OPTIMIZATION OF DNA EXTRACTION PROTOCOL USING NON-PATENTED BULK REAGENTS

A Thesis

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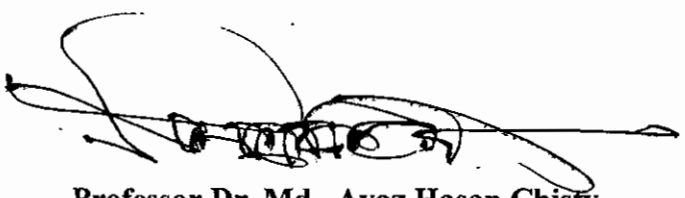

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Declaration

OPTIMIZATION OF DNA EXTRACTION PROTOCOL USING NON-PATENTED BULK REAGENTS

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 Sister.....

Acknowledgement

It is my great pleasure for being able to complete this thesis for which I am completely indebted to the creator of this universe, the Almighty Allah.

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Peya Dey
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Abstract

DNA-based detection methods for detection of penaeid shrimp viruses are used routinely at present in a number of laboratories around the world. Before going on track in this case extraction of DNA is obvious. Different methods have various effects on DNA extraction. An ideal extraction technique should optimize DNA yield, minimize DNA degradation, and be efficient in terms of cost, time and labor. Therefore which method is optimized one for WSSV infected shrimp among commonly used protocols was indispensable to justify. In the present study shrimp pleopod tissue was used, six protocols were applied and two protocols were optimized for DNA extraction. Three types of DNA extraction kits (Purelink Genomic DNA kit, IQ2000 WSSV Kit, DNAzol) and three types of DNA extraction buffer (0.5% SDS + Proteinase K, 0.5% SDS and 1% SDS) were used. All reagents' cost was analyzed for 200 times extraction. The time was analyzed to finish one extraction (excluding the time of PCR and gel elctrophoresis). DNA concentration was higher when Purelink® Genomic DNA kit was used but it was not time saving and procedure was complex than other methods and was an expensive method. Purity of DNA obtained from protocol-6 (DEB-3) was the best and result shows that there was no chance of protein and phenol contamination but it was most expensive method. In the term of cost and time protocol-4 and protocol-5 were standard methods.

Table of Contents

List of tables

List of figures

List of Abbreviations

Abbreviation	Meaning
A_{260} nm	Absorbance at 260 nanometere
A_{280} nm	Absorbance at 280 nanometere
$A_{260/280}$	ratio of A_{260} nm and A_{280} nm
DEB	DNA Extraction Buffer
DOF	Depertment of Fiaheries
DNA	Deoxyribonucleic acid
Nm	Nanometer
µl	Microliter
µg	Microgram
PCR	Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulfate

CHAPTER ONE

Introduction

Chapter 1

Introduction

Fisheries sector contributes 4.39% to the Gross Domestic Product (GDP). More than 11% people of total population earn their livelihood from this sector (DOF, 2013). Shrimp farming and related activities contribute significantly to the national economy of Bangladesh. More than 2 million people are engaged directly and indirectly in upstream and downstream activities related to shrimp industry in the country (DoF, 2012).

A significant area of southern part is under cultivation of shrimp culture. The main cultured species is the tiger shrimp (*Penaeus monodon*). It is a marine shrimp and is cultivated in salty water. The next most important cultured species for export is fresh water variety, giant prawn locally called golda, (*Macrobrachium rosenbergii)*.

Successful shrimp culture depends mainly on the availability of healthy and quality shrimp seeds. Disease outbreaks are recognized as a significant constraint to shrimp production and trade, affecting both the economic development and socioeconomic revenue of the sector in many countries in the world. The sustainability of this important sector, however, largely depends on addressing a number of challenges among which the occurrence of disease is most crucial one.

Being a very sensitive organism, shrimp can be easily infected by bacterial, viral and parasitic pathogens due to lack of suitable environment, nutrition etc. Among the infectious diseases of cultured shrimp, certain virus-caused diseases stand out as the most significant.

White Spot Syndrome disease is a major viral shrimp disease is caused by white spot syndrome virus (WSSV) in black tiger shrimp (*P. monodon*). Although fresh water giant prawn is not prone to WSSV, they can only act as a carrier. Shrimp occurs 100% mortality within a week after the emergence of gross signs.

At present, no treatments are available to control the disease and mortality. The only alternative to reduce the risk of WSSV entry into commercial shrimp production

facilities is the stocking of virus free healthy PL. Good disease screening facilities using sensitive molecular biology based detection techniques have to be implemented.

The effective control and treatment of diseases of aquatic animals requires access to diagnostic tests that are rapid, reliable and highly sensitive. In many cases, post-mortem necropsy and histopathology have been the primary methods for the diagnosis of fish and shellfish diseases. However, these methods often lack specificity and many pathogens are difficult to detect when present in low numbers or when there are no clinical signs of disease (Ambrosia and De Wall, 1990). Direct culture of pathogens is also widely used. However, these methods are time-consuming and costly, and, for shrimp, cell lines suitable for virus culture have not been available (FAO, 1999).

Efforts to overcome these problems have led to the development of immunoassay and DNA-based diagnostic methods including fluorescent and polymerase chain reaction (PCR) amplification techniques. The use of DNA-based methods derives from the premise that each species of pathogen carries unique DNA or RNA sequences that differentiate it from other organisms. The techniques offer high sensitivity and specificity, and diagnostics kits allowing rapid screening for the presence of pathogen DNA are moving rapidly from development in specialized laboratories to routine application. DNA probes are expected to find increasing use in routine disease monitoring and treatment programs in aquaculture, in field epidemiology and in efforts to prevent the spread of pathogens (FAO, 1999).

WSSV virus can be detected by the DNA extraction from hepatopancreas, muscle and pleopod of shrimp with the help of PCR technology (Paul, 1997). In this study muscle from pleopod was used.

DNA Extraction is just isolation and purification nucleic acids that only contain DNA but not RNA from sample using a combination of physical and chemical methods. It is an essential part of a successful PCR amplification. The quality and quantity of DNA also affects the PCR result.

The basic steps involved in all DNA extraction methods consist of the following:

1. **Cell lysis:** The first step involves disrupting the cell to access the DNA. This can be done using chemical or physical methods.

2. **Lipid removal:** Removal or separation of membrane lipids and cell debris. This is usually done by using detergents such as sodium dodecyl sulfate, and by centrifugation.

3. **Deproteinize cell extract:** Protein denaturation is done using a protease such as proteinase K. Subsequently denatured protein is separated from the cell extract.

4. **RNA removal:** Removal of RNA is done by adding an RNase, which rapidly degrades RNA into ribonucleotide subunits. This step is usually optional within most kits.

5. **DNA precipitation/aggregation/elution.**

The most common methods of DNA extraction are:

Chelex DNA extraction

Chelex resin is often used for DNA extraction in preparation for PCR. The ChelexTM protects the sample from DNAases that might remain active after the boiling and could subsequently destroy the DNA. Its advantages are that it is cheap, quick, has a low contamination rate and does not use any dangerous chemicals. However, its disadvantages include being inefficient for use on blood samples, producing low purity DNA samples.

Phenol - Chloroform extraction method

Phenol chloroform extraction is a liquid-liquid extraction. A liquid-liquid extraction is a method that separates mixtures of molecules based on the differential solubilities of the individual molecules in two different immiscible liquids.

Effective at extracting the large amounts of high molecular weight DNA. However, this method does also have some disadvantages including being very labor intensive, being easily contaminated.

Silica based DNA extraction methods

Silica DNA extraction methods have become the staple for many labs as it is quick reliable and produces very high quality DNA. This method is based on the selective adsorption of nucleic acids to a silica-gel membrane in the presence of high concentrations of chaotropic salts. This method is time saving, reliable and produces very high quality DNA.

Homemade DNA extraction protocols are cheap and very efficient to recollect DNA from samples, but are usually time-consuming; commercial kits are generally very fast but too expensive and generate large quantities of contaminant wastes, such as beads, filters, columns and microcentrifuge tubes. An ideal protocol should optimize DNA yield, minimize degradation, and be efficient in terms of cost, time, labor, and supplies (Chen *et al.*, 2010).

In case of shrimp the application of DNA extraction protocol will be viable when the quality and quantity of extracted DNA will be suitable for diagnostic purposes. However the cost of reagents and time consumed to run a protocol are other important subjects to consider any protocol as feasible. It is possible to extract DNA from suspected shrimp PL with a single pre formulated reagent that can be used in the field. The cost for PCR screening could be substantially reduced if bulk reagents are used instead of commercial WSSV detection kits. Therefore the objectives of the study were:

- To develop a DNA extraction protocol for shrimp tissue that involves low-cost, bulk available, non-hazardous chemicals and that is less manipulative
- To compare the extraction protocol with established protocols and commercial kit based protocols.

CHAPTER TWO

Litetrature Review

Chapter 2

Literature Review

2.1. Protocols for DNA extraction

Literally hundreds of protocols for DNA preparation from various sources of tissue have been published over the last few decades. So far there is no common and simple procedure for genomic DNA extraction that can be used on a large scale for different eukaryotic organisms. Usually different tissues require different protocols and different tissue preparation steps (Aljanabi and Martinez, 1997).

Similarly various protocols have been developed to extract DNA for the PCR detection of WSSV in shrimp by different workers (Nonaka *et al.,* 1998).

A simple method of extraction of white spot syndrome viral DNA (WSSV) from infected shrimp was described by Yoganandhan *et al.*, (2003) for the polymerase chain reaction (PCR) detection of WSSV. They used NTE buffer (0.2M NaCl, 0.02M Tris-HCl and 0.02M EDTA, pH 7.4). The DNA preparation using this method was found to be free from the host DNA, RNA and protein. This method of extraction has worked successfully for extracting the WSSV-DNA from different organs (haemolymph, eyestalk, carapace, head muscle, heart, gills, appendages, hepatopancreas, stomach, intestine, abdominal muscle and tail muscle) of WSSV-infected adult shrimp, and WSSV-infected larvae and postlarvae.

Aljanabi and Martinez (1997) extracted DNA from shrimp by using Proteinase K and reported that this method did not require expensive and environmentally hazardous reagents and equipment. Even it could be performed in low technology laboratories.

Bruce *et al.*, (1991) described procedures for the purification of virions and nucleocapsids of *Baculovirus penaei* of penaeid shrimp and subsequent extraction of the viral nucleic acid. *Baculovirus penaei* infected hepatopancrata, from two species of shrimp from different geographical locations in the Americas, were removed and homogenized in a solution of TN buffer (0.01 M Tris-HCl, 0.10 M NaCl, pH 8.0). The homogenized mixture was strained through a 100-mesh screen to remove large

pieces of tissue and centrifuged to concentrate the remaining material. The pellet was suspended in TN buffer and layered on to a handmade CsCl gradient.

In the study of Biswas (2007), optimization of DNA extraction protocol from shrimp tissue was described. In this study DNA was extracted using six different protocols from shrimp muscle and hepatopancreas tissue. DNAzol with and without RNAse treatment, DNA extraction buffer with and without RNAse treatment, Proteinase K isolation technique and SDS isolation method were used to extract DNA. From the study it was found that highest DNA was obtained from proteinase K but it was time consuming, DEB with RNAse aws the best method with satisfactory quantity and quality.

In a study Tsai *et al.*, (2012) detected white spot syndrome virus by polymerase chain reaction performed under insulated isothermal conditions to develop a rapid, low-cost, and user-friendly system for the diagnosis of white spot syndrome virus (WSSV), a PCR assay performed in capillary tubes under insulated isothermal conditions. WSSV-specific ampli-cons were produced from nucleic acid extracts of WSSV-infected Penaeus vannamei in the WSSV iiPCR assay, with sensitivity comparable to that of an OIE-certified commercial nested PCR kit (IQ2000TM WSSV Detection and Prevention System).

Cavalli *et al.*, (2008.) evaluated WSSV in wild shrimp after a major outbreak in shrimp farms at laguna, southern Brazil. In the study 50-100 mg of shrimp gill tissue was used for DNA extraction. DNAzol reagent (Invitrogen) was used, according to manufacturer instructions, with some modification.

Chen *et al.*, (2010) compared five Methods for Total DNA Extraction from Western Corn Rootworm Beetles. From individual western corn rootworm beetles, *Diabrotica virgifera virgifera*, DNA was extracted by the SDS method, CTAB method, DNAzol^{R} reagent, Puregene^{R} solutions and DNeasy^{R} column and were compared in terms of DNA quantity and quality, cost of materials, and time consumed. Although all five methods resulted in acceptable DNA concentrations and absorbance ratios, the SDS and CTAB methods resulted in higher DNA yield at much lower cost and less degradation as revealed on agarose gels. The DNeasy^{R} kit was most time-efficient but was the costliest among the methods tested. The effects of ethanol volume,

temperature and incubation time on precipitation of DNA were also investigated. The DNA samples obtained by the five methods were tested in PCR for six microsatellites located in various positions of the beetle's genome, and all samples showed successful amplifications.

In a recent study Muegue *et al.*, (2013) Optimized PCR protocols for the detection of viral pathogens in shrimp aquaculture in the Philippines. Different tissues of shrimps were used for DNA extraction. They used Proteinase K and DNA extraction buffer with the following composition; 10 mM Tris, 125 mM NaCl, 10 mM EDTA, 0.5% SDS and 4M Urea. For the postlarvae samples, an additional step of eye stalk removal was employed to remove inhibitors.

PCR inhibitors were noted by Belcher & Young (1998) to be present in DNA samples prepared from whole MBV-infected PL Penaeus monodon when using the extraction method recommended by Wang *et al.* (1996) for BP, which incorporates proteinase K. However, when hot phenol was used to extract the DNA, this inhibitory effect was removed.

Tang and Lightner (2000) worked on Quantification of white spot syndrome virus DNA through a competitive polymerase chain reaction. In this study total DNA was extracted, from shrimp hemolymph and tissues, with a High Pure ^{TM} PCR template preparation kit (Roche-Boehringer Mannheim, Germany).

Islam *et al.*, (2006) used the guanidium isothiocyanate (DNAzol) to extract DNA from muscle, hepatopancreas and pleopod tissue of shrimp (penaeus monodon) for PCR operation.

IQ2000TM WSSV Detection and Prevention System which can differentiate infected shrimps into 4 different levels of infection: very light, light, medium, and severe. The diagnostic results produced by IQ2000TM Systems are very helpful for shrimp disease control program in the shrimp farming industry. To provide a more convenient reaction condition and time saving procedure for multi-viral diagnosis, IQ2000TM WSSV Detection and Prevention System can be performed not only by its own PCR reaction condition but also by the Uni-IQ Program, a universal PCR profile which can be used for all IQ2000TM Systems. Under this reaction profile, different

kinds of shrimp viral diseases can be screened at the same batch of PCR reaction. (IQ2000TM WSSV Detection and Prevention System, 2013)

Mega and Revers (2011) worked on developing a rapid, efficient and low cost method for rapid DNA extraction from arthropods. They presented a rapid and efficient method to obtain good quality DNA from small samples of arthropod tissues generating low quantities of hazardous wastes. This new method was compared with another homemade protocol using phenol and other two commercial kits. The quality of DNA obtained was checked by spectrophotometer and evaluated by an AFLP assay. Homemade protocols were more efficient in recollect DNA than commercial kits, without lose any quality of samples. Also, they were less time and fund consuming, with costs ten times cheaper than commercial kits.

2.2. Detemination of quantity of DNA

Reliable measurement of DNA concentration and purity is important for many applications in molecular biology. The concentration of extracted DNA can be determined in three different ways: by examining UV absorbance with spectrophotometer, by fluorimetry, by comparison with DNA standards on agarose gel (Aquadro *et al.*, 1998).

The most common technique to determine DNA yield and purity is absorbance by spectrophotometer (Aquadro *et al.*, 1998). The absorbance of the sample at specific wavelengths is measured, A_{260} (absorbance at 260 nm), to measure nucleic acid, and A_{280} to measure contaminating protein in the sample. Based upon the absorbance readings, the concentration of the sample is determined, and A_{260}/A_{280} ratios are calculated to indicate sample purity. The maximum absorbance of nucleic acids occurs at a wavelength of 260 nm. It is generally accepted that DNA or relative purity will yield an A_{260}/A_{280} ratio of $\geq$1.8 on a scale with a maximum of 2.0. (Leninger, *et al.*,1975).

Agarose gel electrophoresis is a commonly used method to separate DNA and RNA molecules of different sizes but can also be used to assess DNA quality and provides useful information regarding the amount of DNA in a solution. Since DNA has a consistent charge-to-mass ratio, the major factor that influences its migration through a gel matrix is size. (Sambrook and Russell, 2001)

CHAPTER THREE

Materials and Methods

Chapter 3

Material and Methods

The Experiment was conducted at Molecular Biology Laboratory of Fisheries and Marine Resource Technology (FMRT) Discipline of Khulna University from June to December 2013.

3.1. Study area

Sample (fresh shrimp) was collected from a shrimp farm of Satkhira

3.2. Sample collection

Fresh shrimp, *Penaeus monodon* was collected from each farm which was kept in individual polythene bag. Then the bags were kept into ice. As soon as possible, the sample containing polythene bags were transported to Molecular Biology Laboratory. Pleopods of the collected shrimp were cut with sterile stainless blades separately for ensuring no contamination and 25-30 mg tissue from each pleopod was weighed out for extraction of DNA

3.3. Genomic DNA extraction

To optimize DNA extraction protocol for Shrimp sample, six different methods were taken into consideration. Out of six methods, first three were commercial kit based and rests of the three were non-patented bulk reagents based that can be affordable by the farmers of shrimp sector. The six applied protocols were-

- Protocol-1: Purelink® Genomic DNA kit
- Protocol-2: DNAzol treatment
- Protocol-3: IQ2000 WSSV Kit
- Protocol-4: DNA Extraction Buffer -1 (DEB-1)
- Protocol-5: DNA Extraction Buffer -2 (DEB-2)
- Protocol-6: DNA Extraction Buffer -3 (DEB-3)

3.4. Overview of the protocols applied

1. PureLink® Genomic DNA Kits

- ≤25 mg of tissue was placed into a sterile microcentrifuge tube
- 180 µL PureLink® Genomic Digestion Buffer and 20 µL Proteinase K (supplied with the kit) was added to the tube, ensuring the tissue is completely immersed in the buffer mix. Then it was mixed by mixing 180 µL Digestion Buffer and 20 µL Proteinase K for each sample.
- It was incubated at 55°C with occasional vortexing until lysis is complete (1–4 hours).
- To remove any particulate materials, the lysate was centrifuged at maximum speed for 3 minutes at room temperature. Supernatant was transferred to a new, sterile microcentrifuge tube.
- 20 µL RNase A (supplied in the kit) was added to the lysate, was mixed well by brief vortexing, and incubated at room temperature for 2 minutes.
- 200 µL PureLink® Genomic Lysis/Binding Buffer was added and mixed well by vortexing.
- 200 µL 96–100% ethanol was added to the lysate, Mixed well by vortexing for 5 seconds.
- A PureLink® Spin Column was removed in a Collection Tube from the package.
- The lysate (~640 µL) prepared with PureLink® Genomic Lysis/Binding Buffer and ethanol was added to the PureLink® Spin Column.
- Column was centrifuged at 10,000 × g for 1 minute at room temperature.
- The collection tube was discarded and the spin column was placed into a clean PureLink® Collection Tube supplied with the kit.
- 500 µL Wash Buffer 1 prepared with ethanol was added to the column.
- It was centrifuged at 10,000 × g for 1 minute.
- The collection tube was discarded and the spin column was placed into a clean PureLink® collection tube supplied with the kit.
- 500 µL Wash Buffer 2 prepared with ethanol was added to the column.
- The column was centrifuged at maximum speed for 3 minutes at room temperature. Collection tube was discarded.

- The spin column was placed in a sterile 1.5-mL microcentrifuge tube.
- 25–200 µL of PureLink® Genomic Elution Buffer was added to the column.
- It was incubated at room temperature for 1 minute. The column was centrifuged at maximum speed for 1 minute at room temperature. The tube contains purified genomic DNA.
- To recover more DNA, a second elution step was performed using the same elution buffer volume as first elution in another sterile, 1.5-mL microcentrifuge tube.
- The column was centrifuged at maximum speed for 1.5 minutes at room temperature and the tube containing purified DNA was collected.
- The purified DNA was stored at –20°C

2. DNAzol Treatment

- 25-30 mg shrimp sample (from pleopod) was taken into 1.5ml tube
- 1ml DNAzol,was added and grinded it with a disposable grinder and vortex slightly
- The lysate was centrifuged at maximum speed for 5 minutes at room temperature. supernatant (~900 µL) was transferred to a new, sterile microcentrifuge tube
- 500 µl 100% alcohol was added into the tube mixed by inversion and kept it in room temperature for 3-5 minutes. Then it was centrifuged at 10000g for 3 minutes, and then decanted the ethanol and 500 µl of 75% alcohol was added. Finally, was centrifuged at 10000g for 3 minutes, then was decanted and dried.
- The pellet was dissolved by TE buffer.

3. IQ2000 WSSV DNA extraction protocol

DNA extracted by Lysis Buffer

- 500 µl Lysis Buffer was added in a 1.5ml tube.
- Shrimp sample was placed into the tube and grinded it with a disposable grinder.
- The prepared sample was incubated at 95-100°C for 10 minutes, then centrifuged at 12000g (12000 rpm r=5~7cm) for 10 minutes.

- 200 µl of the upper clear solution was transfrred to a fresh 1.5ml tube with 400 µl 95% ethanol.
- Vortexing was done, it was centrifuged at 12000g for 5 minutes, then decanted the ethanol and the pellet was dried.
- Pellet was dissolved by TE buffer.

From protocol 4 to 6, the main extraction buffers were prepared using different reagents which were non-patented and available in the market. The reagents were bought from market separately and prepared proper way with appropriate calculation.

4. DEB-1 protocol

Composition of DEB-1:

1. Tris (10mM)

2. EDTA (25mM)

3. NaCl (100mM)

4. SDS (0.5%)

In this method, 0.5% SDS buffer was used. The methods were as follows

- 25-30 mg shrimp sample was taken (from pleopod) into 1.5ml tube
- 500 µl 0.5% SDS was added in the tube and it was grinded with a disposable grinder.
- It was incubated at 100°C with occasional vortexing until lysis is complete (About10 m)
- The lysate was centrifuged at maximum speed for 3 minutes at room temperature. supernatant (~400 µL) was transferred to a new, sterile microcentrifuge tube
- 800 µl 100% alcohol was added into the tube and kept it in room temperature for 3-5 minutes. It was centrifuged at 10000g for 3 minutes, then the ethanol was decanted and 800 µl of 75% alcohol was added. Finally, it was centrifuged at 10000g for 3 minutes, then was decanted and dried.
- The pellet was dissolved by TE buffer.

5. DEB-2 protocol

Composition of DEB-2:

1. Tris (10mM)

2. EDTA (25mM)

3. NaCl (100mM)

4. SDS (1%)

In this protocol, 1% SDS buffer was used in lieu of 0.5% SDS. The methods were as follows:

- 25-30 mg tissue sample was grinded with 500 µl 1% SDS in a centrifuge tube
- was kept at 100^0C heat block for 10 minutes
- Then it was centrifuged for 10 minutes at 10,000 rpm
- It was kept for 5min
- Was centrifuged for 10min.
- 75% alcohol was added to this
- All alcohol was carefµl ly dried at room temperature.
- 50µl TE was added

6. DEB-3 protocol

Composition of DEB-3:

In this method, 0.5% SDS was used with Proteinase-K. The methods were as follows:

- 25-30 mg shrimp sample was taken (from pleopod) into 1.5ml tube
- 180 µl 0.5% SDS and 20 µl proteinase-k was added in the tube and grinded with a disposable grinder.
- It was incubated at 55°C with occasional vortexing until lysis is complete (About 1 hour)
- The lysate was centrifuged at maximum speed for 3 minutes at room temperature. supernatant (~200 µL) was transferred to a new, sterile microcentrifuge tube

- 400 µl 100% alcohol was added into the tube and kept it in room temperature for 3-5 minutes. It was centrifuged at 10000g for 3 minutes, then ethanol was decanted and add 400 µl of 75% alcohol. Finally, was centrifuged at 10000g for 3 minutes, and then it was decanted and dried.
- The pellet was dissolved by 50 µl TE buffer.

3.5. Determination of DNA purity

DNA quality and quantity were determined spetrophotometrically. The concentration (C) was therefore determined (Aquadro *et al.*, 1998) by:

C = A260 (observed) × 50 µg/ml ×dilution factor for measured sample, given that a 50 µg/ml solution of double stranded DNA has an absorbance of 1.0 at 260 nm.

$$\text{Where, dilution factor} = \frac{\text{Amount of solvent for dilution}}{\text{Amount of original sample}}$$

The concentrations of DNA sample were determined from the absorbance at 260 nm (A_{260}).The DNA purity was found by using following formula.Purity= A_{260}/A_{280}

3.6. DNA amplification in PCR:

Two sets of primers (Lo, 18S) were used for the detection of WSSV. PCR reactions were carried out in a 10 µl reaction mixture that consisted of, 0.5 µl of each primer (10 pmol/ µl), 5 µl of premix, 3 µl 20 times diluted of template DNA.

All of the components were mixed together in one tube. The reaction was carried out in an automated machine, known as a thermocycler, which is capable of rapidly increasing and decreasing the temperature. The cycling conditions for the PCR reaction consisted of an initial 94°C for 25 sec, followed by 32 cycles of 94°C for 20 sec, 62°C for 40 sec and 72°C for 60 sec, with a final extension at 72°C for 5 min.

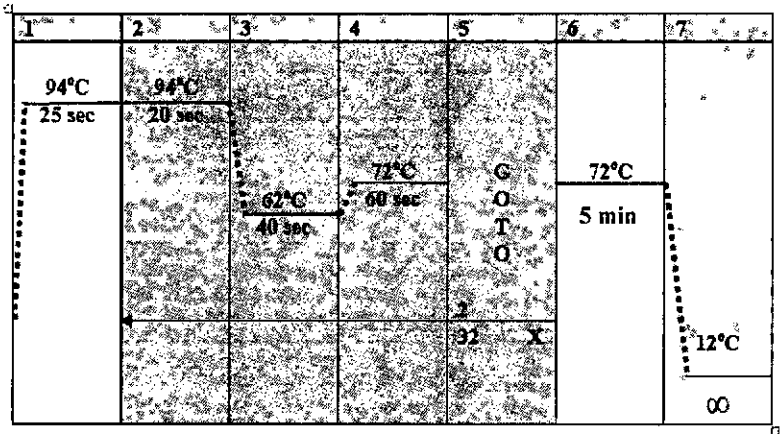

Figure 01: PCR thermal cycling conditions

3.7. Gel electrophoresis

Agarose gels act as a matrix which separate DNA based on size. PCR products were analysed by electrophoresis in1.5% agarose gel stained with ethidium bromide and visualized by ultraviolet transillumination. 100bp marker was used.

- At first 100 mL of 1X TAE was measured
- 1.5g agarose was measured
- Then it was mixed with TAE in a conical flask and was melted completely in microwave
- When agarose was completely dissolved, it was allowed to cool slowly.
- 10 µl Ethidium Bromide was carefully added in this
- Comb was placed in the tray
- Then it was carefully poured in the tray
- Comb was removed from gel by pulling straight up
- Gel was placed into running chamber,
- 5 µL of sample and loading dye was mixed

- Desired DNA ladder, samples were loaded into wells
- Gel was run for about 30 min.

CHAPTER FOUR

Results

Chapter 4

Results

Optimization of the DNA extraction protocols relied on the cost, time consumed concentration and purity i.e. whether the protein, ethanol or RNA contamination (from the ratio of A_{260nm} and A_{280nm}) existed. DNA concentration of DNA obtained by each protocol are listed in Table-1.

Table 1: Concentration(ng/µl) and purity of DNA obtained from six protocols.

Protocol no.	**Protocol name**	**A_{260}**	**$A_{260/280}$**	**Con. (ng/µl)**
01	Purelink® Genomic DNA kit	0.19	1.86	712.5
02	DNAzol treatment	0.17	1.74	637.5
03	WSSV Kit	0.11	1.66	412.5
04	DEB-1	0.13	1.7	487.5
05	DEB-2	0.09	1.62	337.5
06	DEB-3	0.18	1.80	675.0

The cost for each method was estimated based on the price of Chemicals, (excluding disposable items) consumed for 200 reactions. The time required to finish one

extraction, using each method was estimated based on the procedures used in this study, excluding the time for PCR and gel electrophoresis.

The estimated cost in BDT (Tk) and time in hours for each method to extract DNA from shrimp are listed in Table 2. Due to the much lower expenses of the laboratory prepared DEB-1 and DEB-2 these two methods were less costly than the three commercial kits and DEB-3. DEB-3 was the most expensive among the six methods

Table 2: Probable time and cost required for developing different protocols

Protocol no.	**Protocol name**	**Cost(200 reactions)**	**Approximate time consumed for 1 reaction (hour)**
1	Purelink ® Genomic DNA kit	108000 Tk	2
2	DNAzol treatment	68000 Tk	1.5
3	IQ2000 WSSV Kit	73257.36 Tk	1.5
4	DEB-1	144.1668 Tk	0.5
5	DEB-2	148.1668 Tk	1
6	DEB-3	1,32144.166 Tk	1.5

From the study it was observed that DNA concentration was higher when Purelink genomic kit was used and was lower when DNA extraction buffer (1% SDS) was used. But Purelink kit was not cost effective. Among the six protocols DEB-1 and DEB-2 treatment was cost effective than other four methods. Protocol-6 was the most expensive method. Protocol-1 required more time than other fives. Protocol-2, Protocol-3 and Protocol-6 required same time to extract DNA. Protocol-4 required least time.

PCR reaction results:

Typical examples of DNA extraction using the six methods visualized on a 1.5% agarose gel are presented in Figure-3. Bands of DNA were formed at 1447bp (Lo primer) and 447bp (18S primer). All protocols showed positive result for WSSV.

Lane 1-6 indicates DNA obtained from protocol 1-6 respectively. Lane-7 indicates reagent control. If the result of reagent control showed a band at 1447bp or 447bp it means that contamination has occurred.

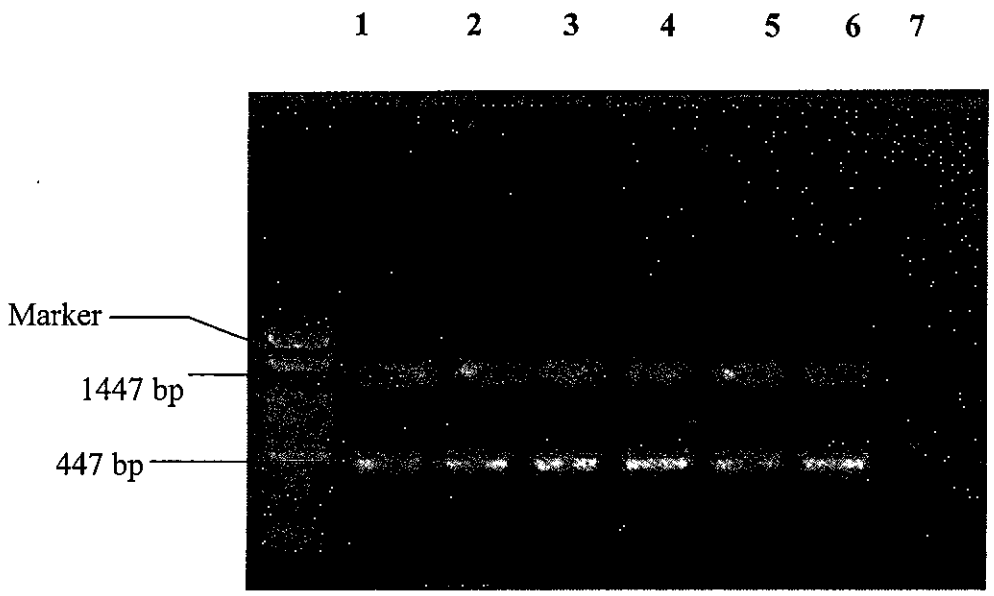

Figure 02: Detection of white spot syndrome virus by polymerase chain reaction in a 1.5% agarose gel

CHAPTER FIVE

Discussion

Chapter 5

Discussion

DNA yield rate, cost, time varied within each extraction method and across the six methods. To compare the efficiency of the DNA extraction methods, cost, time and the DNA yield based on the DNA concentration from each method was calculated. The DNA quality can be said best whether the ratio of A_{260} and A_{280} is around 1.8 (Ligozzi and Fontana, 2003; Hillis *et al.,* 1990). Lower ratios indicate protein or phenol contamination (Hillis *et al.*, 1990)

DNA yield and absorbance ratios for the six methods of DNA extraction are listed in Table 1. The extraction method had a significant effect on the yield of DNA.

The PureLink™ Genomic DNA Kits allow rapid and efficient purification of genomic DNA (gDNA). The kit is designed to efficiently isolate genomic DNA. (PureLink Genomic DNA Mini Kit Catalog, 2007).

DNA concentration was higher (712.5µg/ml) when Purelink ® Genomic DNA kit was used but in this study it required more time than any other method. Cost of this protocol was more than other five protocols.

DNA concentration was 675µg/ml when DEB-3 was used. In this method the ratio of A_{260} and A_{280} was 1.8. Theory says that there is no chance of protein contamination due to the protein digestion by proteinase K (Milligan, 1998).

It is perhaps the most widely used method of isolation DNA, particularly from animal sources, is based on solubilization of the tissue in a detergent buffer usually containing SDS, and followed by proteinase digestion of cellular proteins often with proteinase K (Flournoy *et al.*, 1996; Shibata, 1994; Hillis *et al.*, 1990; Kawasaki, 1990; Apples and Moran, 1984).

DNAzol is a complete and ready to use reagent for the isolation of genomic DNA based on the use of a novel guanidine-detergent lysing solution that hydrolyzes RNA and allows the selective precipitation of DNA from a cell lysate (Gupta and Preet, 2012). In this study it was found that cost of DNAzol was more than protocol-4 and protocol-5 but less than other 3 methods

Working in collaboration with National Taiwan University, Gene Reach has successfully developed IQ2000^{TM} WSSV Detection and Prevention System is very helpful for shrimp disease control program in the shrimp farming industry.

To provide a more convenient reaction condition and time saving procedure for multi-viral diagnosis, IQ2000^{TM} WSSV Detection and Prevention System can be performed not only by its own PCR reaction condition but also by the Uni-IQ Program, a universal PCR profile which can be used for all IQ2000^{TM} Systems.

The validation data for this kit have been certified by the OIE, based on expert review, as fit for:

1) To certify freedom from infection (<10 virions/sample) in individual animals or products for trade/movement purposes;

2) To confirm diagnosis of suspect or clinical cases (confirmation of a diagnosis by histopathology or clinical signs);

3) To estimate prevalence of infection to facilitate risk analysis (surveys/herd health schemes/disease control). (IQ2000^{TM} WSSV Instruction Manual, 2013)

Gene Reach has developed IQ Plus^{TM} WSSV Kit with POCKIT System based on iiPCR technology, which is highly sensitive and specific for WSSV detection, and is comparable to IQ2000TM viral detection system. IQ Plus^{TM} WSSV Kit with POCKIT System is specially designed for on-site viral detection in the farm. The assay has been simplified for easier and faster operation with the use of compact and portable equipments for WSSV detection at pond-side. (IQ Plus™ WSSV Kit with POCKIT System User Manual, 2013). The present study revealed that IQ2000TM WSSV kit was less expensive and time saving than PureLink™ Genomic DNA Kit. DNA concentration from this protocol was 412.5 µg/ml which was higher than protocol-2, protocol-4 and protocol-5 but lower than other two protocols.

Detergents are used to solubilize the cell membranes. Popular choices are SDS (Sodium dodecyl sulfate), Triton X-100, and CTAB, (Ausbel *et al.,* 1998).

SDS is strong anionic detergent. It removes the negative ions from the protein and destroys its confirmation. From the study of (Kabir *et al.*, 2006) it was found that to extract genomic DNA, high salt SDS based DNA extraction method was followed because of the availability of the reagents and less time consuming than others. The present study revealed that DEB-1 and DEB-2 was cost effective and DEB-1 was less time consuming than others. In these two methods the ratio of A_{260} and A_{280} was 1.7 (protocol-4) and 1.62 (protocol-5).It indicates that there was protein contamination.

From the present study it as found that cost of DEB-1 (protocol-4) and DEB-2 (protocol-5) was satisfactory than other four methods. Protocol-4 was most time saving and Protocol-1 was less time saving. Rest all were in the range of 1-1.5 hour.

CHAPTER SIX

Conclusion

Chapter 6

Conclusion

This study has optimized DNA extraction protocols for the PCR detection of the commercially important viral pathogen of shrimp using non patented bulk reagents.

From this study it is concluded that the protocol presented here were good methods to obtain high quality DNA from WSSV infected shrimp tissue. These methods can be used for routine procedures in shrimp health or diagnostic laboratories as a management tool for the prevention and control of viral diseases in shrimp aquaculture in the country through early detection of the pathogen.

By reason of having comparatively easy procedure with the obtained results the DEB-1 (protocol-4) and DEB-2 (protocol -5) were standard methods. These protocols were effective, time saving and most economic. DEB-3 was the most expensive among the six methods.

DNA concentration was higher when Purelink® Genomic DNA kit was used. It was not time saving, as well complex procedure than other methods and an expensive method. Purity of DNA obtained from protocol-6 (DEB-3) was the best and result shows that there was no chance of protein and phenol contamination but it was most expensive method. Most of all, it is concluded that protocol-4 and protocol-5 were the best methods.

CHAPTER SEVEN

References

Chapter 7

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ANNEX

Cost for DNA extraction

Protocol-1: PureLink® Genomic DNA Kits:

Price of 1 (50 run) DNA extraction kit: 27,000 Tk

Then, price of 4 kits (200 run): 108000 Tk

Protocol-2: DNAzol treatment

DNAzol extraction buffer

Price of 100 ml = 34000 Tk

Then, Price of 1 ml = $\frac{34000}{100}$ Tk

Then, Price of 200 ml (200 run) = $\frac{34000\times200}{100}$ Tk

= 68000 Tk

Protocol-3: IQ2000 WSSV Kit 73,257.36 Tk for 100ml (200 reactions)

Protocol-4: DEB-1protocol

EDTA

In 1000 ml, 1000 mM (1 mole) = 292.24 g

In 1000 ml, 1mM = $\frac{292.24}{1000}$ g

In 1000ml, 25mM = $\frac{292.24\times25}{1000}$ g

= 7.306 g

In 1000ml, 25mM EDTA = 7.306 g

In 1ml, 25mM EDTA = $\frac{7.306}{1000}$ g

In 100ml, 25mM EDTA= $\frac{7.306\times100}{1000}$ g

= 0.7306 g

Price of 100g EDTA = 1800 Tk

Then, price of 1g EDTA = $\frac{1800}{100}$ Tk

Then, price of 0.7306 g EDTA = $\frac{0.7306 \times 1800}{100}$ Tk

=13.1508 Tk

NaCl

In 1000 ml, 1000 mM (1 mole) = 58.5g

In 1000 ml, 1mM = $\frac{58.5}{1000}$ g

In 1000ml, 100mM = $\frac{58.5 \times 100}{1000}$ g

= 5.85g

In 1000ml, 100mM NaCl = 5.85g

In 1ml, 100mM NaCl = $\frac{5.85}{1000}$ g

In 100ml, 100mM NaCl = $\frac{5.85 \times 100}{1000}$ g

= 0.585g

Price of 1000g NaCl = 1600 Tk

Then, price of 1g NaCl = $\frac{1600}{1000}$ Tk

Then, price of 0.585 g NaCl = $\frac{1600 \times 0.585}{1000}$ Tk

= 0.936 tk

Tris-HCl

In 1000 ml, 1000 mM (1 mole) = 157.60g

Chemicals/reagents	Cost for 200 runs (BDT Tk)
1. Tris (10mM)	126.08 Tk
2. EDTA (25mM)	13.1508 Tk
3. NaCl (100mM)	0.936 Tk
4. SDS (0.5%)	4 Tk
Total	144.1668Tk

Protocol-5: DEB-2

EDTA

In 1000 ml, 1000 mM (1 mole) = 292.24 g

In 1000 ml, 1mM $= \frac{292.24}{1000}$ g

In 1000ml, 25mM $= \frac{292.24 \times 25}{1000}$ g

$= 7.306$ g

In 1000ml, 25mM EDTA = 7.306 g

In 1ml, 25mM EDTA $= \frac{7.306}{1000}$ g

In 100ml, 25mM EDTA= $\frac{7.306 \times 100}{1000}$ g

$= 0.7306$ g

Price of 100g EDTA = 1800 Tk

Then, price of 1g EDTA = $\frac{1800}{100}$ Tk

Then, price of 0.7306 g EDTA = $\frac{0.7306 \times 1800}{100}$ Tk

$=13.1508$ Tk

NaCl

In 1000 ml, 1000 mM (1 mole) = 58.5g

In 1000 ml, 1mM $= \frac{58.5}{1000}$ g

In 1000ml, 100mM $= \frac{58.5 \times 100}{1000}$ g

$= 5.85$g

In 1000ml, 100mM NaCl = 5.85g

In 1ml, 100mM NaCl $= \frac{5.85}{1000}$ g

In 100ml, 100mM NaCl = $\frac{5.85 \times 100}{1000}$ g

$= 0.585$g

Price of 1000g NaCl = 1600 Tk

Then, price of 1g NaCl = $\frac{1600}{1000}$ Tk

Then, price of 0.585 g NaCl = $\frac{1600 \times 0.585}{1000}$ Tk

$= 0.936$ Tk

Tris-HCl

In 1000 ml, 1000 mM (1 mole) = 157.60g

In 1000 ml, 1mM $= \frac{157.60}{1000}$ g

In 1000ml, 10mM $= \frac{157.60 \times 10}{1000}$ g

$= 15.76$g

In 1000ml 10mM tris= 15.76g

In 1ml, 10mM tris $= \frac{15.76}{1000}$ g

In 100ml, 10mM tris= $\frac{15.76 \times 100}{1000}$ g

$= 1.576$g

Price of 100g Tris = 8000 Tk

Price of 1g Tris = $\frac{1600}{1000}$ Tk

Price of 1.576 g Tris = $\frac{8000 \times 1.576}{100}$ Tk

= 126.08 Tk

1% SDS

To prepare 100ml solution 1 g SDS is needed

Price of 1000g (1kg) SDS = 8000Tk

Then, Price of 1g SDS $= \frac{8000}{1000}$ Tk

Then, Price of 1 g SDS $= \frac{8000 \times 1}{1000}$ Tk

= 8Tk

Price of 100ml = 8tk

Chemicals/reagents	Cost for 200 runs (BDT Tk)
1. Tris (10mM)	126.08 Tk
2. EDTA (25mM)	13.1508 Tk
3. NaCl (100mM)	0.936 Tk
4. SDS (1%)	8 Tk
Total	148.1668 Tk

Protocol-6: DEB-3

Proteinase K:

Price of 1ml (1000 µl) = 33000 Tk

Then, For 200run, Price of 4ml (4000 µl) = 33000×4 Tk

= 1, 32000 Tk

Chemicals/reagents	Cost for 200 runs (BDT Tk)
1. Tris (10mM)	126.08 Tk
2. EDTA (25mM)	13.1508 Tk
3. NaCl (100mM)	0.936 Tk
4. SDS (0.5%)	4Tk
5. Proteinase K:	1, 32000 Tk
Total	1,32144.166 Tk

Determination of DNA concentration:

$C = A_{260}$ (observed) × 50μg/ml × dilution factor for the measured sample.

Where, Dilution factor = $\frac{amount\ of\ solvent\ for\ dilution}{amount\ of\ original\ sample}$

In this study dilution factor was 75 (1500/20)

1. **Concentration of DNA obtained from protocol-1:**

   C =0.19 × 50 × 75

   =712.5 μg/ml

2. **Concentration of DNA obtained from protocol-2:**

   C = 0.17 × 50 × 75

=637.5 μg/ml

3. Concentration of DNA obtained from protocol-3:

C = 0.11× 50 × 75

=412.5 μg/ml

4. Concentration of DNA obtained from protocol-4:

C = 0.13 × 50 × 75

=487.5 μg/ml

5. Concentration of DNA obtained from protocol-5

C = 0.09 × 50 × 75

=337.5 μg/ml

6. Concentration of DNA obtained from protocol-6

C = 0.18 × 50 × 75

= 675 μg/ml