IDENTIFICATION OF RISK FACTORS FOR WSSV (White Spot Syndrome Virus) INFECTION IN SHRIMP (*Peneaus monodon*) FARMS OF KHULNA AND BAGERHAT DISTRICTS

A Thesis By

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Approved as to style and content
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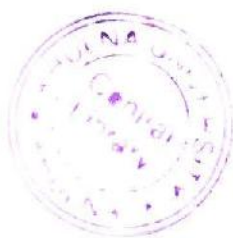

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DECLARATION

Identification of risk factors for wssv (white spot syndrome virus) infection in shrimp (*peneaus monodon*) farms of khulna and bagerhat districts

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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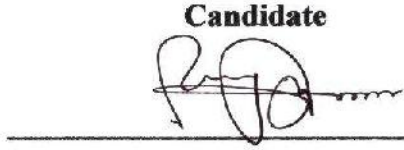

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ABSTRACT

In shrimp culture, various diseases, white spot syndrome virus (WSSV) in particular, has been emerged as a serious constraint to shrimp culture in Bangladesh. In this study, initiative has been taken assessing the riskfactors for WSSV outbreak under which 72 farms were investigated belonging 4 Upazilas of Bagerhat (Kochua, Rampal, Fakirhat) and Khulna (Paikgacha) during 2010-11. Major scenario depicted improved traditional culture method with the stocking density of 15,000~20,000/ha for bagda. Color of soil of most of the *ghers* in the study area found black. Water quality parameters *viz.*, temperature, Dissolve oxygen, salinity, pH, ammonia and alkalinity ranged of 20.7~34.7^0 C, 3~8.7 mg/l, 1~15 ppt, 7~10, 0.3~1.9 mg/l and 17.1~136 mg/l respectively. Water depth of the maximum *gher* was found within the range of 0.45 to 1.8 m. PCR test has been carried out to confirm WSSV infection. About 20 factors were considered for assessing the association of WSSV outbreak. Study revealed significant correlation with some factors like accessibility of cattle (r=0.630, $p \leq .01$) and linked up with other ghers (r=0.754, $p \leq .01$) within a cluster (Spearman's rho correlation coefficient test). Pearson Correlation coefficient for salinity found to have significant negative correlation with the risk of WSSV infection (r= -0.727, $p \leq .01$), followed by temperature (0.624, $p \leq .01$) and average depth (-0.618, $p \leq .01$), however, feeding kept 30.6% farms away from the outbreak followed by sludge removal (26.39% farms). On the contrary, uses of river water directly into the ghers caused 38.9% risk of being attacked which is absolutely nill and 1.4% for the underground and rain water respectively. Therefore, ensuring proper gher management practice, virus free Pl, awareness buildup at the farmer level and community based farm management development can act as preventive measures in reducing the risk of WSSV infection.

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List of Abbreviations

Abbreviation	Elaboration
µl	Micro liter
bp	Base pair
DO	Dissolve Oxygen
DNA	Deoxy Ribose Nuclic Acid
MHC	Major Histocompatibility Complex
PCR	Polymerase Chain Reaction
PL	Post Larvae
UV	Ultraviolet Transilluminator
WSSV	White Spot Syndrome Virus

Chapter One

INTRODUCTION

Diseases cause devastating losses to the shrimp industry world-wide (Wongteerasupaya *et al.*,1995). In Asia alone, the industries suggest annual losses of about 4 billion US$. Most of these pandemic diseases are of viral origin and one of the two most lethal is the White Spot Syndrome Virus, WSSV (Flegel, 2009).

White Spot Syndrome (WSS) is a viral infection of penaeid shrimp. The disease is highly lethal and contagious, killing shrimps quickly and thus become a fatal issue in the shrimp sectors world-wide. Outbreaks of this disease have wiped out within a few days the entire populations of many shrimp farms throughout the world. Natural epidemics of this disease have been reported throughout the world, especially in Asia. In mainland China, severe epizootics related to a WSSV infection that previously occurred in Taiwan in early 1992 have occurred every year since 1993, causing high mortalities in cultivated P. chinensis and resulting in great economic loss (Xu, Piao, Wang, Wang, Xiang & Wang 2000). In Korea, massive mortalities have occurred among the penaeid shrimp P. orientalis (Park, Lee, Lee & Lee1998) since1993, with an isolate originated from the same ancestor as the Taiwan, Thailand and China isolates (Moon *et al.*,2003). Since 1994, WSSV has been detected in cultured P. monodon in peninsular Malaysia (Wang, Hassan, Shari¡, Zamri & Chen 1999b), while the Indian subcontinent has been affected by WSSV epizootics, with outbreaks occurring in post larvae (Manivannan *et al.*,2002) and cultured P. monodon (Selvin & Lipton 2003). In Thailand, epidemics with WSSV showed seasonal fluctuation in the percentage of individuals infected, ranging from a low percentage (0-6%) from January to May to a higher

percentage (6-18%) for the rest of the year, with a peak from September to November (Withyachumnarnkul *et al.*,2003). In the Americas, mortalities of cultured P. vannamei (Boone), induced by WSSV, have occurred in Ecuador since 1999 (Rodriguez *et al.*,2003). In Mexico, the worst cases of this disease were also detected in 1999 (Galaviz-Silva *et al.*,2004), when WSSV caused severe damage to the shrimp industries of both Central and South America (Global Aquaculture Alliance 1999a, 1999b)

There is no treatment for WSSV and prevention is the best way to avoid outbreaks (Menasveta, 2002). Several studies have investigated the effect of disinfectants on WSSV (Chang *et al.*,1998; Maeda *et al.*,1998; Balasubramanian *et al.*,2006). The use of vaccines and immunostimulants to control WSSV has also been explored (Citarasu *et al.*,2006; Satoh *et al.*,2008; Sajeevan *et al.*,2009). Lotz(1997) and Mohan *et al*(2005) suggested biosecurity measures to exclude the pathogen or reduce its risk. Measures adopted by the shrimp industry include the stocking of shrimp larvae confirmed- WSSV- negative with polymerase chain reaction (PCR) test, use of disinfectants, closed culture system to reduce water exchange, birds' scares, crab fence, foot/tire bath, and limited access to the farm. In 2007, Islam *et al.* developed a PCR-based WSSV screening protocol in a diversity of species in Bangladesh and suggested PCR screening of WSSV infection for shrimp brood and larval stocks and other potential virus carriers that could be an effective tool in battle against the fatal virus that has almost devastated the shrimp commerce of the country. Most of the studies done on WSSV risk factors dealt with the carrier organism (Lo *et al.*,1996; Kanchanaphum *et al.*,1998; Suppamataya *et al.*,1998; Otta *et al.*,1999; Corsin *et al.*, 2001; Hossain *et al.*,2001; Yan *et al.*,2004; Liu *et al.*,2006), transmission (Suppamataya *et al.*,1998; Corsin *et al.* 2001; Peng *et al.*,1998), effect of water physico-chemical parameters (Vidal *et al.*,2001; Guan *et*

al.,2003; Rahman *et al.*,2006; Reyes *et al.*,2007), and genetic studies (Wongteerasupaya *et al.*,2003; Dieu *et al.*,2004; Syed Musthaq *et al.*,2006). Only a few studies reported risk factors related to pond culture. WSSV infection has been positively correlated with proximity of the pond to the sea and negatively to ponds closely located within a given cluster (Mohan *et al.*,2008). Sludge removal, ploughing, liming, complete system dry-out between culture cycles, water filtration through 300 μm mesh screen and phosphorus application through fertilization were reported to reduce risk of WSSV infection (Corsin *et al.*,2001; Velasco *et al.*,2002; Mohan *et al.*,2008). Corsin *et al.* (2001) found no association between stocking density and WSSV infection at harvest. However, Pienado-Guevara and Lopez-Meyer (2006) reported that removal of 40% and 50% of shrimp population with low level WSSV infection may improve survival. Ingestion of infected shrimp or fresh feed and use of commercial feeds have been associated with WSSV infection (Chou *et al.*,1995, Chou *et al.*,1998; Corsin *et al.*,2001). WSSV still continues to plague the shrimp industry, despite the bulk of information available. Most of these information resulted from experimental or laboratory-based studies relating to transmission and to the physicochemical properties of the water.

Epidemiology is another approach of understanding a contagious disease. Factors related to pond location and management practices seem to affect WSSV epidemiology but these have hardly been investigated. A survey is appropriate to gather information from a population which is beyond the control of the investigator on an experimental unit. The relationship between a disease and the hypothesized causal factors in a certain disease occurring situation, like between WSSV and farm practices, can be investigated using both questionnaire biological samples in a cross-sectional survey.

The virus has a wide host range and is highly virulent and leads to mortality rates of 100% within days in the case of cultured penaeid shrimps. Most of the cultured penaeid shrimps (Penaeus monodon, Marsupenaeus japonicus, Litopenaeus vannamei, Fenneropenaeus indicus, etc.) are natural hosts of the virus. Several non-penaeid shrimps were also found to be severely infected during experimental challenges. Many crustaceans like crabs (Scylla spp., Portunus spp.), spiny lobsters (Panulirus spp.), crayfish (Astacus spp., Cherax spp., etc.) and freshwater shrimp (Macrobrachium spp.) are reported to get infected with variable severities depending on the life stage of the host and presence of external stressors (temperature, salinity, bacterial diseases, pollutants, etc.). Clinical signs of WSSV include a sudden reduction in food consumption, lethargy, loose cuticle and often reddish color, and the presence of white spots of 0.5 to 2.0 mm in diameter on the inside surface of the carapace, appendages and cuticle over the abdominal segments.

Disease progression is characterized by cessation of shrimp feeding followed within a few days by the appearance of moribund shrimp swimming near the surface at the edge of rearing ponds. The cuticular inclusions range from minute spots to discs several millimeters in diameter, which may coalesce into larger plates. These are most easily observed by removing the cuticle over the cephalothoraxes, scraping away any attached tissue with the thumbnail and holding the cuticle up to the light. However, the appearance of white spots in the cuticle is unreliable even for preliminary diagnosis of WSD as similar inclusions can be produced by high alkalinity and other environmental conditions and bacterial white spot syndrome (Wang *et al.*,2002). Therefore, preliminary diagnosis must include histopathological examinations. Farms and hatcheries have few defenses against rampaging protozoa, fungi and bacteria, but it is the viral diseases that pose the greatest threat.

The causative agent of white spot disease (WSD) is white spot syndrome virus (WSSV); a double-stranded DNA virus assigned by the International Committee on Virus Taxonomy to its own new genus, Whispovirus, in the family Nimaviridae (Mayo, 2002a,b). The Virions are large (80–120 · 250–380 nm), rod-shaped to elliptical, and with a trilaminar envelope (Inouye *et al.*,1994; Wang *et al.*,1995; Durand *et al.*,1997; Kanchanaphum *et al.*,1998; Van Hulten *et al.*,2001). The virus received different names during the first years after it appeared, such as hypodermal and haematopoietic necrosis baculovirus, rod-shaped nuclear virus of Penaeus japonicus (RV PJ) (Inouye *et al.*,1994; Nakano *et al.*,1994), systemic ectodermal and mesodermal baculovirus (Wongteerasupaya *et al.*,1995), white spot baculovirus (Chou *et al.*,1995; Lo *et al.*,1996) and Chinese baculovirus (Lu *et al.*,1997). All these isolates are now recognized as one virus: WSSV. White spot syndrome virus has a large circular double-stranded DNA genome of 292967 bp (van *et al.* 2001a); however, di¡erent genome sizes have been reported from diverse virus isolates (Chen,Wang, Huang, Peng, Chen, Lin, Chen, Dai,Yu,Wang, Lo & Kou 2002).

Besides clinical signs of infection including characteristic white spots in the cuticle (Chou *et al.*,1995; Wang *et al.*,1999), histopathological signs by light and electron microscopy have also been extensively described for WSSV-infected animals (Chou *et al.*,1995; Wongteerasupaya *et al.*,1995; Lightner, 1996; Durand *et al.*,1997; Wang *et al.*,1999). In addition to antisera for immuno-detection (Zhan *et al.*,1999; Van Hulten *et al.*,2001a), DNA hybridisation probes have been developed in several laboratories (Chang *et al.*,1996; Durand *et al.*,1996; Wongteerasupaya *et al.*,1996; Lo *et al.*,1997) to detect virus-infected cells. Molecular primers based on these probes have been developed for WSSV detection by

polymerase chain reaction (PCR) (Chang *et al.*,1996; Lo *et al.*,1996b). These molecular techniques are highly specific and have a high degree of sensitivity.

In Bangladesh, Shrimp sector is playing a significant role in foreign exchange earning, employment generation and poverty reduction in Bangladesh. The two main areas of shrimp production are located in the Southwestern part - Khulna, Satkhira and Bagerhat districts; and the other area is located in the Southeastern part of the country consisting of Chittagong and Cox's bazaar districts (Rahman, 1998). About 75% of the total shrimp farms are located in Khulna, Bagerhat and Satkhira districts. Penaeus monodon is the major species cultured in Bangladesh. Of all the exportable agro-based primary commodities, shrimp is by far the most important. Traditionally shrimp farming began by trapping tidal waters in nearby coastal enclosures known as 'Gher' where no feed, fertilizers or other inputs were applied. Due to an increasing demand from both national and international markets farmers started to switch over into improved extensive and semi-intensive systems.

Although the total production of shrimp is increasing due to horizontal expansion of culture area but per unit production is quite low compared to neighboring and other developed countries mainly due to occurrence of diseases and lack of appropriate culture technology. Various diseases particularly, WSD caused by WSSV in brackish water shrimp culture has emerged as a serious constraint affecting shrimp production in Bangladesh.

In aquaculture systems, a risk factor is a crop-related factor, which simply increases or decreases the probability of occurrence of an adverse event happening during a specified time period (MPEDA/NACA, 2003). WSD is associated with some adverse events during the shrimp cropping period. If a high prevalence of WSSV in seed batches stocked in ponds

increases the probability of occurrence of WSD then the high prevalence of WSSV in seed batches is called a risk factor to WSD. MPEDA/NACA (2003) further noted that epidemiology investigates the statistical and biological significance of the relationship between the adverse event and the hypothesized risk factor to determine whether the hypothesized risk factor is a risk factor or not. The risk factor study of research considers shrimp disease outbreak and poor production as adverse crop events for the epidemiological analyses. In a farm situation, a number of "component causes" (risk factors) along with the "necessary cause" might become "sufficient cause" to produce WSSV outbreaks.

As there are few comprehensive studies on shrimp/prawn diseases in Bangladesh, associated risk factors and their control strategies, this study was conducted to infer the potential risks for the WSSV outbreaks in an epidemiological manner. During the study, data (Water quality parameters, management strategy e.g., pond preparation, liming, feeding, dike condition, cattle accessibility, etc.) collected from the sampling sites were analyzed by SPSS to figure out the risk factors where each ghers claimed WSSV positive/negative were confirmed by PCR test unless the identical opaque spherical spots appear on the sample shrimp confirming WSSV infection. Study revealed that accessibility of outer animals, lower depth, water sharing, linked up with other ghers, water source, etc. in a word poor farm management system and lack of awareness increased the risk of disease outbreak. As in every year, shrimp production largely hampered due to WSSV outbreak, this study will be helpful minimizing the risk and enhancing the production.

Objective

Objective of the study was to identify the risk factors for WSSV outbreaks in shrimp culture farms and to suggest the ways to overcome outbreaks.

Chapter Two

METHODOLOGY

2.1. Study area and experimental design

A total of 72 shrimp gher/farms (both affected and unaffected) were investigated randomly from four upazilas *viz.* Paikgacha, Kochua, Rampal and Fakirhat of Khulna and Bagerhat district. Random sampling was carried out for the month of January to June, 2011. These months were considered as the most crucial in WSSV infection and proliferation in the region for the last few decades. Investigation of the gher/farms carried out according to the following design (Table -2.1).

Figure 2.1: Red circle indicating the sampling area at Bagerhat and Khulna District

Table 2.1. Study area, water type and sample number

Sl. No.	Upazila	Type of water	No. of sample/month
01	Fakirhat	Farmer *gher*	3
02	Kochua		3
03	Paikgacha		3
04	Rampal		3
	Total Sample		**12 gher/month×6 month= 72**

During each sampling the following activities were performed:

- ❑ Questionnaire survey by interviewing the farmers on general aqua-ecology and management practices etc
- ❑ Water quality analysis
- ❑ Preservation of shrimp samples for investigation of WSSV.
- ❑ Statistical analysis for identification of the risk factors associated with WSSV infection.

Table. 2.2: List of different types of data considered

Sl. No.	Categorical Data	Numerical (Parametric) Data
1	Month	Temp.
2	Dike Condition	DO
3	Pond Drying	Salinity
4	Ploughing	Ph
5	Sludge Removal	Ammonia
6	Water Source	Alkalinity
7	Water Sharing	Stocking Density
8	Cattle Accessibility	Avg. Depth
9	Water Exchange	
10	Pl Source	
11	Liming	
12	Feeding	

2.2. Questionnaire survey

Each sampling comprised of the data regarding water quality parameters, general aqua-ecology and management practices etc. in a pre-tested well structured questionnaire comprised of several groups of variables viz., pond preparation, water management, culture practices and bio-security measures etc. that constitute the categorical part for the statistical analysis. The farmers were interviewed as per questionnaire on the spot in order to collect information regarding the gross aqua-ecology, farm management practices and previous history of shrimp farm/*ghers*.

2.3. Water quality analysis

Water sample collected and tested on the spot using a water test kit (HACH FF-2) within 10.00 A.M. Water quality parameters such as water temperature, salinity, pH, dissolved oxygen, total alkalinity and total ammonia were measured for each sampling site for statistical analysis.

2.4. White spot syndrome viral disease investigation

100 shrimp from each *gher* were examined during each sampling period for clinical signs and symptoms by eye observation. Twenty five shrimp from the observed samples were collected for PCR test (Genei's kit). Before DNA extraction, samples from each gher given an ID number. Under an ID no. all the shrimps were finely chopped and a required amount of tissue mass was taken for DNA extraction.

2.4.1. Chemicals used

1. DNA Extraction buffer
2. First PCR pre-mix (Contains reaction buffer with MgC12, dNTP's & WSSV specific

external primers)

3. Nested PCR pre-mix (Contains reaction buffer with MgC12, dNTP's & WSSV specific internal primers)
4. Taq DNA Polymerase
5. Positive control DNA
6. Negative control DNA
7. Gel loading buffer
8. DNA molecular weight marker

2.4.2. General Precautions

The nested PCR is highly sensitive and the reliability of the test is based on the well protection from contaminating each other. Following were made in reducing the cross contamination.

1. All the steps were carried out with gloves on.
2. The workbench was cleaned with 0.5 – 1 % freshly prepared sodium hypochloride on a regular basis.
3. Use of dedicated pipettes for PCR and contamination avoided through pipette tips.
4. Three separate areas were separated with dedicated pipettes and reagents for each area, e.g., first area for sample preparation, second sterile area (laminar hood) where the PCR mix is made and a third area where the gel electrophoresis is done.

2.4.3. Procedural precautions

1. All the reaction components kept on ice, the moment they are removed from the freezer.
2. The extraction buffer and gel loading buffer thawed at room temperature.

3. Fresh tips for each sample and reagent were used.
4. Used tips, and other reagents were discarded into a jar containing10-20% chlorax.
5. The positive control DNA kept separate from other kit components.
6. Taq Polymerase, PCR pre mix vials and the other vials spinned down for 30 seconds at 10,000 rpm before opening to recover the entire volume.

2.4.4. DNA Extraction

1. Shrimps were washed with sterile distilled water finely chopped.
2. From the chopped sample 20-30 mg of tissue samples were collected using a sterile forceps
3. 1.5ml Eppendorf vial taken and labeled as sample = 1.
4. First sample taken in this tube and 200 µl of extraction buffer were added
5. The tissue were homogenize with the help of the tissue grinder till most of the tissue is ground
6. Another 800 µl of extraction buffer added to this vial, sample #1 and the homogenization was repeated.
7. The same procedure applied for other samples after changing gloves for each sample. Labeled vials as sample # 2,3,4,5, etc.
8. The vials kept at 95°C for10 minutes in a water bath
9. All the vials centrifuged for 10 minutes at 10,000 rpm. The DNA samples are now ready.
10. Pipetting out 50-100 µl of each of the supernatants containing sample DNA into the 0.5ml vials
11. Labeled as SD # 1,2,3,4,5, etc. for sample DNAs.

2.4.5. DNA amplification:

2.4.5.1 First PCR:

Materials required:

1. First PCR Premix: 1 vial
2. Taq DNA Polymerase: 1 vial for both first & Nested PCR
3. Positive control DNA: 1 vial
4. Negative control DNA : 1 vial
5. Sample DNA vials SD # 1,2.3,4..... etc.
6. PCR vials: Label them as F# 1,2,3 ... etc.
7. Disposable gloves.

Master mix for first PCR as follows.

First run reaction volume	-	25 µl/vial
Mix the following		
First PCR pre mix	-	23 µl
Taq Polymerase	-	1 µl
Sample	-	1 µl

One vial of thawed first PCR premix was mixed well, determining the number of samples including 1 negative control, 1 positive control and 1 reagent control. The total volume of the master mix was calculated and 1 additional volume (24 pl) added for pipetting losses. 24µl of First PCR master mix aliquot into each of the required no. of PCR vials F # 1,2,3 .... etc. Then 1µl of each sample DNA added to the PCR vials F# 1,2,3... etc. 1µl of Negative control DNA added to the negative control vial. Then 1µl of Positive control DNA was added to the

positive control vial. The last vial remains as reagent control without any sample DNA marked as blank.

The contents of the PCR vials mixed by gentle tapping with the fingers and run the First PCR as follows:

95 °C x 3 minutes followed by

25-28 cycles of: 95 °C x 30 seconds

58 °C x 30seconds

72 °C x 30 seconds

Last cycle of 72 °C x 5 minutes

All the vials F # 1,2,3.... kept carefully at -20°C until nested PCR is done.

2.4.5.2 Nested PCR

Materials required:

1. Nested PCR premix: 1 vial
2. Taq DNA Polymerase
3. Tubes containing first PCR products F # 1,2,3...
4. PCR vials. Label them as N # 1,2,3...
5. Disposable gloves.

Nested PCR reaction volume - 25 µl/vial

Nested PCR pre mix - 23 µl

Taq Polymerase - 1 µl

First PCR product - 1 µl

Vials containing first PCR products centrifuged for 30 seconds at 10,000 rpm. The amount of master mix required, calculated as mentioned in the step 1 of First PCR. 24µl of Nested PCR

master mix aliquot into each of the vials.1µl of first PCR products added from vials F# 1,2,3.... to vials N # 1,2,3..... For reagent control and negative control in Nested PCR 1µl added from the negative control and reagent control vials of First PCR respectively. For positive control in Nested PCR 1µl added from the positive control DNA provided in the kit to 24 µl of the master mix.

The contents of the PCR vials mixed by gentle tapping with the fingers and performed the nested PCR as follows:

95 °C x 3 minutes followed by

25-28 cycles of: 95 °C x 30 seconds

58 °C x 30 seconds

72 °C x 30 seconds

Last cycle of: 72 °C x 5 minutes

The nested PCR products are then stored carefully till the gel electrophoresis performed.

2.4.6. Analysis of PCR products by gel electrophoresis:

Materials required:

1. DNA Molecular Weight Marker
2. Gel loading buffer
3. Submarine gel electrophoresis system with power supply
4. Agarose
5. 1 X TAE Buffer
6. Ethidium Bromide
7. U V Transilluminator

Sample preparation for gel electrophoresis:

To make the samples ready for electrophoresis 5 µl of 6X loading dye are added to the first & nested PCR vials F# 1,2,3... and N # 1,2,3 ... and mixed well.

The electrophoresis apparatus was assembled and 2.0% agarose gel was prepared by adding 2g of agarose to 100 ml of 1X TAE buffer. The agarose was then boiled in a beaker until it became clear without any agarose particles. 10µl of 10mg/ml ethidium bromide dye solution for 100 ml of agar cooled to 60 °C and poured into the gel box. The total thickness of the gel maintained not more than 0.8cm. The reservoir buffer (1 X TAE) was added and then the combs carefully removed once gel was solidified. In each pocket 8-10 µl of the samples (change the pipette tips for each sample) and 5µl Ready-to-use marker were loaded. The gel was run at 100-120 volts and stopped the electrophoresis when the deep blue dye reached around 2/3 rd of the gel (loading dye had bromophenol blue which was deep blue and xylene cyanol which was light blue). The gel was then removed and was ready for visualization on a mid wave UV Transilluminator to read the final result.

2.5. Statistical analysis

Statistical analysis was done by using SPSS (Windows Evaluation Ver. 14.0, released September 5, 2005). For the categorical data, nonparametric correlation test, spearman's rho has been performed inferring the degree of WSSV association whereas, for the parametric data, Pearson correlation coefficient has been considered. A positive correlation coefficient indicates that an increase in the corresponding variable will increase the risk of WSSV incidence. On the contrary, a negative correlation coefficient implies that an increase in the level of the respective variable will reduce the risk of WSSV occurrence.

Chapter Three

RESULTS

Tthe presence of WSSV in to the shrimp was made confirmed by Polymerase Chain Reaction (PCR) test; the test was performed when the symptoms of the disease (Opaque white spherical spot in cephalothorax, abdomen and telson mainly) could be reckoned visibly through appearance of spherical opaque spots in cephalothorax, abdomen and telson. PCR test obtained no presence of WSSV in the month of January and February (Fig. 3.1. A and B) but in early March, 2 ghers (in case of 2 and 3 sample) in Rampal Upazila showed WSSV positive result at 300 bp for nested PCR, however, external sign was visually absent (Fig 3.1. C).

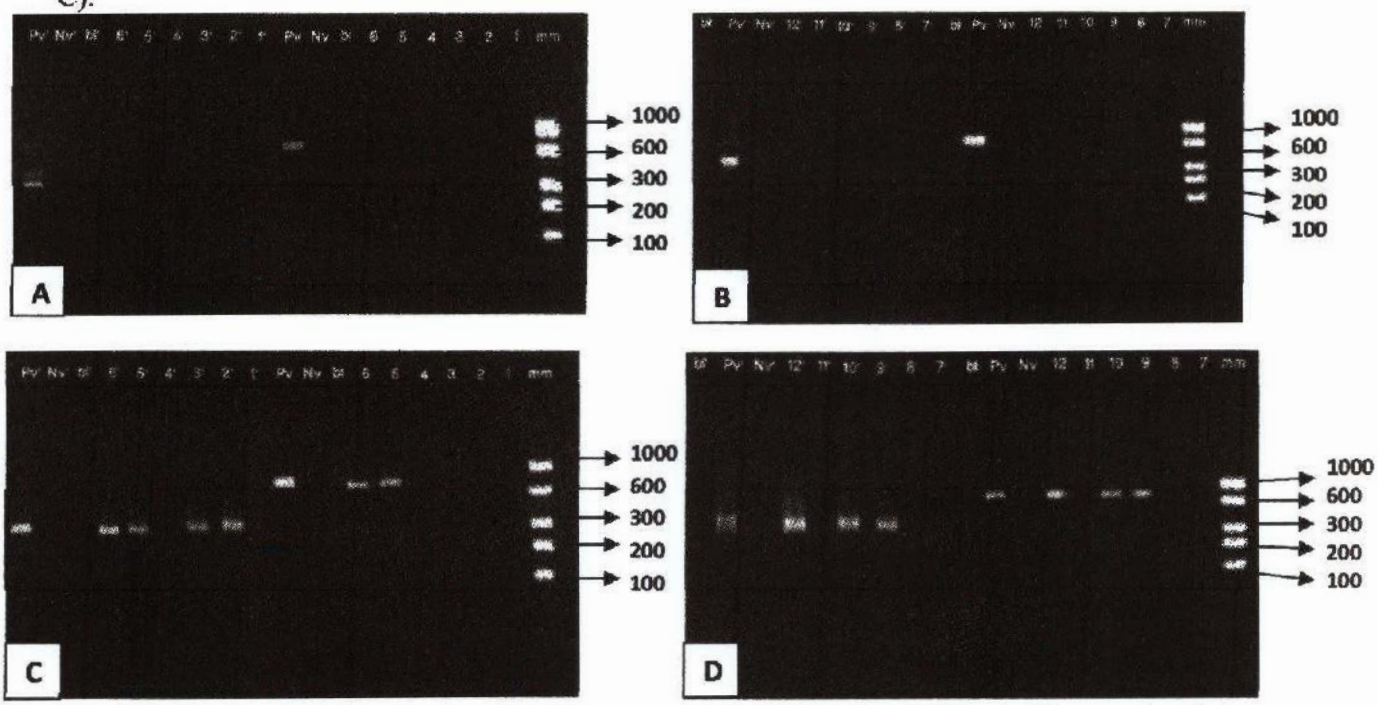

Figure 3.1. DNA Band attained by UV radiation (mm: molecular marker; 1~12: samples for 1st step PCR; bl: reagent controll; Nv: negative; Pv: positive; 1'~12': samples for nested (2nd step) PCR; bl': nested reagent controll; Nv': nested negative control; Pv': nested positive control).

In the same month, among the rest of the ghers 5, 6, 9, 10 and 12 showed WSSV positive both at 600 and 300 bp for first step and nested PCR respectively, along with identical opaque white spherical spot as external sign(Fig 3.1. C and D).

Regarding the parametric and non-parametric data analyses, six water quality parameters of the 8 parametric data collected throughout the crucial period of disease occurrence from the four sampling sites were analyzed by Pearson correlation test using SPSS (Table 3.1.).

Salinity and Temperature found to have significant correlation (r= -.727 and .624, $p \leq 0.01$)} with WSSV infection followed by average depth (r= -.618, $p \leq 0.01$). Study depicted, increase of average temperature from 21^{0}C at January to 33^{0}C at June, number of infected farm/gher reached from nil to 10 (Figure 3.2.) .

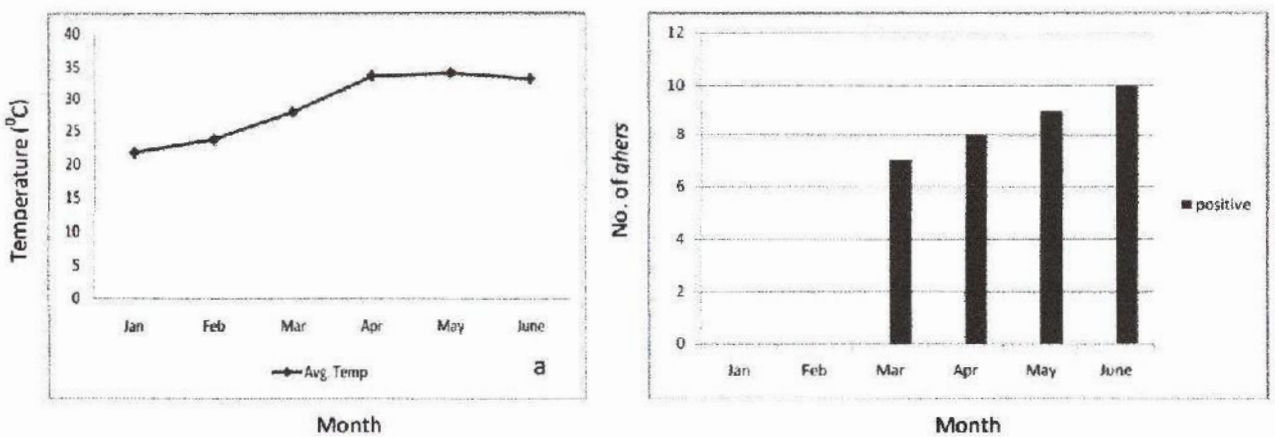

Figure 3.2. a) Trend of average temperature from January to June; b) Number of infected ghers throughout the study period

On the other hand, for the categorical data, Spearman's rho test was performed to infer the level of significance (Table 3.2.). Sharing same water in several ponds/ghers (Linked up with other ghers) showed significant influences (r=.754, $p \leq 0.01$) in WSSV infection followed by infection due to the accessibility of cattle or other animals into the ghers (r= .630, $p \leq 0.01$).

Table 3.1. Pearson correlation coefficient for parametric data.

		Tem	DO	Salinity	pH	Ammonia	Alkalinity	Avg.Depth	Stocking Density
Pearson Correlation	WSSV Infection	**.624(**)**	.070	**-.727(**)**	-.261(*)	.175	-.256(*)	**-.618(**)**	.043
	Sig. (1-tailed)	.000	.280	.000	.013	.071	.015	.000	.359
	N	72	72	72	72	72	72	72	72

** Correlation is significant at the 0.01 level (1-tailed).
* Correlation is significant at the 0.05 level (1-tailed).

Table 3.2. Spearman's rho correlation coefficient for non-parametric data.

		WSSV Infection	Pond Drying	Ploughing	Sludge Removal	Linked with	Water Sharing	Water Source	Accessibility of Cattle	Feeding	Liming
Spearman's rho WSSV Infection	Correlation Coefficient	1.000	-.273(*)	-.276(**)	-.179	**.754(**)**	.308(**)	**.416****	**.630(**)**	-.219(*)	-.302(**)
	Sig. (1-tailed)	.	.010	.009	.067	.000	.004	.000	.000	.032	.005
	N	72	72	72	72	72	72	72	72	72	72

* Correlation is significant at the 0.05 level (1-tailed).
** Correlation is significant at the 0.01 level (1-tailed).

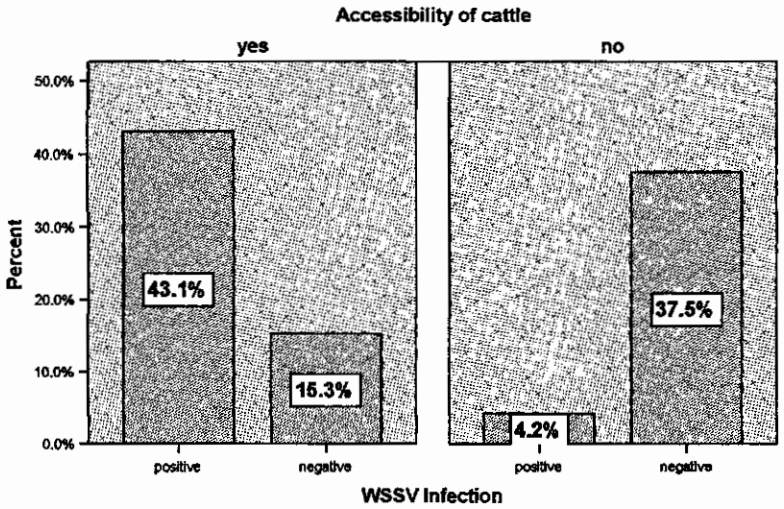

Fig 3.3. Percentages of WSSV infection and cattle accessibility

Among the ghers investigated, 43.1% found to be infected by WSSV where accessibility of cattle was frequent compared to the ghers free from cattle grazing (4.2%) (Fig. 3.3.).

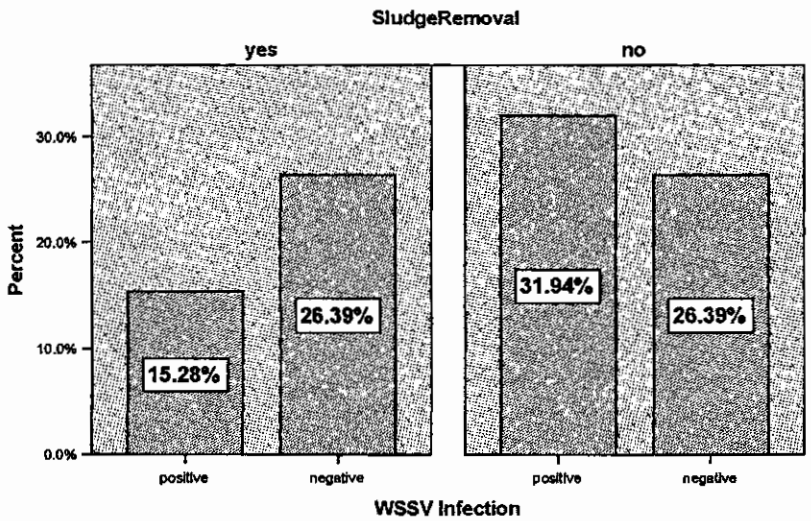

Fig 3.4. percentages of WSSV infection and sludge removal

Removal of sludge (black soil) was also found to have influences on WSSV infection. Sludge removed farms were found to be infected at 15.28% whereas farms which did not remove sludge were found to be infected at 31.94% (Fig. 3.4.).

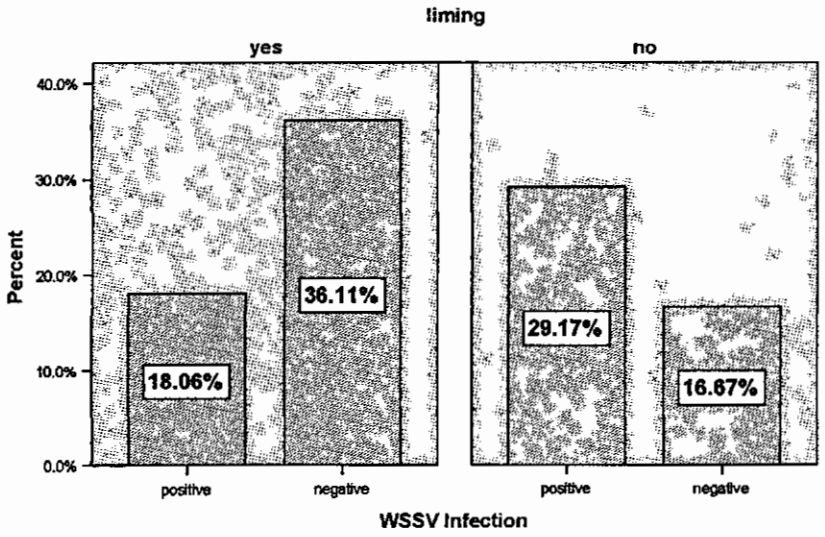

Fig 3.5. Percentages of WSSV infection and liming

Liming prior to the beginning of culture found to have influence reducing the risk of WSSV infection up to 36.11% whereas the risk raised up to 29.17% in the ghers that did not apply lime during as well as at the beginning of culture (Fig.3.5.).

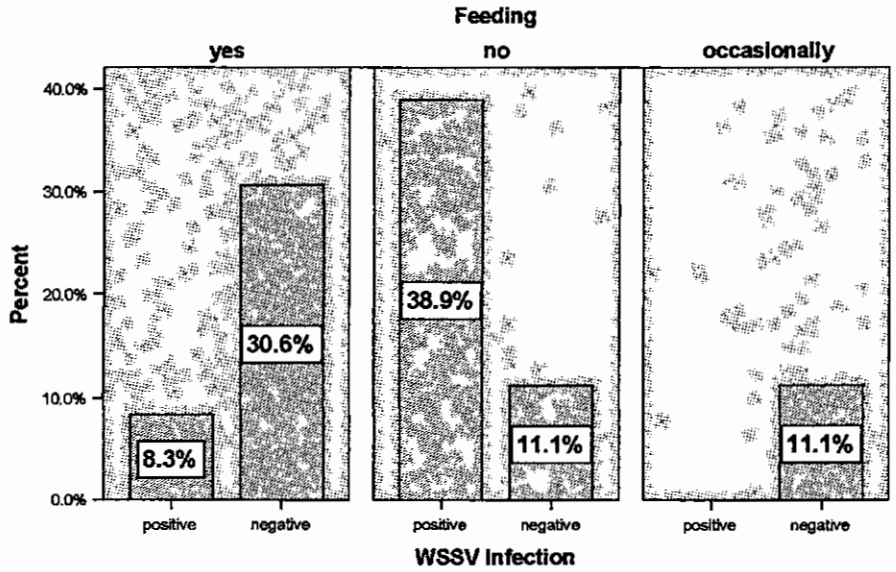

Fig 3.6. Percentages of WSSV infection and feeding

Feeding, however, is an important issue in any kind of culture practice, farmers in the study area found to be reluctant in feeding showed 38.9% risk of being infected by WSSV which can be reduced to 8.3% by providing supplementary feed (Fig3.6.).

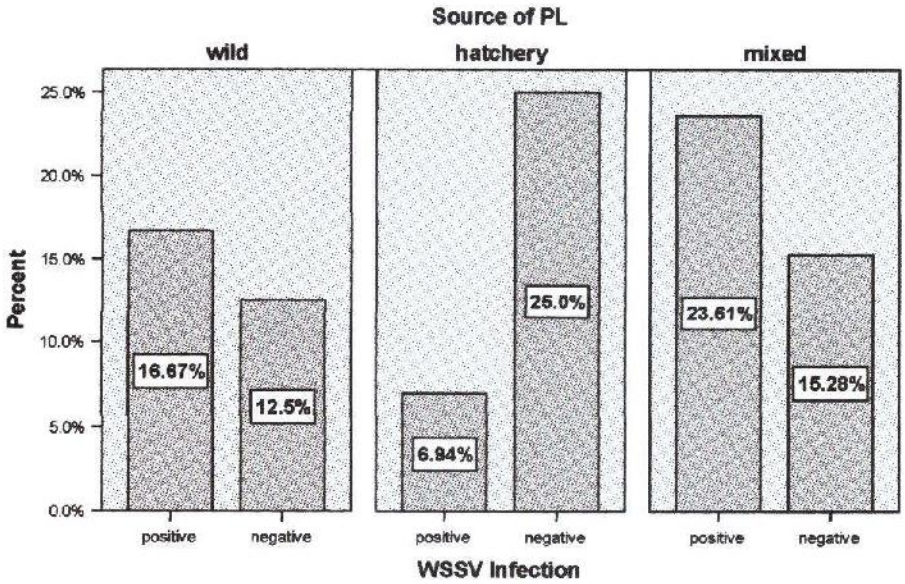

Fig 3.7. percentages of WSSV infection and source of PL

Another important factor found to be the source of PL. Risk of WSSV infection to the hatchery PL was up to 6.94% which could be up to 16.67% and 23.61% of the PL of wild source and a mix of wild and hatchery source respectively (Fig.3.7.).

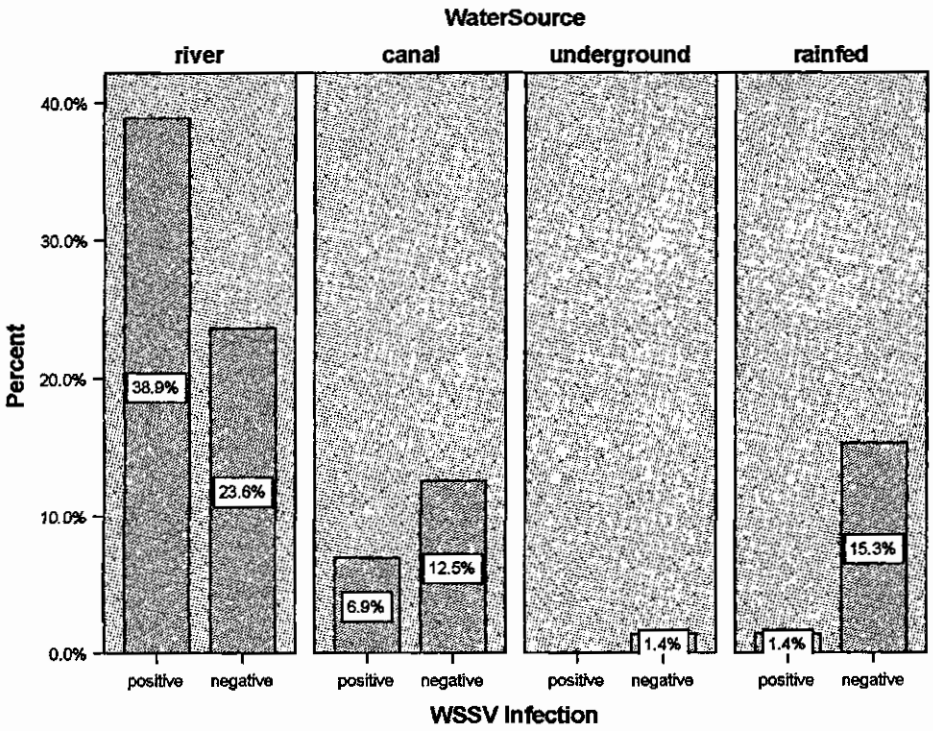

Fig 3.8. Percentages of WSSV infection and source of water

The most interesting thing found in the study was the source of water. WSSV risk was found up to 38.9% and 6.9% for the ghers having water directly from the rivers and canals which was just nil and 1.4% for the underground and rain fed water respectively (Fig. 3.8.).

Chapter Four

DISCUSSION

As an invertebrate, shrimp lack the key components of the vertebrate adaptive immune response (e.g. immunoglobulins, major-histocompatibility-complex (MHC) antigens, T-cell receptors) that provide a versatile mechanism for natural protection and allow for conventional vaccination against viruses (Arala-Chaves & Sequeira 2000; Johnson *et al.* 2008). Therefore, strategies for health management in shrimp aquaculture are primarily based on the principles of pathogen exclusion and the avoidance of environmental conditions that induce stress, stimulate viral replication or facilitate disease transmission. Studies revealed rapid changes in temperature due to heavy rain have influence on viral proliferation.

Temperature fluctuation and low temperature are identified as risk factors for WSSV infection, while an increase in temperature can be a risk factor for an outbreak in pond-cultured *P.monodon* (Tendencia, *et al.* 2010). As the temperature raises high to 33-35^{0}C in the month of April, May and June, number of infected ghers increased also. This is because, sudden rain at those months reduced the temperature rapidly as most of the gher found to have too low water depth to resist abrupt change in water temperature. According to Tendencia *et al.*, (2010a) climate is one of the WSSV risk factors. Climate as a risk factor referred to stocking of shrimp during the cool months. Low atmospheric temperature which affects water temperature is an important WSSV risk factor. Exposure to low water temperature could lead to WSSV infection in pond cultured *P. monodon* (Tendencia *et al.*, 2010b). This has been attributed to the increase in viral replication and the decrease in the immune response of shrimp at low temperature (Vidal *et al.*, 2001; Reyes *et al.*, 2007).

Salinity found to be another important factor supposed to have significant association with WSSV outbreak. Tank-based studies have reported that fluctuations in salinity and temperature couldweaken the shrimp's immune systemand affect viral replication. In *Marsupenaeus japonicus*, the immune response becomes weaker as the deviation from the original salinity they were kept in becomes greater (Yu *et al.*, 2003). Acute salinity change of greater than 4 ppt in 1 h, as well as continuous small salinity adjustments, could lead to rapid WSSV proliferation and decreased disease-resistance ability of *Fenneropenaeus chinensis* which makes the shrimp more susceptible to viral infection than they previously were, resulting in WSSV outbreak (Liu *et al.*, 2006). In the current study, being traditional in culture pattern, the gher structures of the most of the ghers could not withstand against salinity fluctuation due to sudden rain causes WSSV outbreak.

Effective pathogen exclusion practices require attention to all points in the shrimp production cycle, from spawning to harvest, at which viruses may be encountered or recycled into the environment (Fegan & Clifford 2001). According to the study, there is a significant correlation between accessibility of cattle in to the shrimp and WSSV proliferation. Accessibility of cattle into the ghers poses increase the risk of viral infection up to 43.1% which is quite alarming. Moreover sharing of same water and common flow-through system is found to be another threat in the study area having significant influence on WSSV outbreak. Mohan *et al.* (2008) reported that WSSV infection is negatively correlated to ponds closely located within a given cluster. It is possible that water that is allowed to enter the pond is mixed with discharge of other farms or of the farm itself. This water could be contaminated. This argument also holds for having the same receiving water source as a risk factor. Contamination of water can also come from the sludge removed from the pond

bottom. However, according to Tendencia *et al.* (2011), biosecurity measures did not prevent WSSV occurrence. Biosecurity measures aim to exclude pathogens and carrier organisms from the culture environment; e.g. farmers installed birds scare and crab fence to prevent the entry of birds and crabs. Though, these biosecurity measures often do not reach their purpose. Birds still fly over the strings that are supposed to scare them, might defecate above the pond and the faeces contaminated with WSSV particles could infect the cultured shrimp. Crab fence, usually made of nets, is installed on the pond dike to prevent the entry of crabs; however, crabs could still enter the pond by creeping through the net or by making holes through the dike. Farmers should optimize their biosecurity measures. Limited access to farm, foot baths and tire baths, and hand disinfection was strictly implemented by farm management. These measures concern humans as carriers. But the question is whether human can really carry WSSV particles that could infect the cultured shrimp was always questionable. This question is not just anecdotic; no scientific evidence proves that humans can transmit WSSV or particles carrying WSSV, although Corsin *et al.* (2005) found that sharing of personnel between ponds is associated with WSSV. Stocking of WSSV negative fry could prevent the entry of WSSV into the culture system, but shrimp once inside the pond can be infected in several ways.

Shrimp cultured in ponds with a larger size had a greater risk of WSSV infection. During the study, it was found that pond with larger size were more susceptible to viral attack compared to the small manageable size. Larger ponds are more difficult to manage. The inefficiency in farm management may lead to disease occurrence in cultured shrimp due to stress. Stocking density was a risk factor also and it might be recommendable to stock relatively less shrimp in larger ponds (Tendencia *et al.*, 2011).

In agreement with Tendencia *et al.* (2011), sludge removal was found to be a risk factor of WSSV infection in the shrimp farms. Sludge is made up of accumulated organic matter such as excess feed and faeces. Organic matter harbours micro organisms that could be pathogenic to shrimp. In most instances, sludge removed from the pond bottom was placed on the dike. This might allow harmful micro organisms present in the sludge to be washed back into the pond. The amount of sludge that accumulates at the pond bottom was affected by stocking density which was also a risk factor. The higher the stocking density the more organic matter due to faeces and uneaten feed may accumulate at the pond bottom.

Feeding live molluscs to shrimp poses a risk for WSSV infection which might transmitted in two ways to the system. Molluscs being filter feeders can serve as WSSV carriers, and molluscs could ingest WSSV particles from the soil and the water column, which could be transferred to the shrimp when the latter feed on them. Feeding commercial pellets as risk factor in polyculture farms might be a consequence of sludge accumulation at the pond bottom due to the absence of pond preparation in these farms (Tendencia *et al.*, 2011). However, feeding showed negative correlation with WSSV infection in the current study. Farmers provide feed to the farms are likely to have 30% less chance by being infected by WSSV.

Source of post-larvae (PL) also found having impact on viral infection in the shrimp farming area of the country because WSSV is highly infective not only for marine penaeid shrimp (Rodriguez *et al.* 2003), but also infect many other crustaceans and marine crabs (Hameed, Balasubramanian, Musthaq & Yoganandhan 2003). There are reports that freshwater species such as the cray fish *Pacifastacus leniusculus* (Jiravanichpaisal, Bangyeekhun, Soderhall & Soderhall 2001) and *Macrobrachium rosenbergii* (Chakraborty, Otta, Kumar, Hossain,

Karunasagar, Venugopal & Karunasagar 2002) are also susceptible to infection.WSSV genomic DNA can be vertically transmitted to *Artemia* cysts, but it is lost during hatching (Li, Zhang, Chen & Yang 2003).

Study revealed that farmers using hatchery produced PL was capable to some extent in reducing the risk of infection by nearly about 10% than the farmers using wild PL in the farms. Because of the same reason, water from different source as of being contaminated with virus particles, also found posing risk of WSSV infection into culture system. Direct rain fed ponds and farmers using underground water found to be less susceptible for viral infection. Evaporation process for rain water and different filtration stages for underground water might have impact reducing the association of WSSV in the farming system. Further study is required confirming the risk factors responsible for massive infection of WSSV in the shrimp industry.

Therefore, the main effects of the protective and risk factors are on water or pond bottom quality and immune response of shrimp. Rapid changes in salinity and temperature can be minimized by ensuring proper depth in the culture pond/gher. Intensive study is required to confirm the optimum water depth minimizing the abrupt changes in salinity and temperature due to sudden rainfall. Some WSSV risk factors such as feeding live molluscs to the shrimp should be avoided. Proper liming and feeding can be maintained to ensure good health condition of the shrimp. Sludge removed from the pond should be disposed in a place where washing back into the system is prevented. The effect of other risk factors can be mitigated by the implementation of the identified protective factors.

Chapter Five

CONCLUSION

Shrimp export is the second largest source of earning foreign currency in Bangladesh. However, in recent years, the production of cultured shrimp has markedly decreased as a result of serious viral disease outbreaks. Especially, the increased severity of widespread White Spot Syndrome Virus (WSSV) infection is the most serious threat to stable aquacultural production. Several studies to control the disease have been done. However, tank experiments identified WSSV risk factors related to the physico-chemical properties of the water, a few studies reported pond level WSSV risk factors. In this study initiative taken to find out the risk-factor associated with WSSV outbreak in the shrimp farms which will be helpful reducing the risk of massive disease outbreak. Study revealed Proper pond/gher preparation, stocking of SPF PL and maintaining optimum water depth (not less than 3 feet) can withstand against the invasion of viral pathogen into the culture area. Flow-through system within the ghers along with cattle grazing in the culture area also found to have fatal impact on viral spreading from which some management strategy can help to get rid out of the issue with ease. In a word, it can be said that proper farm management could have adequate impact on preventing mass mortality due to WSSV infection as there is no sustainable treatment has yet been developed. Therefore, proper initiatives to be taken to grow awareness among the farmers which can play vital role in minimizing the loss through WSSV outbreak of shrimp sector.

Chapter Six

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Dyke repairing: Yes No

5. Stocking management

Source of pl:

Size of pl:

Stocking density:

Time of stocking:

Stocking frequency:

Nursed pl: Yes No

Stocking strategy: Poly culture Mono culture

Species composition:

Netting: Yes No

6. Post-stocking management

Culture method: Traditional Semi-intensive

Intensive

Culture period:

Source of feed: Natural Supplemental

Feeding frequency:

Type of feed: Self formulated Readymade

Feeding ingredients (for self formulated feed):

Rate of feeding (g/body weight):

Disease occurrence: Yes No

Protecting measure:

Protection of outer animal by polythene: Yes No

Presence of unwanted animals: Yes No

Use of aerator: Yes No

Fig. Sample Collection

Fig. Questionnaire session

Fig. WSSV infected sample

Fig. Sample Preparation

Fig. Loading sample into PCR

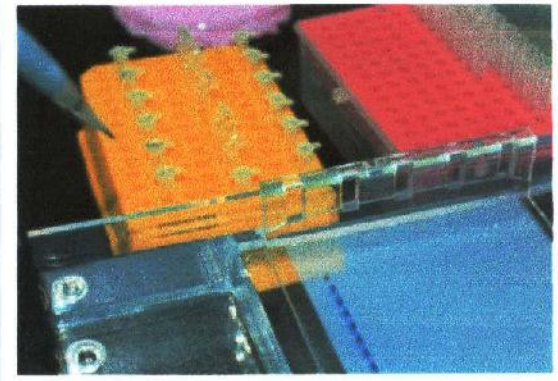

Fig. Gel loading after PCR