Tissue tropism of White Spot Syndrome Virus in artificially infected shrimp collected from South east Coastal part of Bangladesh.

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Khulna, Bangladesh

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DECLARATION

Tissue tropism of White Spot Syndrome Virus in artificially infected shrimp collected from South east Coastal part of Bangladesh.

The results submitted in this 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.

04-04-'13

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Abstract

White spot is a common symptom that frequently reported from a variety of culturing farms and ghers in Bangladsh specially in Khulna region which is caused by a DNA virus of White spot syndrome virus (WSSV). This virus causes a serious economic losses of shrimp *Penaeus monodon* and the incidence of the disease is severe. In this study, polymerase chain reaction (PCR) was used to determine the pattern of WSSV virus multiplication in tissues of shrimp and in addition the entrance of virus route as well. Viral solution was isolated from moribund WSSV infected shrimp using PBS solution and injected in 3rd and 4th segment of pleon. Every time 3 samples were collected randomly from tanks after each 3,6,12 and 24 hour post inoculation (hpi). Two different pairs of primers yielding amplicons ranging from 298 and 941 bp were used. The results revealed that none of the sample showed positive result at one step PCR test using 941 bp. The samples were then subjected to 2 step PCR using 1ul of first step amplified product as a DNA template and the results of PCR analysis showed that all the tissue or organ of gill, hemolymph, abdominal muscle, pleopods, and the tail muscle investigated. WSSV in the injected shrimp was particularly prevalent in the gills(60%), followed in order of decreasing prevalence by hemolymph(57%),abdominal muscle(50%), pleopods(46%), and the tail muscle(23%).Since no mortality occurred during experimental period the virus in shrimp was considered in the carrier state. This suggests that disease outbreaks do not occur if shrimp defense mechanisms manage to contain low intensity viral infections under low-stress culture conditions. Conversely, outbreaks may occur under stressful conditions.

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List of Abbreviations

Abbreviation	Elaboration
µl	Micro liter
bp	Base pair
kbp	Kilo base pair
mM	Mili Mole
ng	Nano gram
nm	Nano meter
PCR	Polymerase Chain Reaction
PhoA	Alkaline phosphatase
pmol	Pico Mole
rcf	Rotation Centrifugal Force
rpm	Rotation Per Minut
UV	Ultraviolet Transilluminator

Chapter One

INTRODUCTION

Chapter One

Introduction

The Shrimp industry of Bangladesh is one of the most important contributors for economic sustenance at present and is large export commodity of the country.

Tiger shrimp is the main cultured species, (locally known as *bagda* shrimp),scientifically named as *Penaeus monodon* (about 77% of export earning of shrimp sector). There is ample demand in the international market for shrimp and Bangladesh is blessed with an environment congenial for shrimp production. However, the industry is fraught with many obstacles at present (USAID, 2005).

Shrimp is the second largest foreign exchange earner after garments. This sector employs nearly 1.5 million people in forward and backward linkage activities like hatching, nursing, producing and harvesting, processing, exporting and other logistic support services (DoF, 2011). It has the highest potential for further development, enough to culture shrimps to earn US$ 1.5 billion by 2010 (BFFEA, 2007). Bangladesh already among the top 10 exporters of shrimp in the world accounts for some 3% global production. The EU (45%), USA (35%) and Japan (4%) are the worlds major importers of shrimp from Bangladesh (Uddin, 2008).

Shrimp farming in the south and southeastern coastal belt of Bangladesh began in the early 1970s. From less than 20,000 ha of brackish water ponds in 1980, the area under cultivation expanded to approximately 140 000 ha by 1995. The last complete survey to estimate the total area under shrimp cultivation was carried out in 1993–94; it has not been updated since then. Paul (2007) estimated that the total area under farming has expanded to 203 071 ha in 2003–2004.

The major shrimp producing districts are Bagerhat, Satkhira, Pirojpur, Khulna, Cox's Bazar and Chittagong, recently farmers especially in the Bagerhat and Pirojpur districts have begun shrimp farming in their paddy fields. Traditionally shrimp farming began by trapping tidal waters in nearby coastal enclosures known as *'Gher'* where no feed, fertilizers or other inputs were applied, with an increasing demand from both national and

international markets farmers started to switch over into improved extensive and semi-intensive systems.

Shrimp farm concentrated largely in tropical developing countries for export to the west, has experienced spectacular growth over recent decades. Currently, aquaculture industry in Bangladesh and other parts of the world especially southwest region has been facing serious problems due to microbial diseases. In aquatic environments, diseases in fishes and shrimps are caused by opportunistic pathogens (Lundin, 1996).

WSSV-infected shrimp may rapidly develop white spots (ranging from 0.5–3.0 mm in diameter) on the exoskeleton, appendages and inside the epidermis. Since these spots are not always present, and since similar spots can be produced by some bacteria, high alkalinity and stress, they are not considered a reliable sign for preliminary diagnosis of this disease. Other signs of WSSV include lethargy, sudden reduction in food consumption, red discoloration of body and appendages and a loose cuticle.

White spot syndrome (WSS) is one of the most important shrimp diseases. It is of global occurrence and it affects most of the commercially cultured shrimp species (Lightner et al. 2001). The clinical signs of this disease include white spots in the exoskeleton and epidermis, lethargy, a pink to reddish-brown coloration, the gathering of affected shrimp around the edges of ponds throughout the day and a rapid reduction in food consumption.

The causative agent of WSSV is an enveloped, non-occluded, rod-shaped DNA virus known as white spot syndrome virus (WSSV) (Wang et al. 2003, Lightner 2001). WSSV is also referred to as white spot syndrome baculovirus (WSBV) (Lo et al. 1996), and it is apparently identical or closely related to penaeid rod-shaped DNA virus, hypodermal and hematopoietic necrosis baculovirus (HHNBV) (Cai et al. 1995), and systemic ectodermal and mesodermal baculovirus (SEMBV) or white spot virus (WSV) (Wongteerasupaya et al. 1995).Because these viral agents appear to be very similar in morphology, histopathology and genome structure (Lo et al. 1996), they have been regrouped and are now often collectively referred to as WSSV (Lightner 1996). In cultured shrimp, WSSV infection is characterized by a wide range of target tissues, rapid disease onset and high mortality. During the viremic phase of infection, the virus is present in many organs.

There was a combined study took using currently available nucleic acid diagnostic tools and conventional histological observations using light (LM) and transmission electron (TEM) microscopy to examine the sites for virus multiplication in *Penaeus monodon* (black tiger shrimp). Sixteen parts excised from shrimp specimens were examined: pleopods, gills, stomach, abdominal muscle, hemolymph, gut, heart, pereiopods, lymphoid organs, epidermis, nervous tissue, hepatopancreas, testes, ovaries, spermatophores, and eye stalks. All these tissues/ organs were found to support WSSV replication (Lo et al. 1997). In lightly infected specimens, WSSV was particularly prevalent in pleopods, followed in order of decreasing prevalence by gills, hemolymph, stomach, abdominal muscle, reproductive organs, midgut, heart, periopods, lymphoid organ, integument, nervous tissue, and the hepatopancreas (Lo et al. 1997). WSSV tissue distribution data from lightly infected specimens helps to suggest the most appropriate tissue source for polymerase chain reaction (PCR) testing. Thus, in the present paper, a larger sample size was used to reconfirm WSSV prevalence in pleopods, gills, hemplymph, stomach, abdominal muscle, heart, periopods and integument.

The present study was designed to assess the current status of WSSV infection in the wild by providing a scientifically sound and statistically rigorous analysis of WSSV incidence, and to analyze if there is a correlation between areas. This was accomplished by using a conventional DNA extraction method, sensitive molecular diagnostic tools (one-step PCR). These diagnostic techniques were selected to provide for rapid screening of large numbers of individuals, to allow for the most sensitive detection of viral DNA in the samples (Durand and Lightner 2002).

PCR is an in vitro method for selective, repeated duplication of a specific segment of DNA. The concept of PCR was developed by Kary Muillis during the early 1980s. The development of nucleotide sequencing methods, the storage of this information in a computer researchable database, invention of automated oligonucleotide synthesis methods, discovery of thermostable DNA polymerase have all contributed to the rapid implementation and widespread use that PCR enjoys today (Karunasagar et al. 1999).

The polymerase chain reaction (PCR) has seen numerous recent applications to pathogen detection and shrimp pathology research. In PCR, small, often undetectable, amounts of DNA can be amplified to produce detectable quantities of the target DNA. This is

gel electrophoresis, by reaction with a specific DNA probe of PCR products blotted directly onto a membrane or to the PCR products in southern transfers (Perkins and Martin, 1999).

Objective of the study

The study was carried out detection of WSSV of brackish water cultured shrimp, *P. monodon*, around Bagerhat district using the most powerful technique of (PCR) with different primers with the intention of knowing the prevalence of the WSSV infection in different organ. It would be important to have the data on the prevalence of this virus and the level of infections as well in the farmed species of *P. monodon*. Considering this point the present study was designed with the following specific objective:

- To detect the entrance route and measure the tissue distribution of white spot syndrome virus.

Chapter Two

LITERATURE REVIEW

Chapter Two

Literature Review

The hypodermal and hematopoietic necrosis virus (HHNBV) is considered as the etiological agent of the prawn explosive epidemic disease (SEED) suffered in China during 1993–1994 (Cai et al. 1995), and a year later it was informally named as systemic ectodermal and mesodermal baculovirus (SEMBV) in Thailand due to its morphology, size, and histopathological profile (Wongteerasupaya et al. 1995). The virus has also been taxonomically affiliated as the following: Chinese baculovirus, red disease, white spot disease, and white spot baculovirus. However, presently the virus is referred to as White Spot Syndrome Virus. (Hossain et al. 2001) reported that wild-caught asymptomatic marine shrimp such as Metapenaeus doson, Parapenaeopsis stylifera, Solenocera indica and Squilla mantis carry WSSV. (Vijayan et al. 2005a) stated that it has been found that WSSV could be accumulated in the digestive tract of polychaetes, where they remain infectious, and thus, these worms may serve as a passive vector of WSSV in aquatic systems. Furthermore, it was demonstrated that shrimp can be horizontally infected via ingestion of WSSV infected polychaete worms, attaining prevalence rates of up to 83%. (Durand et al. 2002) reported that the head had a higher WSSV load than did the tail. Since the abdomens of shrimp represented 58% of the total body weight, the total virus load on a per weight basis turns out to be similar in the cephalothracies (49%) and abdomen (51%) of the same shrimp with acute phase WSSV infection. (Vijayan et al. 2005b) Histopathological investigation on the Y-organ collected from moribund shrimps revealed the presence of intranuclear basophilic inclusions, characteristic of WSSV. More than 70% of the Y organ cells were infected, suggesting the degenerated state of the organ. The cellular integrity of the Y-organ was completely destroyed by the WSSV. In lightly infected specimens, WSSV was particularly prevalent in pleopods, followed in order of decreasing prevalence by gills, hemolymph, stomach, abdominal muscle, reproductive organs, midgut, heart, periopods, lymphoid organ, integument, nervous tissue, and the hepatopancreas (Lo et al. 1997). The virus–receptor interaction is a multistep process in itself, multiple attachment receptors may be used sequentially, or in a cell-type-specific manner, and co-receptors may also be involved (Hasson et al. 2006). In an infected individual, the co-receptor-determined tropism of the virus may change from macrophages or dendritic cells in mucosal tissues to a tropism for T cells. This change is

due to mutations emerging in the viral envelope genes during progression of the infection (Cunninghum et al. 2002)

(Lo et al. 1996) hypothesize that disease outbreaks do not occur if shrimp defense mechanisms manage to contain low intensity viral infections under low-stress culture conditions. Conversely, outbreaks may occur under stressful conditions. it is possible to detect 10 to 50 copies of target DNA in PCR, and the sensitivity of the 2-step amplification protocol is about 10^3 to 10^4 times greater than that of l-step amplification alone (Lo et al. 1998). We also concluded from our study on WSSV tissue distribution in shrimp that the virus must replicate very rapidly from the carrier state to transition state, i.e. by a factor greater than 10^3 or well over the sensitivity threshold of l-step WSSV diagnostic PCR (McBeath et al. 2000). The best sources for PCR template preparation during nondestructive screening of asymptomatic carrier brooders, would be (a small piece of) the gills or (a small aliquot of) the hemolymph (Lo et al. 1996). In lightly infected specimens, WSSV was particularly prevalent in pleopods, followed in order of decreasing prevalence by gills, hemolymph, stomach, abdominal muscle, reproductive organs, midgut, heart, periopods, lymphoid organ, integument, nervous tissue, and the hepatopancreas (Lo et al. 1997). WSSV tissue distribution data from lightly infected specimens helps to suggest the most appropriate tissue source for polymerase chain reaction (PCR) testing (Leong et al. 1995).

Chapter Three

MATERIALS AND METHODS

Chapter Three

Materials and Methods

3. Design of Experiment:

3.1.1 Preparation of viral inoculums:

Preparation of the viral inoculums For the inoculum preparation, soft tissue (15 g) from the cephalothorax of WSSV-infected *Penaeus monodon* (with prominent white spots on the exoskeleton) was homogenized in 150 ml PBS (288.75 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PHO_4, pH 7.3), then centrifuged at 3000 rpm for 10 min at 4°C. The supernatant was centrifuged at 8000 rpm for 10 min at 4°C. The final supernatant was filtered through a 0.45-µm filter and stored at −80°C until used. Before storage, the presence of WSSV in the tissue sample and the final supernatant fluid was determined by nested-PCR assay (Syed-Musthaq et al., 2003)

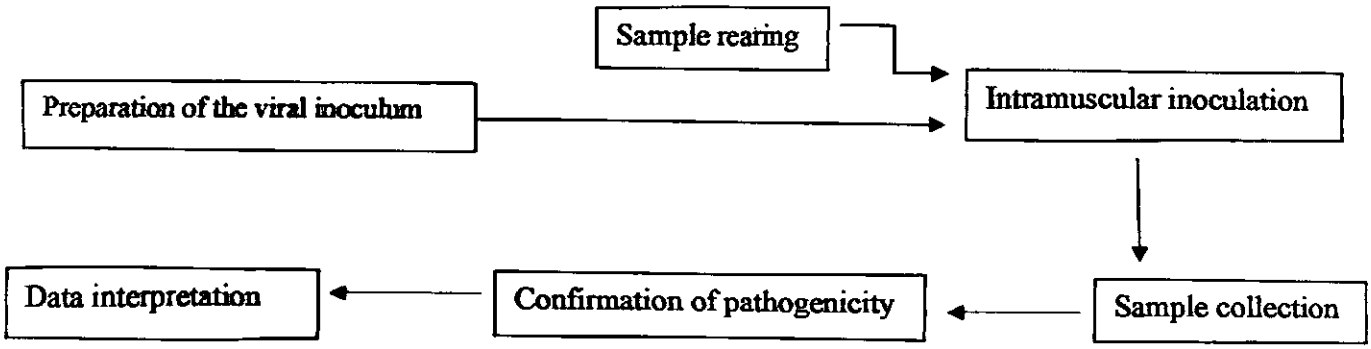

Fig: 3.1 Flow chart of design of experiment

3.1.2 Experimental animals-shrimp – *Penaeus monodon*:

Firstly, few ghers were chosen for sample then random sampling done from selected ghers in order to check WSSV and at the result all sample showed negative result.One of the selected ghers marked for sampling.

About 50 samples of SPF *Penaeus monodon*, juvenile shrimp were collected from Khulna division for the study. The shrimps used in culture were imported from gher through water drum. Shrimp *Penaeus monodon* were maintained in 1.5 ft×1 ft×1 ft size aquarium tank at room temperature (28-30°C) with salinity between 3 and 4 ppt. Natural estuarine water was used for all the experiments. Total 10 pieces of aquarium taken and each aquarium contains 5 shrimps. So total 50 samples were used for this experiment. Another aquarium was kept as a control treatment.

The species, *Penaeus monodon* were fed with formulated shrimp feed (30% protein) at 10% body weight, once in a day. They were kept in these tanks for 7 d for acclimatization prior to the experiments. About five shrimps were picked out randomly from those shrimps for PCR examination to exclude viral infection, because only healthy individuals were used. Further, a representative sample of these animals was subjected to nested PCR.

3.1.2.1 Sample size:

Sample age was 1 month and body weight was 8gm ±2 gm/pcs.

3.1.2.2: Water quality:

In the aquarium same water quality maintained as the gher from where the sample were collected. Water quality chart were given below:

Parameter	Gher	Aquarium
Salinity	4 ppt	4 ppt
Temp	28°C	27°C
DO	3.5 mg/l	5 mg/l
P_H	6.5	7.5

Table 3.1: Parameter table

Reftractometer, Thermometer, Aerator, and P_H used to maintain the water quality. Because of using of artificial aeration system, aquarium showed higher DO reading than gher.

3.1.2.3 Intramuscular inoculation procedure

Experiment was performed using the intramascular route. In experiment, 3 groups of 10 shrimp (Maximum body weight = (8±2) g, n=50) were inoculated with 25µl of viral solution. In addition, 5 shrimp were mock-inoculated with same dose PBS used as controls. Shrimp were injected between the 3rd and 4th segments of the pleon. Before and after injection, this surface was wiped with 70% ethanol.

3.1.2.4 Sample collection:

Every time 3 samples were collected randomly from tanks after each 3,6,12,24 hour post inoculation (hpi). Then preserved the sample in -80°C for pathogen confirmations. Abdominal muscle, pleopods, heart, integument, pereiopods, eyestalks, Gills, Stomach taken as probable route of WSSV.

3.2 Laboratory Work for detection of Pathogen:

3.2.1 DNA Extraction

According to the Invitrogen manual for DNAzol reagent, viral DNA was extracted from the sample. DNAzoL reagent is a complete and ready-to-use reagent for the isolation of genomic DNA isolation solid and liquid samples. The DNAzoL reagent procedure is based on the use of a novel guanidine-detergent lysing solution which permits selective precipitation of DNA from a cell lysate. DNAzoL Reagent is an advanced DNA isolation reagent that combines both reliability and efficiency with simplicity of the isolation protocol. The DNAzoL reagent protocol is fast and permits isolation of genomic DNA from a large number of samples of small or large volumes.

During the isolation, a biological sample is lysed (or homogenized) in DNAzoL reagent and the genomic DNA is precipitated from the lysate with ethanol. Following an Ethanol wash, DNA is solubilized in 8 mM NaOH. The procedure can be completed in about 30 min with DNA recovery of 70-100%. The isolated DNA can be used without additional purification for applications PCR. (Invitrogen, Life Technologies, USA; Ausubel et al. 1990)

Procedure

- At the very beginning of extraction process, 2 ml (1 ml in each tube) cultured sample was centrifuged at 10000 rcf for 5 minutes with a centrifuge machine (Vision Micro High Speed Refrigerated Centrifuge, Model no: VS-15000 CFN II). At the end of centrifugation, clear supernatant layer was removed by a pipette (1000 µl) and deposited plate layer was kept for further procedure.
- ***Lysis of cells:*** 1000 µl DNAzol reagents were added to each tube of a sample. The samples were were dissolved completely by gentle pipetting in a hand-held Teflon homogenizer. A loosely fitting homogenizer Used with a tolerance of 0.1-0.15 mm or higher.
- ***Centrifugation:*** The dissolved cells were centrifuged for 10 min at 10,000 rcf at 4°C or room temperature. Following centrifugation, the resulting viscous supernatant was carefully transferred to a fresh tube. This step removes insoluble tissue fragments, RNA, and excess polysaccharides from the lysate/homogenate. This process was performed in order to minimize RNA carry-over into the DNA.
- ***DNA Precipitation:*** DNA from the lysate was precipitated by the addition of 0.5 ml of 100% ethanol per 1 ml of DNAzoL Reagent used for the isolation. Samples were mixed by inversion and store them at room temperature for 1-3 minutes. Then it was centrifuged for 10 minutes at 10000 rcf in cool temperature. DNA should quickly become visible as a cloudy precipitate. Carefully the supernatant was decanted leaving the DNA pellet near the top of the tube. The tubes were placed upright for 1 min and was aspirated the remaining lysate from the bottom of the tubes.
- ***DNA Wash:*** The DNA precipitate was washed twice with 0.8-1.0 ml of 75% ethanol. At each wash, the DNA in ethanol suspended by inverting the tubes 3-6 times. The tubes were stored vertically for 0.5-1 min to allow the DNA to settle to the bottom of the tubes and remove ethanol by pipetting. At each wash, the DNA in ethanol was vortexed and was suspended by centrifugation for 10 minutes at 10000 rcf.
- ***DNA Solubilization:*** After removing the ethanol, the DNA was air dried by storing in an open tube for 5-15 seconds. (If the DNA is exposed to air for more than a few seconds, it

will be much more difficult to dissolve.). If the DNA was not properly dried, it was kept on a heatblock at 55°C for 5 minuts. The DNA was dissolved in 8 mM NaOH by slowly passing the pellet through a pipette tip. Use of the 8 mM NaOH was assured full solubilization of the DNA precipitate. 100 µl of 8 mM NaOH was added to the DNA precipitate to dissolve. Then it was centrifuged for 3 minutes at 10000 rcf.

3.2.1.1 Qualitative and quantitave determination of extracted DNA

3.2.1.1.1 Agarose gel electrophoresis determination of DNA integrity

Integrity of extracted DNA sample was studied by agarose gel electrophoresis as described by Sambrook et al. (1989). Briefly, electrophoresis was performed using a horizontal electrophoresis apparatus (Bioneer, Korea) at a constant volt of 100 with a 1% agarose gel containing 1.2 µl ethidium bromide in 120 ml 1x TAE buffer. Supply of power for electrophoresis was continued until DNA migrated near about the two-third of the gel towards the positive electrode. DNA bands were visualized by UV illumination and photographed using the High Performance UV Transilluminators, (Ultra-Violet Products Ltd., UK). The detailed procedure was mentioned in appendix. A.

3.2.1.1.2 Spectrophotometric method determination of DNA Purity

The concentration of DNA samples were determined from the absorbance at A_{260} (absorbance at 260 nm) using a double beam spectrophotometer (Hitachi U-2910 spectrophotometer, Japan) against NaOH blank. 2000 µl (2 ml) of 8 mM NaOH was taken in blank cuvette whereas a mixture of 4 µl solubilized DNA and 1996 µl of 8 mM NaOH was taken in another cubette. The resulting data against each absorbance was found on the program's page. DNA sample containing cubette was washed properly before loading next sample. Thus, a list of data of the samples against two absorbance was found and saved. Absorbance at A_{280} had been determined as same technique. The protocol used in this experiment is designed for a double-beam spectrophotometer. The DNA purity was found by using following formula.

1) Purity = Absorbance at A_{260} /Absorbance at A_{280}

3.2.2 Components for DNA amplification in PCR

3.2.2.1 Description of primers

Oligonucleotide primers: The DNA fragment to be amplified was determined by selecting primers. Primers are short and artificial DNA strands that were complementary to the beginning and the end of the DNA fragment to be amplified. They anneal to the DNA template at these starting and ending points, where the DNA-polymerase binds and begins the synthesis of the new DNA strand. In this study, 2 pairs of oligonucleotide primers (Both forward and reverse) named, 146 F2, 146 R2, Lo 3-4 were procured from Bioneer, Korea. Sequences of the 2 PCR primer pairs, their corresponding size of expected amplification products were shown in Table 3.2.

Primer name	Primer sequence	Size (bp)	Ref.
146F2 146R2	5'-GTA ACT GCC CCT TCC ATC TCC A-3' 5'-TAC GGC AGC TGC TGC ACC TTG T-3'	941	Lo et al. (1996)
Lo 3-4	5'-ACCTGGCGTAGTTCTTGC-3' 5'-GGTAGATTCTGGTATTAGG-3'	298	Lo et al. (1996)

Table 3.2: Primers and expected size of PCR-amplified gene targets of WSSV

3.2.2.2 Top DNA polymerase enzyme

The enzyme was isolated room recombinant E. coli. Strain containing the DNA polymerase gene from *Thermus thermophillus*. It exhibits highest activity at pH 9.0 and 72°C (Bioneer, Korea). To prepare 1 unit Top DNA Polymerase from purchased 5 units, it was diluted with enzyme dilution buffer (50 mM Tris-HCL, 0.1 mM EDTA, 1mM DTT, Stabilizers, 50% Glycerol, pH 8.2) at 4: 1 ratio (Buffer : Enzyme).

3.2.2.3 Deoxynucleotide Triphosphates (dNTPs) mixture

These molecules correspond to the four bases present in DNA (adenine, guanine, thymine and cytosine) and are substrate for the DNA polymerase. Each PCR requires four dNTPs (dATP, dGTP, dTTP, dCTP), which are used by the DNA polymerase as building blocks to synthesize new DNA. In the present experiment, 10 mM dNTP mixture (each dNTP 2.5 mM) was used (Bioneer, Korea).

3.2.2.4 Buffers used for PCR

- **10x Reaction buffer** (Tris; pH 9.0, 15 mM $MgCl_2$)
- **Enzyme dilution buffer** (50 mM Tris-HCl, 0.1 mM EDTA, 1mM DTT, Stabilizers, 50% Glycerol, pH 8.2)

Component	Final volume	Final concentration
1. **A *Taq* Polymerase (2.25units/µl; Top DNA Polymerase, Bioneer, Korea)**	0.6 µl	0.027 unit/50 µl
2. **10X Reaction Buffer (Tris; pH 9.0, 15 mM $MgCl_2$)**	5 µl	1x
3. **10 mM dNTP mixture (each dNTP 2.5 mM)**	1 µl	200µM
4. **Primers**	1 µl (Forward 0.5 µl + Reverse 0.5 µl)	10 pmol
5. **Template DNA**	10 µl	75-160 ng
6. **Di- ionized water**	32.4 µl	

Table 3.3: The component of reaction mixture and their compositions in 50 µl reaction mixture together with final concentration for the 1 step PCR.

3.2.3 DNA amplification by a thermal cycler

Desired DNA amplification and target gene amplification were conducted by PCR analysis. In this method, following DNA extraction from samples with forward and reverse primers and *Taq* polymerase. PCR amplification was performed in a Gene Cycler under the following conditions: In brief, heat denaturation at 94°C for 2 min followed by 35 cycles of heat denaturation at 94°C for 1 min, primer annealing at 58°C for 1 min and DNA extension at 72°C for 1 min. This is followed by incubation at 72°C for 10 min followed by cooling at 4°C (Table 3) and the PCR product was analyzed by agarose gel electrophoresis.

Before PCR, made PCR sample by the following way, in Brief, 3 µl DNA extract were directly used as template in each PCR-tube for the reaction. The reaction mixture consisted of 1 µl primer (Forward 0.5 µl + Reverse 0.5 µl), 5 µl 10x Reaction buffer, 1 µl dNTPs, .6 µl 2.25 unit Top DNA Polymerase enzyme as well. These all components were mixed in 39.4 µl distill water and thus a total of 50 µl PCR sample was prepared in a 0.2 ml PCR-tube (Eppendrof, Humburg, Germany) to operate in a thermal cycler.

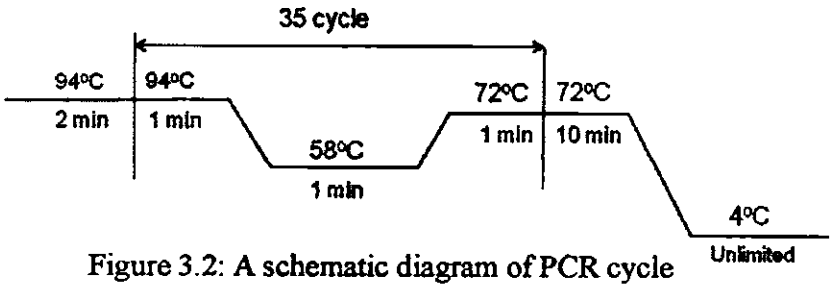

Figure 3.2: A schematic diagram of PCR cycle

In PCR running, control reactions consisted of:

1) Positive control: Reaction mixtures with DNA extracted from positive sample that always showed the positive response if all chemical and mixture is right.

However, in the present experiment both experimental template (samples of DNA) and three control reactions were assigned as template.

3.2.4 Master mix for Nested PCR:

Component	Final volume	Final concentration
7. A *Taq* Polymerase (2.25units/µl; Top DNA Polymerase, Bioneer, Korea)	0.6 µl	0.027 unit/50 µl
8. 10X Reaction Buffer (Tris; pH 9.0, 15 mM $MgCl_2$)	5 µl	1x
9. 10 mM dNTP mixture (each dNTP 2.5 mM)	1 µl	200µM
10. Primers	1 µl (Forward 0.5 µl + Reverse 0.5 µl)	10 pmol
11. Template DNA	1 µl	
12. Di- ionized water	41.4 µl	

3.2.5 Evaluation of PCR products

After completion of the thermal cycling, a sample of 4 µl from each PCR product was analyzed electrophoretically by running on a 2% agarose gel following the procedure described in the "Agarose gel electrophoresis determination of DNA integrity" and the amplified product size was determined by comparison with a 100 bp DNA size marker.

Chapter Four

RESULTS

Chapter Four

Results

In this experiment work, a total of 30 samples were taken to observe whether WSSV can replicate in all the organ samples tested or not. The results revealed that none of the sample was showed positive result at one step PCR test. The samples were then subjected to 2 step PCR *using 1ul of one step amplified product as a DNA template and the results of PCR analysis* showed that the presence of WSSV in all the organs of gill, hemolymph, abdominal muscle, pleopods, and the tail muscle investigated (Table-4.1).

Organ	Sample	Time (hpi)										%	Avg
	N= 30	3 hr	6 hr	12hr	1 d	2 d	3 d	4 d	5 d	6 d	7d		
Gill	1	-	-	-	+	+	+	+	+	+	+	70	60
	2	-	-	-	-	+	+	+	+	+	+	60	
	3	-	-	-	-	+	-	+	+	+	+	50	
A.Muscle	1	-	-	-	-	+	+	-	+	+	+	50	50
	2	-	-	-	-	-	+	+	+	+	+	50	
	3	-	-	-	-	+	+	+	+	+	-	50	
Pleopod	1	-	-	-	-	-	+	+	+	+	+	50	46
	2	-	-	-	-	-	-	+	+	+	+	40	
	3	-	-	-	-	-	+	+	+	+	+	50	
Tail muscle	1	+	+	+	-	-	-	+	+	+	+	40	23
	2	+	+	-	-	-	-	-	-	+	-	10	
	3	+	+	-	-	+	-	+	-	-	+	20	
Hymolymph	1	-	-	-	+	+	+	+	-	+	-	50	57
	2	-	-	-	+	+	+	+	+	+	+	70	
	3	-	-	-	-	+	+	-	+	+	+	50	

Table 4.1: WSSV tissue tropism of intra-muscularly infected *Penaeus monodon* as revealed by 2- step WSSV diagnostic PCR

From the Table 4.1, it can be seen that WSSV in the injected shrimp was particularly prevalent in the gills, followed in order of decreasing prevalence by hemolymph, abdominal muscle, pleopods, and the tail muscle. During the experimental period, WSSV prevalence was highest in Gill (60%) then at hymolymph (57%) where as pleopod showed lower magnitude of prevalence of 46%.

Other then injected area, WSSV virus was first detected at 24 hpi in the gills. In case of hemolymph, 17 samples (out of 30) were showed positive result simultaneously by nested test suggesting the viral replication must be occurring in the samples of haemolymph and virus are dispersed rapidly (within 24h pi) to other organs of gill, abdominal muscle and pleopod through haemolymph circulation.

Though virus was injected at tail region however, tail muscle showed positive result at very high prevalence rate (100%) up to 12h pi and a significant decline was observed (Table 4.1) which implies that the injected virus are not capable of infecting all types of cells or organs. This result also suggesting that tail muscle is not suitable for early screening for WSSV infection.

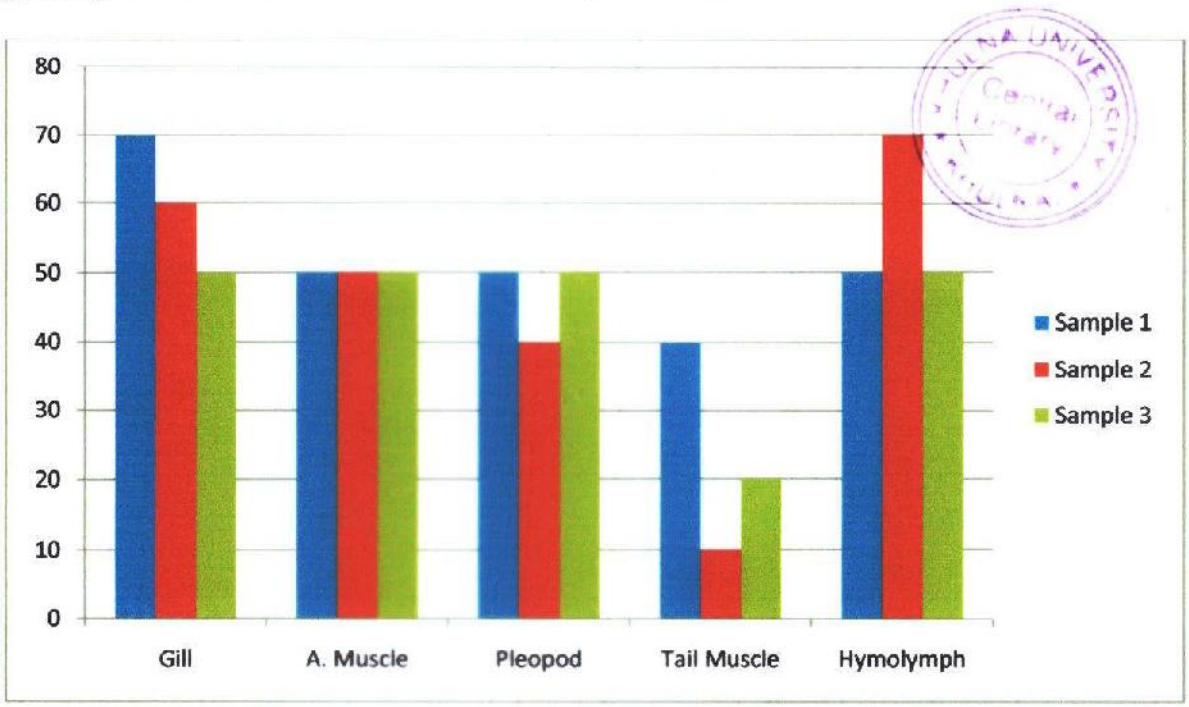

Chart 4.1: Tissue tropism chart of WSSV infected shrimp

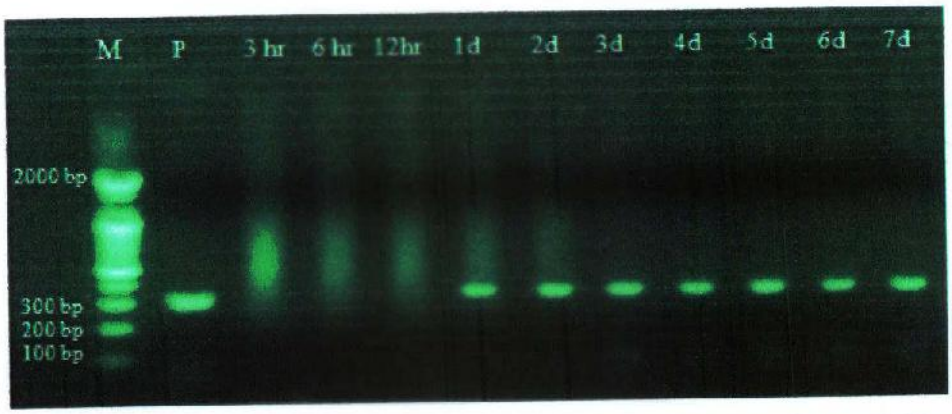

Fig 4.1: WSSV-specific DNA fragments of gill amplified by 2-step PCR using prns 146 primer and 298 bp primer sets with P: positive control. M: marker

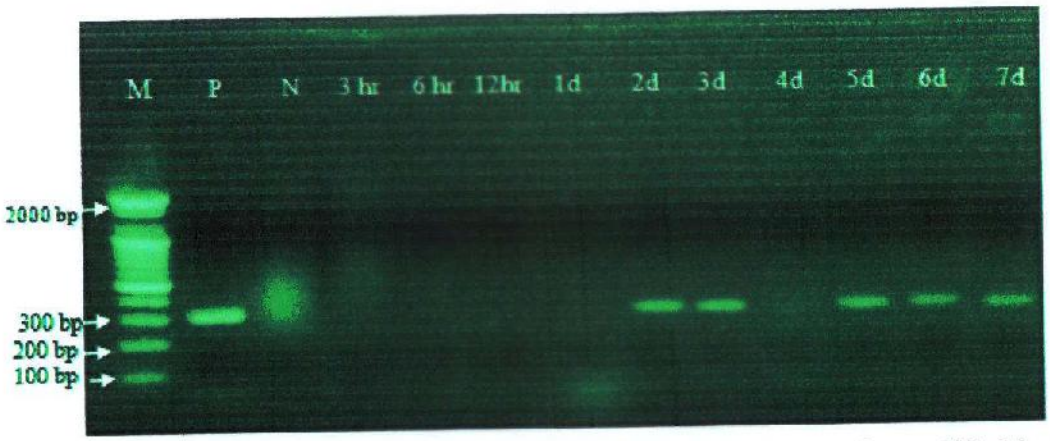

Fig 4.2: WSSV-specific DNA fragments of abdominal muscle amplified by 2-step PCR using 146 primer and 298 bp primer sets with P: positive control. M: marker. N: negative control

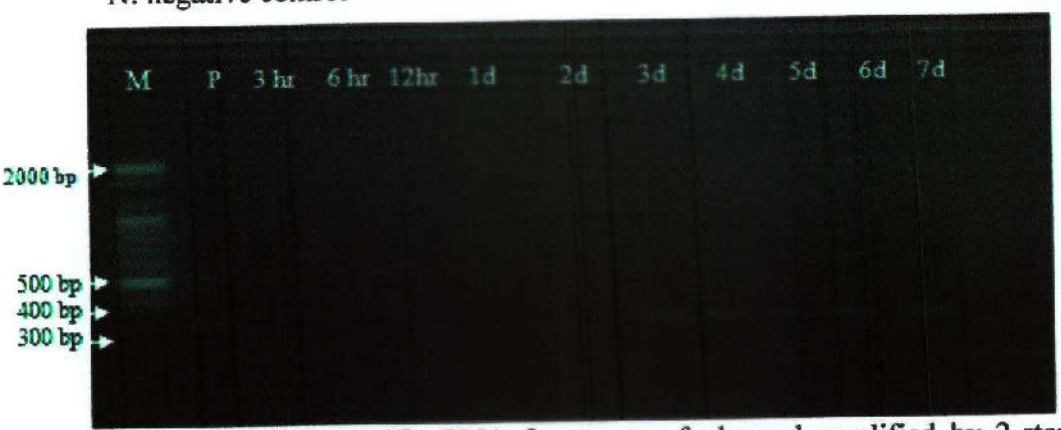

Fig 4.3: WSSV-specific DNA fragments of pleopod amplified by 2-step PCR using 146 primer and 298bp primer sets with P: positive control. M: marker.

Chapter Five

DISCUSSION

Chapter Five

Discussion

In this experiment total 30 healthy shrimp were taken and artificially WSSV injected into the sample for experiment. No mortality seen during the experiment and showed no positive result in 1 step PCR. So nested PCR had done for pathogenecity confirmation and most of the sample had shown positive result. It confirms that all shrimp look like healthy though they were in carrier state.

Table 4.1 shows positive and negative results. Since No mortality occur during experimental period so the virus in shrimp was in the carrier state. Although the carrier state might persist for months, but Lo et al. proved that the highly infected (transition) state usually lasts for only a few hours and once a specimen becomes 1- step PCR positive, it will die within a few days at the most (Lo et al. 1997). So the transition interval is short-lived and during this time the disease/infection can progress rapidly (Lo et al. 1996).

In the present study, primer set (pms) 146 F2/R2 and 298 bp primer sets and the PCR reaction conditions described by Lo et al. (1996) were utilized for WSSV diagnostic PCR. With this 2-step WSSV diagnostic PCR, it is possible to detect 10-50 copies of target DNA in a PCR reaction, and the sensitivity of the 2-step amplification protocol is about 10^3 to 10^4 times greater than that of 1-step amplification alone.

This experiment conclude from the foregoing that to produce results seen in Table 4.1, the virus must have replicated very rapidly, by a factor greater than 10^3 (i.e., to well over the sensitivity threshold of 1-step WSSV diagnostic PCR). In 1997 Lo et al. did his experiment with these pms146 primer sets and concluded that 1-step positive diagnosis with clinically normal animals were a very clear indication of the severe infection (Lo et al. 1997).

In very lightly infected shrimp WSSV was particularly prevalent in gills (60%), followed in order of decreasing prevalence by hemolymph, abdominal muscle, pleopods, tail muscle. The fact that the percentage of WSSV-positive samples remained fairly low in tail muscle, it suggested that the virus might not be able to replicate-or at least not be able to replicate. However, the percentage of WSSV-positive samples increased Table 4.1. This suggested that

the virus was able to replicate at these oragns. Therefore, there are other possible explanations that found equally well account for the observed data. Since all the tested organ were WSSV positive and hymolymph showed early positive result so it might be certain that all these infections were the direct result of systemic transmission. From this experiment it showed that when a shrimp infected by WSSV then the virus mix with blood stream and spread out over the various organ of the body but massive movement occur toward the gill so the gill rapidly became the main location of viral replication. As a result shrimp die within a short time.

This order, which is based on a larger sample size, is slightly different to the order reported in paper (Lo et al. 1997). However, pleopods, gills, hemolymph, and abdominal muscle were still the most prevalently infected organs in this studies. Thus, in the carrier state, in these organs that the virus most frequently appears and replicates. Also, because of a problem with false negatives Lo et al. said that, WSSV is likely to be more prevalent in eyestalks. Therefore, for non-destructive screening of asymptomatic carriers, I recommend that the best sources for PCR template preparation would be (a small piece of) gills or (a small aliquot of) the hemolymph. There is also some evidence to suggest that an ablated eyestalk would be a good alternative, provided that the compound eye is removed before use (Wang et al. 2003)

Since no mortality occur under such favorable (low-stress, or at least low-density-stress) conditions, so I hypothesize that the shrimp defense mechanisms were able to contain the virus. It can be said that disease outbreaks do not occur if shrimp defense mechanisms manage to contain low intensity viral infections under low-stress culture conditions. Conversely, outbreaks may occur under stressful conditions.

Chapter Six

CONCLUSION

Chapter Six

Conclusion

The phenomenal growth of the shrimp farming industry in less than two decades has created various problems. Disease epizootics of economic significance are a major constraint to the industry, not only because they affect the quantity of harvest, but because disease also affects the quality and regularity of production. Identification of disease causing agents is the primary need of shrimp disease study. Due to lack of highly sensitive detection methods, the management of viral disease in shrimp aquaculture has been a challenge.

rapid development of molecular biological techniques offers significant advantages or workers involved in shrimp disease diagnosis. The application of PCR based techniques is beginning a new era of excellence in the field of shrimp disease analysis. The findings reported in this study describe a versatile, reliable and highly sensitive PCR system for the rapid detection of WSSV in shrimp farm samples. The PCR method described in this study has high throughput and involves a turnaround time of less than 18 h which is much quicker and more reliable than existing traditional culture-based techniques. Overall, the data indicated that the PCR scheme is a potentially very useful and powerful technique for the microbiological assessment of sample. This method would be particularly useful for the assessment of WSSV. Therefore, in context of Bangladesh, this could be an ideal protocol for PCR Confirmation test of WSSV for shrimp brood and larval stocks and other potential viral carriers.

In context of Bangladesh, this could be an ideal protocol for PCR confirmation test of WSSV for shrimp brood and larval stocks and other potential viral carriers. The primers should be chosen taking into consideration the purpose of diagnosis. If PCR is used for confirmatory diagnosis of overt disease, primers yielding larger fragments can be used but if the purpose of the PCR is to screen for WSSV and in cases where a low virus load is expected, use of primers yielding smaller amplicons would give more accurate results. This experiment showed that the presence of WSSV in all the organs of gill, hemolymph, abdominal muscle, pleopods, and the tail muscle investigated. WSSV in the injected shrimp was particularly prevalent in the gills, followed in order of decreasing prevalence by hemolymph, abdominal muscle, pleopods, and the tail muscle.

During the experimental period, WSSV prevalence was highest in Gill (60%) then at hymolymph (57%) where as pleopod showed lower magnitude of prevalence of 46%.

Since no mortality occur under such favorable (low-stress, or at least low-density-stress) conditions, so it is hypothesized that the shrimp defense mechanisms were able to contain the virus. It can be said that disease outbreaks do not occur if shrimp defense mechanisms manage to contain low intensity viral infections under low-stress culture conditions. Conversely, outbreaks may occur under stressful conditions.

From our tissue tropism result, as virus was first detected in the gill and hemolymph (non-injected organ). It might suggested that the injected virus was transported to gill (within 24h) through heaolymph indicating systemic infection and gill is the possible route of entrance for this viral infection as well.

At present, the main goal of the shrimp industry is to meet the growing demand in a sustainable manner without damaging the environment. Further research is required to enable high-quality validation and trial of these methods as we move into the next century of shrimp health research and development.

Chapter Seven

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APPENDICES

Chapter Eight

Appendix

Appendix A

Agarose gel electrophoresis determination of DNA integrity

Integrity of extracted DNA sample was studied by agarose gel electrophoresis as described by Sambrook et al. (1989). Briefly, electrophoresis was performed using a horizontal electrophoresis apparatus (Bioneer, Korea) at a constant volt of 100 with a 1% agarose gel containing 1.2 µl ethidium bromide in 120 ml 1x **TAE buffer.** Supply of power for electrophoresis was continued until DNA migrated near about the two-third of the gel towards the positive electrode. DNA size was determined by comparison with a DNA size marker (100 bp DNA ladder, Bioneer, Korea). DNA bands were visualized by UV illumination and photographed using the Gel Doc 1000 documentation system (Bio-Rad).

Materials

1. Electrophoresis buffer; TAE buffer (40 mM Tris- Acetate & 1mM **EDTA,** pH 8.0);
2. Agarose powder;
3. Ethidium bromide (NOTE: Ethidium bromide is a known mutagen and thus handled as a hazardous chemical — hand gloves were used while handling);
4. Gel loading buffer (BlueJuiceTM Gel Loading Buffer: 65% (w/v) sucrose, 10 mM Tris-HCl (pH 7.5), 10 mM EDTA, and 0.3% (w/v) bromophenol blue, Bioneer, Sheul, Korea);
5. DNA size marker;
6. An electrophoresis chamber, power supply, gel casting tray and sample combs (around which molten agarose is poured to form sample wells in the gel);
7. Electric balance (Mettler Toledo, B303-S, Germany);
8. Micro wave oven (Walton, Japan);
9. Digital camera (Canon G9, Japan); and
10. Conical flask, beaker, cylinder, parafilm, micro-pipette, micro tips and tissue paper.

Procedure

1. **TAE buffer preparation:** To prepare 1000 ml 1x TAE buffer solution from 50x TAE buffer 20ml TAE buffer and 980 ml distill water was mixed up to make 1000 ml 1x solution.
2. **Agarose gel preparation**
   a. Gel casting tray and samples comb were installed.
   b. An amount of 1.5 g Agarose powder was weighted out using the electric balance and taken in a conical flask, shake for a few times and then heated in a microwave oven for 2 minutes. To melt the powder properly, heated in the oven and shaking at a regular time (30s) interval.
   c. The mixture was cooled to about 60°C by falling cool tap water on the wall of the conical flask with continuous jerking and by keeping at room temperature
   d. 1.5 µl Ethidium bromide was added to the solution and mixed well;
   e. The solution was poured into the gel casting tray containing sample comb and were allowed to solidified at room temperature; (Note: with decreasing temperature gel will be solidify rapidly thus step 1.4 to1.6 was done quickly without consuming high time.
   f. After gel has solidified, the comb was removed, using care not to rip the bottom of the wells.
   g. The gel could be stored at -4°C for further use.

3. The gel, still in its plastic tray, was inserted horizontally into the electrophoresis chamber and was covered by adding approximately 450 ml TAE buffer;
4. 4 µl of the extracted DNA sample was mixed with 2 µl of loading buffer on a parafilm and were poured into a sample wells. By maintaining sample's serial number, this was done for every sample separately. To avoid cross-contamination, separate micro-tips were used for every sample.
5. Approximately 2 µl of colored DNA size marker was poured into first sample wells on gel ;
6. The electrophoresis chamber was covered with the lid;
7. The power supply was switched on, only volt was fixed at a constant value of 50, the machine was run until DNA migrated to the two-third of the gel towards the positive electrode (DNA was usually blue colored based on loading buffer);

8. After 25 to 30 minutes The power supply was switched off and the lid of the electrophoresis chamber was removed;
9. The gel, still in its plastic tray, was removed from the electrophoresis chamber and placed on an ultraviolet (UV) transilluminator.
10. The UV transilluminator was switched on, DNA banding pattern was visible;
11. The resulting banding pattern was captured by using the digital camera and software.

Appendix-A

Spectrophotometric method determination of DNA concentration and purity:

The concentration and purification of DNA samples were determined from the ratio of absorbance at A_{260} (absorbance at 260 nm) and A_{280} (absorbance at 280 nm) using a spectrophotometer (Spectrophometer, Hitachi High-Technologies Co., Japan) against 8 mM NaOH solution. The protocol used in this experiment was designed for a double-beam spectrophotometer. Briefly, the concentration and the purity of DNA samples were determined by the following procedure (Ausubel, 1994).

Materials

- DNA samples;
- Double-beam spectrophotometer
- Matched quartz semi-micro spectrophotometer cuvette (1-cm-pathlength);
- Micro-pipette, micro tips and tissue paper; and
- Distilled water (neutral pH).

Procedure

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

1. Spectrophotometer was warmed up before use.
2. Spectrophotometer's software was opened
3. 2000 µl of 8 mM NaOH was taken in blank cuvette;
4. DNA sample (4 µl solubilized DNA + 1996 µl of 8 mM NaOH) was taken in another cubette.
5. Resulting data against each absorbance was found on the program's page.
6. DNA sample containing cubette was washed properly before loading next sample.
7. A list of data of the samples against two absorbance was found and saved.
8. The machine was turned off before the program was closed .

9. Calculation;
   a) Double-stranded DNA concentration (C), µl/ml =Absorbance at A_{260} *50*500;
   b) Purity = Absorbance at A_{260} /Absorbance at A_{280}

Appendix-C

Description of Ladder:

100 by DNA Ladder is specially designed for determining the size of double strand DNA from 100 to 2,000 base pairs. The DNA Ladder consists of 13 double strand DNA fragments ranging in sizes from 100 to 1,000 by in 100 by increments, and additional fragments of 1,200, 1,600, 2,000 bp. The 500, 1,000 and 2,000 by bands are two to three times brighter for easy identification (Bioneer, korea).

Application: Size confirmation of DNA fragments.

Features:

- Ready-to-use
- Recommended Loading: 1.5 – 2.0 µl/5 mm lane width
- Typical Loading Number of Lanes: 125 - 166 (5 mm lane width)
- Range (bp): 100 - 2,000
- Number of Bands: 13

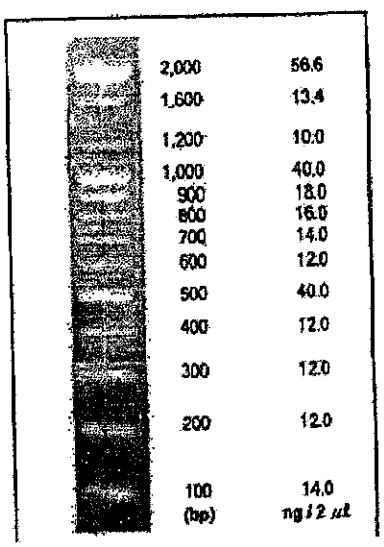

Fig-8: 1% agaros gel strained with Ethidium Bromide