using the Lo *et al.* (1996) outer primers combination (combination-1, 16S rRNA and LoF1R1). At least 30 cycles of PCR amplification was found to be optimum for the combination of 16S rRNA and Lo F1R1 primers whereas the rest of the combinations yielded the best banding pattern after carrying out at least 32 cycles PCR amplification. Time required for the assays ranged between approximately 1h 18 min to 1h 40 min.

In addition to the above four combination of multiplex protocols, another four combination of semi-nested multiplex protocols were optimized using either forward or reverse or both of the inner (nested) primer (Fig. 07). The amplification conditions and banding pattern intensity was not found satisfactorily as optimized in the first four combinations. For these combinations, initial denaturation at 95°C for 01 min, denaturation at 95°C for 40 se, annealing at 58°C for 30 sec extension at 72°C for 45 sec and a final extension at 72°C for 05 min were found to be optimum with at least 32 cycles that required approximately 2h and 10 min. For each of the combination, two of the expected three bands were amplified. Another one was not found because there might have lower infection of the virus. All the three bands can be found when laboratory technician will screen WSSV affected shrimp of higher level infection. Among the protocols, the multiplex protocol using 16S rRNA and Lo F1R1R2 can be suggested for the detection of the virus in terms of banding sensitivity.

The study found satisfactory results for the multiplex protocol development and thus the study recommended following any one of the optimized combination for the simultaneous detection of WSSV with shrimp DNA. Each of the protocol ensured the diagnosis of WSSV with the shrimp as internal positive control. In addition, the protocols of semi-nested multiplex PCR can also be used to determine the viral severity of the affected shrimp. Laboratory technicians can use any one of the above mentioned protocols based on their objectives of analysis.

However, among the first four combinations the study recommended to follow the multiplex PCR protocol using the combination of 16S rRNA and Lo F2R2 primers (combination 2) in terms of sensitivity of the viral detection. Hossain *et al.* (2004) reported that primers containing

smaller amplicon sizes are much more sensitive than the primers containing larger amplicon sizes. A similar type of finding was also reported by Nunan and Lightner (2011) by optimizing a single step PCR protocol for the detection of WSSV using the nested primer developed by Lo *et al.* (1996). They revealed that the assay based on smaller amplicon size of the inner primer allowed the gradation of viral infection, with highly infected animals being positive in non-nested PCR and lightly infected animals being positive only in inner (nested) PCR. The presence of WSSV can often be detected in apparently healthy PL by PCR (Lo *et al.*, 1996b; Magbanua *et al.*, 2000; Otta *et al.*, 2003). Ayub *et al.* (2008) reported that the PL samples from the affected tanks were positive for WSSV by one-step PCR, while those from the healthy tanks (not showing mortality) were positive only by nested PCR. In this study, among the two WSSV specific primers, the Lo F2R2 had smaller amplicon size being an inner primer of Lo F1R1 (Fig. 01).

As a result, this study suggested following the multiplex protocol based on the 16S rRNA and Lo F2R2 primer combination for the detection of WSSV with simultaneous presence of the internal control. Because of higher sensitivity of the inner (nested) primer being a smaller amplicon size, it may be useful in confirming the early stages of WSSV infection when the virus concentration is relatively low or before the manifestation of infection. Therefore, the samples that would be proved as negative by this multiplex protocol may only be certified as non-infected and the samples that would be proved as positive by this multiplex protocol may be certified as infected which would be useful to predict either an outbreak of the WSSV disease or the completion of the culture.

Again, among first four of the optimized multiplex PCR protocol, the combination of 16S rRNA and Lo F1R1 can also be used because of reduced cycling number, lower time consumption and good banding pattern with stronger intensity. It was showed that there need at least 30 cycles for this combination whereas each of the other combinations need at least 32 cycles which was time consuming. Again, the banding intensity of this protocol was comparatively stronger than the other combination. The recommended protocol also showed wide range of specificity amplifying different types of virus affected shrimp samples (Fig. 08 and 09). Thus ,this study also suggested following the protocols developed by the combination of 16S rRNA and Lo F1R1that can be used for rapid and routine screening whether WSSV positive or negative, if sensitivity is not a major concern.

Using the primers, two amplicons corresponding to 1447/941/1196/1192 bp and 414 bp were obtained in a single combination reaction amplified with the genomic DNA of infected shrimp. The amplification was done manipulating thermal cycling conditions and reagents combinations and 56°C-58°C for 30 sec of at least 30-32 cycles PCR amplification were observed. However, in terms of time and resource use this study strongly recommended the multiplex PCR assay using the 16S rRNA primer (for shrimp presence) and Lo F2R2 (for WSSV presence) primer combination. The study revealed that, when using the recommended multiplex protocol, an annealing temperature of 58°C for 30 sec at least 32 PCR cycles would result in good amplification efficiency.

The recommended multiplex PCR protocol has different dimensions in comparison with the worldwide developed and followed protocols using the same WSSV specific primer of the study. These protocols mainly based on two step nested PCR without amplifying host DNA. In comparison, a nested PCR assay developed by Lo *et al.* (1996) used an annealing temperature of 55°C and 60°C for the first and second PCR respectively. On the other hand, the one step PCR developed by Nunan and Lightner (2011) had an annealing temperature of 62°C using the Lo *et al.* (1996) second step/nested primers. Both of these assays had at least 40-60 cycles and required 3-5 hours for amplification. These assays were developed only for confirming the presence of WSSV.

Likewise, the OIE Manual of Diagnostic Tests for Aquatic Animals (2009) provides a standardized approach to the diagnosis of the diseases listed in the Aquatic Code. The nested PCR protocol in the OIE Manual of Diagnostic Tests for Aquatic Animals (2009) was developed by Lo *et al.* (1996) and this procedure is considered the reference standard for detection of WSSV by PCR (Nunan and Lightner, 2011). Most of the detection laboratories follow the OIE manual or its little modification worldwide. For example, OIE validated and certified WSSV detection kit named 'IQ2000^{TM} WSSV detection system' is frequently used by the WSSV screening laboratory of our country notably WorldFish under the AIN project (personal communication). The 'IQ2000^{TM} WSSV detection system' follows two step nested PCR amplification where a total of at least 45 cycles PCR amplification has been performed and approximately 25 µl of PCR mixture has been used. This procedure needs approximately 03 hours and 30 minutes which only detects the viral presence without confirming the presence of

host (shrimp). On the other hand, the optimized and recommended multiplex PCR technology of this study requires only 1 hour and 35 minutes to perform a single run; thus saving time and effort.

The recommended multiplex PCR protocol revealed the simultaneous detection of WSSV DNA and shrimp DNA in a single tube. The simultaneous detection of the protocol strongly ensures the presence of the virus in that host sample. The Lo *et al.* (1996) two step WSSV detection PCR, the modified Lo *et al.* (1996) procedure and Nunan and Lightner (2011) procedure established the protocols for detecting only WSSV. Likewise, most of the OIE recommended protocols (and Kit) can only detect WSSV presence employing two step PCR. The presence of the host is detected in another tube/reaction. In addition, all the above procedures suggest perform reaction allowing 20-25 µl PCR mixture in a reaction. In contrast, the recommended multiplex PCR assay needs only 10 µl PCR mixtures in a reaction and it can detect the presence of shrimp and WSSV simultaneously by performing a single reaction/run; this reduces the use of the resource. Again, a cost effectiveness analysis of the multiplex protocol revealed that this protocol was 35 times cheaper than the imported kit protocol (Table 09).

The recommended single step multiplex PCR test minimizes cross-contamination due to the fact of not having to open an amplified PCR sample for use as template in the second nested step. The OIE manuals, the Lo *et al.* (1996) procedures and the modified Lo *et al.* (1996) procedures requires two steps for nested PCR amplification and as a result there needs opening of the PCR tube, containing the amplified product from the first step PCR, and possibly introducing contamination into WSSV-free samples (Nunan and Lightner, 2011).

Regarding specificity, seven templates from a highly affected shrimp having seven different tissues were tested using another recommended protocol (Fig. 10). Furthermore, nine different random samples were tested using the protocol. All the tests were performed allowing a separate reaction having a WSSV positive sample. These tests revealed the higher degree of specificity for the recommended protocol.

Thus, it can be concluded that, the developed multiplex PCR protocol had at least six different dimensions. The dimensions were:

- It can detect the presence of WSSV with the presence of host (shrimp) simultaneously in a single tube reaction
- It has higher degree of specificity
- It has higher sensitivity
- No chance for cross-contamination because no need to open the tube for second step
- Time saving
- About 35 times cost effective

Chapter Six

CONCLUSION

6. Conclusion

In this study, a single step PCR assay to detect WSSV in shrimp was optimized to be used as multiplex PCR. The use of the assay enable to detect simultaneous presence of both WSSV and its internal positive control (shrimp) in a single reaction tube; thus, saving both time and resource during analysis. All the reagents and chemicals used for the protocol development had been procured from local suppliers. Using the primers developed by Lo *et al.* (1996) and Dieu *et al.* (2004), the study optimized and developed eight (08) different multiplex PCR protocol based on the eight (08) different combinations of the primers. Of the eight multiplex PCR protocol, first four could able to confirm simultaneous presence of WSSV with its host whereas another four protocols could able to confirm the presence of the both gene as well as to detect the infection level.

This study strongly showed that laboratory technicians can follow any one of the developed protocols for their routine work. Considering time, effort, cost, sensitivity and specificity, the study recommended following the multiplex protocol with the primer combination of 16S rRNA and Lo F2R2 (combination-2). If sensitivity is not the major problem, laboratory technicians can follow the multiplex protocol with the primer combination of 16S rRNA and Lo F1R1 (combination-1). Finally, the study also recommended following one of the semi nested multiplex PCR protocols with the primer combination of 16S rRNA and Lo F1R1R2 (combination-6) that would detect the severity of the infection.

In summary, it can be said that this study has adapted, optimized and validated a faster, non-stop single step multiplex PCR system targeting both WSSV and shrimp DNA in a single tube without employing costly imported kit. The developed multiplex PCR system using the published primers targeting both WSSV- and shrimp-specific DNA in a single tube can be used for rapid and routine screening of WSSV in shrimp PL, juvenile, adult. This can be used for screening the shrimp larvae/PL/adult for WSSV viral infection before stocking them in grow-out ponds or during different stages of their life cycles; thus preventing huge economic losses due to the WSSV virus. In addition, this multiplex PCR assay is useful for epidemiological study of the WSSV virus in shrimp and carriers in the country.

Chapter Seven

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7. References

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Chapter Eight

ANNEX

8. Annex

Annex I:

Spectrophotometric absorbance, concentration and purity of five extracted DNA from shrimp

Sample no	Absorbance		Concentration (µg/ml)	Purity (A_{260}/A_{280})
	260 nm	280 nm		
Sample-1	0.0401	0.0221	201	1.81
Sample-2	0.0395	0.0226	198	1.75
Sample-3	0.0411	0.0229	206	1.79
Sample-4	0.0406	0.0227	203	1.79
Sample-5	0.0410	0.0220	205	1.86
Average	**0.0405**	**0.0225**	**203**	**1.80**

*Dilution factor 100

- Each of the DNA was diluted to 20 times for each PCR mixture cocktail. So, each of the mixture get the concentration of approximately 10 ng/µl.

Annex-II:

Reagents, their preparation and cost

DNA Extraction Buffer (DEB)

Required amount:

An amount of 500 µl buffer was used for one (1) sample DNA extraction (Per run or per reaction). So, expected amount of buffer for 200 run was 100 ml (500 µl × 200 = 1, 00000 µl = 100 ml)

Composition: DEB-1 composed of 10 mM Tris-HCl, 25 mM EDTA, 100 mM NaCl and 0.5% (w/v) Sodium dodecyl sulfate

Preparation for expected amount

Tris-HCl (10 mM)

Molecular weight of Tris-HCl = 157.60 g (i.e., 1 Mole = 157.60 g/L). So, 157.60 g Tris-HCl incorporates in 1 L or 1000 ml water for the concentration of 1 mole or 1000 mM.

So, needed amount for 1000 mM Tris-HCl was = 157.60g

Needed amount for 10 mM Tris-HCl was = $\frac{157.60\times10}{1000}$ g = 1.58 g

As a result, for 10 mM Tris-HCl, 1.58 g was dissolved in 1000 ml

Now, 1000 ml water with 1.58 g Tris-HCl equivalent to 10 mM

100 ml water with $\frac{1.58\times100}{1000}$ g = 0.16 g Tris-HCl equivalent to 10 mM.

As a result, Tris-HCl of about 0.16 g was dissolved in 100 ml (200 run) distilled and sterile water for 10 mM final concentration.

EDTA (25 mM)

Molecular weight of EDTA= 292.24 g (i.e., 1 Mole = 292.24 g/L). So, 292.24 g EDTA incorporates in 1 L or 1000 ml water for the concentration of 1 mole or 1000 mM.

So, needed amount for 1000 mM EDTA was = 292.24 g

Needed amount for 25 mM EDTA was = $\frac{292.24 \times 25}{1000}$ g = 7.31 g

As a result, for 25 mM EDTA, 7.31 g was dissolved in 1000 ml

Now, 1000 ml water with 7.31 g EDTA equivalent to 25 mM

100 ml water with $\frac{7.31 \times 100}{1000}$ g = 0.73 g EDTA equivalent to 25 mM.

As a result, EDTA of about 0.73 g was dissolved in 100 ml (200 run) distilled and sterile water for 25 mM final concentration.

NaCl (100 mM)

Molecular weight of NaCl = 58.5 g (i.e., 1 Mole = 58.5 g/L). So, 58.5 g NaCl incorporates in 1 L or 1000 ml water for the concentration of 1 mole or 1000 mM.

So, needed amount for 1000 mM NaCl was = 58.5 g

Needed amount for 100 mM NaCl was = $\frac{58.5 \times 100}{1000}$ g = 5.85 g

As a result, for 100 mM NaCl, 5.85 g was dissolved in 1000 ml

Now, 1000 ml water with 5.85 g NaCl equivalent to 100 mM

100 ml water with $\frac{5.85 \times 100}{1000}$ g = 0.59 g NaCl equivalent to 100 mM.

As a result, NaCl of about 0.59 g was dissolved in 100 ml (200 run) distilled and sterile water for 100 mM final concentration.

Sodium dodecyl sulfate, SDS (1% or 0.5%)

Electrophoresis grade SDS was taken and 1 gm/0.5 gm from the reagent was dissolved in 100 ml of water. Heat to 65-70°C was adopted for dissolution. pH was adjusted.

TE buffer

Needed amount

Each reaction required 100 µl. So 200 reaction required = (100 µl × 200) = 20, 000 µl = 20 ml.

Composition

TE buffer was prepared with 10 mM Tris-Hcl and 1 mM EDTA dissolving de-ionized distilled water.

Preparation for expected amount

Tris-HCl (10 mM)

From the previous calculation,

1000 ml water with 1.58 g Tris-HCl equivalent to 10 mM

20 ml water with = $\frac{1.58\times20}{1000}$ = 0.03 g Tris-HCl equivalent to 10 mM

As a result, Tris-HCl of about 0.03 g was dissolved in 20 ml (200 run) distilled and sterile water for 10 mM final concentration.

EDTA (25 mM)

From the previous calculation,

Required amount for 1000 mM EDTA was = 292.24 g

Required amount for 1 mM EDTA was = $\frac{292.24\times1}{1000}$ g = 0.29 g

As a result, for 1 mM EDTA, 0.29 g was dissolved in 1000 ml

Now, 1000 ml water with 0.29 g EDTA equivalent to 1 mM

20 ml water with $\frac{0.29\times20}{1000}$ g = 0.01 g EDTA equivalent to 1 mM.

As a result, EDTA of about 0.01 g was dissolved in 20 ml (200 run) distilled and sterile water for 1 mM final concentration.

Ethanol (75%)

For 100 ml 75% ethanol, 75 ml 100% ethanol was mixed with 25 ml de-ionized distilled water.

PCR amplification recipes

Primers

Primer Dilution

Primers were received as dry, powder form (not visible because of low amount) from the suppliers. Appropriate amount of distilled water corresponding to the weight of individual primer (200-400 µl depending on nucleotide numbers) was added to obtain 100 pmole/µl stock solutions. A ten times (10) dilution was applied to obtain 10 pmole/µl working solution.

Required amount

Required concentration

Maximum 01 µl of each 10 pmole/µl diluted primer (F/R) was used

Premix

Premix was imported from TAKARA BIO INC. Japan. The premix was composed of the following recipes with concentration

Table: Recipes in imported premix with its final concentration

Recipes	**Final concentration**
Taq polymerase	0.25 units
PCR reaction buffer	1 X
$MgCl_2$	2 mM
dNTP's	200 µM

The premix was concentrated as 2X for which 5 µl of premix mixture was taken into the 10 µl PCR reaction (1 reaction) mixture to get the final concentration given the above table.

Gel Preparation

The gel preparation procedures are as follows:

- Agarose gel with a concentration of 1.5% was taken into a conical flask
- Flask containing the agarose was heated in a microwave oven for ~ 1-2 minutes to dissolve until appearing a translucent mixture
- Ethidium bromide (EtBr) with a concentration of 10 µg/ml was mixed by swirling the flask when temperature is 40-45°C.
- After leaving the gel at room temperature for 2 minutes for cooling, it was poured slowly into the gel tray. The comb of the tray was inserted with a correct position.
- The gel was allowed to set for at least 30 min to 1 hour and the newly prepared gel for electrophoresis was placed in the electrophoresis tank which was previously filled with TAE buffer.

Cost

Tris-HCl

Required for: Preparation of DNA extraction buffer for lysis

Unit cost: Tk. 3,000 for 100 g

Required amount: About 0.16 g was needed for 200 reactions

Total cost:

100 g Tris-Hcl costs = 3,000 Tk.

$$0.16 \text{ g Tris-HCl costs} = \frac{3000 \times 0.16}{100} \text{ Tk} = 4.80 \text{ Tk.}$$

$$0.03 \text{ g Tris-HCl costs} = \frac{3000 \times 0.03}{100} \text{ Tk} = 0.9 \text{ Tk} = 1 \text{ Tk}$$

EDTA

Required for: Preparation of DNA extraction buffer for lysis

Unit cost: Tk. 800 for 100 g

Required amount: About 0.73 g was needed for 200 reactions

Total cost:

100 g EDTA costs = 800 Tk.

0.73 g EDTA costs = $\frac{800\times0.73}{100}$ Tk = 5.84 Tk.

0.01 g EDTA costs = $\frac{800\times0.01}{100}$ Tk. =0.08 Tk = 0.10 Tk

NaCl

Required for: Preparation of DNA extraction buffer for lysis

Unit cost: Tk. 1,600 for 1000 g

Required amount: About 0.59 g was needed for 200 reactions

Total cost:

1000 g NaCl costs = 1,600 Tk.

0.59 g NaCl costs = $\frac{1600\times0.59}{1000}$ Tk = 0.94 Tk. = 1 Tk.

SDS

Required for: Preparation of DNA extraction buffer for lysis

Unit cost: Tk. 3,000 for 500 g

Required amount: About 1 g (Protocol 5) was needed for 200 reactions

Total cost: 500 g SDS costs = 3,000 Tk.

1 g SDS costs = $\frac{3000\times1}{500}$ Tk = 6 Tk.

Ethanol (100% + 75%)

Required for: DNA precipitation and wash for protocols 2 to 6

Unit cost: Tk. 2,800 for 2500 ml

Required amount: Average 200 ml for protocol 2 and 3 (Precipitation + Wash)

Average 250 ml for protocol 4 to 6 (Precipitation + Wash)

Total cost:

2500 ml Ethanol costs = 2,800 Tk.

$$200 \text{ ml Ethanol costs} = \frac{2800 \times 200}{2500} = 224 \text{ Tk.}$$

$$250 \text{ ml Ethanol costs} = \frac{2800 \times 250}{2500} = 280 \text{ Tk.}$$

Primers

In the optimized PCR

F primer was given to 1 ul of 10 pmole/ul (maximum)

R primer was given to 1 ul of 10 pmole/ul (maximum)

So, total primer was needed to 2 ul of a specific primer pair

Hence, it was possible to carry out 3000 reactions with a single primer pair imported from suppliers.

Now, required amount for 200 reactions = 400 µl of a primer pair

According to the local suppliers, 1 base pair of a primer set cost = 200 tk. (100 tk/base)

In the study, the total base pair of Lo F1+R1 = 22, Lo F2+R2 = 21 and 16s rRNA F+R = 20.

So, cost for Lo F1+R1 = 22×200 = 4400 Tk

Cost for Lo F2+R2 = 21×200 = 4200 Tk

Cost for 16s rRNA F+R = 20×200 = 4000 Tk

Hence, the actual cost for 200 reactions of Lo F1+R1 = $\frac{4400 \times 400}{6000}$ = 294 Tk.

$$\text{The actual cost for 200 reactions of Lo F2+R2} = \frac{4200 \times 400}{6000} = 280 \text{ Tk.}$$

$$\text{The actual cost for 200 reactions of 16s rRNA F+R} = \frac{4000 \times 400}{6000} = 267 \text{ Tk.}$$

Premix

One (1) set of Premix had 25000 µl solutions that cost 34,500 Tk.

Again, for 200 reaction, there was needed = 200×5 µl = 1000 µl

Now, 25000 µl premix cost = 34,500 Tk.

$$1000 \text{ µl premix cost} = \frac{34500 \times 1000}{25000} \text{ Tk} = 1{,}380 \text{ Tk.}$$

Gel electrophoresis recipes

DNA molecular weight marker (100 bp)

Required amount: about 2.5 µl can be used for on an average 20 PCR amplified sample/reaction

So, for 200 reactions, 25 µl was needed

Cost: 250 µl cost about 19,000 Tk

$$25\ \mu l \text{ cost about} = \frac{19000 \times 25}{250} \text{ Tk} = 1900 \text{ TK.}$$

Gel loading buffer

Required amount

About 0.25 µl per reaction, so 50 µl needed for 200 reactions

Cost: 1 ml 0r 1000 µl cost about 9000 Tk

$$\text{So, } 50\ \mu l \text{ cost about} = \frac{9000 \times 50}{1000} \text{ Tk} = 450 \text{ Tk.}$$