Detection of viral pathogens in shrimp broodstock of commercial shrimp hatchery in Cox's Bazar using polymerase chain reaction

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Detection of viral pathogens in shrimp broodstock of commercial shrimp hatchery in Cox's Bazar using polymerase chain reaction

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DECLARATION

Detection of viral pathogens in shrimp broodstock of commercial shrimp hatchery in Cox's Bazar using polymerase chain reaction

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

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Dedicated to...

My Beloved Parents

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Abstract

Bangladesh enters into international shrimp market in early 1970s and now it occupies 2nd position in foreign currency earning. However, this sector facing a serious problem with White Spot Syndrome Virus (WSSV) which causes 100% mortality of shrimp in the shrimp farm within two weeks. Shrimps are also infected by Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Monodon Baculo Virus (MBV), Hepatopancreatic Parvo Virus (HPV) and Yellow Head Virus (YHV) which may cause serious problem in farms. Therefore, the present study was conducted to identify these viral pathogens in brood stocks of *Penaeus monodon* and to assess the impact of temperature fluctuation on viral pathogen intensity to wild shrimp brood stocks using polymerase chain reaction (PCR). The study was conducted in five shrimp hatcheries of Cox's Bazar area from January to March, 2014. Samples of pleopod, gill and feces were collected from 20 shrimp broods from each hatchery in every month. Among the tested viral pathogens, the highest and lowest prevalence was observed in IHHNV as 95% (Zamzam hatchery) and WSSV (Modern hatchery) and HPV (Zamzam hatchery) as 20%, respectively. However, there is no significant difference was found on viral contamination rate among the hatcheries except IHHNV ($P>0.05$). The significant difference was found among the virus prevalence rate with temperature changes ($P<0.05$). The trend of monthly infection rate was sequenced as March>February>January. The percentage of prevalence of WSSV, IHHNV, MBV and HPV were showed significant variation with the temperature changes ($P<0.05$). Tukey post-hoc HSD test stated that MBV and IHHNV, YHV, and WSSV and HPV were showed significantly ($p<0.05$) highest, moderate and lowest percentage of prevalence. However, there were no significant differences of contamination rate between MBV and IHHNV, and WSSV and HPV ($P>0.05$) in different hatcheries during the study period. This research signify the percentage of total carriage of different viruses by wild brood stock to hatchery and it will be a effective guidelines for national policy makers to take further steps on reducing the viral pathogens intensity in shrimp hatchery industries.

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List of Abbreviations

Abbreviation	Elaboration
WSSV	White Spot Syndrome Virus
IHHNV	Infectious Hypodermal and Haematopoitic Necrosis Virus
MBV	Monodon Baculo Virus
HPV	Hepatopancreatic Parbo Virus
YHV	Yellow Head Virus
DNA	Dioxyribonucleic Acid
RNA	Ribonucleic Acid
rpm	Rotation Per Minute
mg	Mili Gram
ml	Milli Liter
DoC	Days of Culture
DoF	Department of Fisheries
DEPC	Diethylpyrocarbonate
bp	Base Pair
EtBr	Ethidium Bhromide
NPV	Nuclear Polyhedrosis Virus
ANOVA	Analysis of Variance
ANCOVA	Analysis of Covariance
PCR	Polymerase Chain Reaction
TBE	Tris, Boric acid, EDTA
µl	Micro Liter
UV	Ultra Violate

Chapter-1

Introduction

1. Introduction

1.1 Background of the study

Aquaculture represents the second largest export industry in Bangladesh. Total 97% of the shrimp produced being exported, contributing about 4.39% to national GDP and employing approximately 1.2 million people for production, processing and marketing activities (Haque *et al.,* 2012). Since the availability of good quality shrimp post larvae (PL) is important in successful shrimp culture, commercial shrimp hatchery expanded gradually from 1996 to 2000 in Kolatoli and Sonapara of Cox's Bazar Zone to meet the growing demand of shrimp culture. Shrimp post larvae first see the light in this world during January 1992 (DoF, 2012) by pioneer hatchery in Kolatoli, Cox's Bazar. At present, there are about 57 shrimp hatcheries in Bangladesh with a capacity to produce 15 billion PL per annum and most of them are established in Cox's Bazar for its geographical importance and suitable environment for PL production. Present demand of Shrimp PL is about 10 billion per year, where 2-3 billion comes from natural sources and the rest 8-7 billion PL demand are met up by commercial hatcheries. Recently, wild fry collection has been strictly prohibited by government that result increasing demand of hatchery produced PL (Debnath *et al.,* 2014). In hatchery, the post larvae is caught at the age of day 15 or 16 by a net and transported by car, bus (80% survive) or airplane (100% survive) to the nursery in the cultivation areas in the south-west of Bangladesh. Then the shrimps are reared in nursery and released in traditional and semi-intensive farms. However, the most important problems in shrimp culture is that shrimp PL are often affected by viral disease that causes mass mortality as well as a huge moneytary loss of farmers.

There are more than 1100 viruses from invertebrates have been reported. More than 30 viral diseases are now known to occur in crustaceans, including penaeids. Amongthe White Spot Syndrome Virus (WSSV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), Monodon Baculo Virus (MBV), Hepatopancreatic Parvo Virus (HPV) and Yellow Head Virus (YHV) are the most harmful viruses reported for farm and hatchery of *P. monodon* for their etiological features.

The first reports of WSSV are in *P. japonicas* in Taiwan (city I-Lan), 1992 (Chen, 1995). WSSV is reported to spread quickly in other species, especially *P. monodon* in most parts of Asian countries and the United States in 1993 (Momoyama *et al.,* 1994; Flegel *et al.,*1995; Galaviz, 1999; Lo *et al.,* 1999a). In Iran it is observed in *P. indicus* from Choebdeh area in 2004 and within 3-10 days, mortality in the farms reached up to 90% and bringing large economic losses to the shrimp farming industry of Iran (Tokhmafshan *et al.,* 2004). In Bangladesh, an outbreak of WSSV of cultured black tiger shrimp occurred in semi-intensive farms in Cox's Bazar in 1994 (Larkins, 1995) and quickly spread to Khulna region of the country. Since then shrimp farming industry in the country has been seriously affected by WSSV resulting in huge loss in every year. As a result, shrimp productions from a unit area have also been declined in Bangladesh. According to the Department of Fisheries (DoF), Bangladesh suffered a 44.3% production loss in 1996, leading to a reduction in 42.3% foreign income of shrimp exports (Siriwardena, 1997).

IHHNV was first discovered in blue shrimp *Penaeus stylirostris* and white shrimp *P. vannamei* (also called *Litopenaeus stylitrostris* and *L. vannamei*) in the Americas in the early 1980's (Lightner *et al.,* 1983b) and it was believed to be introduced by importation of live experimental stocks of the black tiger shrimp *P. monodon* from Asia (Lightner and Redman, 1991, Lightner, 1999b). It is important to understand that IHHNV was unknown before this jump from *P. monodon* because it generally produces no adverse effects in *P. monodon,* including no gross or histological signs of disease (Chayaburaku *et al.,* 2005) but produce a runting syndrome in the giant tiger shrimp, *Penaeus monodon* reducing the latter's market value by10%–50% (Lightner and Redman, 1998). Although IHHNV has been reported to occur in several species of wild and cultured penaeid shrimp throughout the world, it has been reported to cause acute epizootics and mass mortality only in *P. stylirostris*, especially in juveniles and sub-adults (Lightner, 1996a). By contrast, it does not cause mortality in *P. vannamei,* but rather reduced, irregular growth and cuticular deformities, gross signs collectively referred to as "runt-deformity syndrome" (Bell and Lightner, 1984; Bell and Lightner, 1987; Brock and Maim, 1994). *P. indicus*and *P. merguiensis* (also called *Fennero penaeus indicus* and *F. merguiensis*) appear to non-susceptible (Bell and Lightner, 1987; Lightner, 1993).

Monodon baculo virus (MBV), also called spherical baculo virus, was first observed in 1977 in adult *P. monodon* that were laboratory reared in Mexico from postlarvae imported from Taiwan (Lightner & Redman, 1981; Lightner *et al.,* 1983b). The virus was soon found to occur commonly in *P. monodon* populations from Taiwan, the Philippines and French Polynesia (Lightner *et al.,* 1983b) and has subsequently been shown to be enzootic in *P. monodon* throughout its geographical range from east Africa and Madagascar in the west, through the Middle East and the Indian subcontinent, South-East and East Asia, to Australia and islands of the South Pacific. It has also been reported in many countries to which *P. monodon* shrimp have been introduced, including West Africa, the Mediterranean, Tahiti, Hawaii, North and South America and the Caribbean (Lightner, 1996b).

HPV has been knownto infect several penaeid shrimp in Asian, Australian and African regions since 1985 (Lightner, 1993).HPV was first reported from Thailand infecting *P. monodon*in 1993 (Flegel and Sriurairatana, 1993). Shrimp infected with HPV usually show non specific gross signs of disease. However, infection isstatistically related to retard growth in *P. monodon* (Flegel *et al.*, 1999). HPV virions are icosahedral, approximately 22 nm in diameter and contain ssDNA of 6 kb in size. This probably comprises overlapping genes for at least 2 polypeptides. The virus was classified in the family *Parvo viridae* based on these characteristics (Bonami *et al.*, 1995; Spann *et al.,* 1997; Sukhumsirichart *et al.,* 1999). Detection was traditionally dependent upon the histological demonstration of basophilic intranuclear inclusions in tubular epithelial cells of the hepatopancreas (HP) by hematoxylin and eosin (H&E) or Giemsa staining (Lightner, 1996a).

YHV of black tiger shrimp *P. monodon* was first discovered in Thailand in 1992,although it is now known to have caused extensive losses on the estern coast of the Gulf of Thailand as early as 1991 (Flegel *et al.,* 1995). Shrimp infected with YHV often show light yellow coloration of the dorsal cephalothorax area and have a pale or bleached appearance. It is a RNA virus. Gill is mainly affected by this virus and it also called gill associate virus.

There is no report about the outbreak of disease affected by other viruses (MBV, HPV, IHHNV, and YHV) in our country but prevalence of these viruses already recorded from hatchery and farm level, which are influenced by biological agent. Viruses are often called infectious disease

agent. They can be present in the water or in sediment as part of the normal flora. The two major characteristics of an infectious agent are (a) capability for direct transmission and (b) ability to multiply in the host tissue. The mode of their transmission is either (a) vertical or (b) horizontal. In horizontal transmission, infectious agents come in contact with the hosts through the water, the feeds or through carrier animals that are in the environment. In vertical transmission, infectious agents transfer from parent to offspring. The female or male brood stock may be carriers of diseases, and transfer them to their offspring through the egg or sperm. Virus normally introduced in commercial hatchery by wild broodstocks, which are collected from commercial fishing ground of Bay of Bengal. These wild broodstocks are frequently affected by viral pathogens such as WSSV, IHHNV, MBV, HPV and YHV. In Bangladesh, most of the hatcheries don't conduct any preliminary viral test before stocking the wild brood. As a result, vertical transmission occurred through broodstock to offspring and their presence and number are largely influenced by environmental factors like temperature, salinity, dissolved oxygen, pH, and availability of food (Debnath *et al.*, 2014). The elevation of water temperature, current speed and heavy rainfall are reported to accelerate the susceptibility of WSSV infectionin *P. monodon* (Iqbal *et al.*, 2011). Changes of temperature over the year have a prominent effect on intensity of viral pathogens to broodstock shrimp. However, the effect of temperature on the prevalence of IHHNV, MBV, HPV and YHV is not yet understood.

Therefore, the present study was under taken to know the present status of WSSV, IHHNV, MBV, HPV and YHV prevalence in broodstock of *P. monodon* collected from Bay of Bengal and used in hatchery for spawning. The results presented in the thesis are part of a larger epidemiological study on WSSV, IHHNV, MBV, HPV and YHV. Besides, some practical issues regarding the methodology for effective screening of broodstock for WSSV, IHHNV, MBV, HPV and YHV by PCR are identified and discussed. Several diagnostic PCR methods have been developed for screening and early detection of the disease (Lightner, 1999b; Lo *et al.*, 1996b; Takahashi *et al.*, 1996; Kim *et al.*, 1998).

1.2 Objectives of the study were

- To detect the contamination of WSSV, IHHNV, MBV, HPV and YHV of *P. monodon* brood in shrimp hatchery
- To know the temporal variation of virus prevalence.

Chapter-2

Literature Review

2. Literature Review

2.1 Viruses

Yan *et al.* (2004) reported that the occurrence of WSSV in rotifer eggs, identified as *Brachionusurceus*, from shrimp culture-pond sediments, was detected by PCR dot blot hybridization. Since WSSV was detected in previously chlorinated eggs, it was hypothesized that WSSV does not bind to the egg surface, but is possibly transmitted vertically. Cell membranes from *B. urceus*, specifically bind purified WSSV *in vitro*, suggesting that this rotifer may be a passive host of WSSV, and may represent a feasible WSSV vector for crustaceans. Vidal *et al.* (2001) reported that serious WSSV epizootics are less probable to occur during warm periods, and it has been proposed that management practices that increase pond temperature may offer an interesting opportunity to manage this disease. Lotz *et al.* (2002) reported that transmission by ingestion of infected tissue is over an order of magnitude higher than cohabitation transmission. Vijayan *et al.* (2005) 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%. Hossain *et al.* (2001) reported that wild-caught asymptomatic marine shrimp such as *Metapenaeusdoson, Parapenaeopsis stylifera, Solenocera indica and Squilla mantis* carry WSSV. 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.

Infectious hypodermal and hematopoietic necrosis virus (IHHNV) was first detected in juvenile *P. stylirostris* imported into Hawaii from commercial hatcheries in Costa Rica and Ecuador in 1981 (Lightner *et al.*, 1983a, b). IHHNV is now classified in the genus Brevidensovirus of the family *Parvo viridae* as *P. stylirostris* denso virus (PstDNV) 24 (Flegel, 2006).

The disease was highly lethal in *P. stylirostris* juveniles and resulted in mortalities of up to 90% in affected populations. IHHNV has been listed as an economically significant pathogen by the Gulf coast research marine shrimp farming consortium (Lotz *et al.,* 1995) and is an important disease causing agent of cultured penaeid shrimp causing severe mortalities in *Litopenaeus stylirostris* from 1980to 1990 (Lightner *et al.*, 1983a, b).

The infectious hypodermal and hematopoietic necrosis virus (IHHNV) 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 baculo virus (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.

Monodon baculo virus (MBV), a nuclear polyhedrosis virus (NPV) of the family *Baculo viridae*, was first reported in *P. monodon* shrimp in Taiwan (Lightner and Redman, 1981). As with all NPVs, it possesses a double-stranded circular DNA genome of 80-100 x 106 Da with a rod shaped enveloped particle often found occluded within protein aceous bodies. The latter is composed primarily of the protein polyhedrin. MBV has been implicated in the collapse of the shrimp farming industry in Taiwan in the mid-1980s. Similar catastrophic mortalities due to MBV have been reported in Mexico (Lightner *et al.,* 1983b) and Thailand (Flegel, 2006) .Even though MBV is not a serious pathogen for the tiger prawn, it should be eliminated from the farming system because it is unlikely that shrimp could carry such heavy infections. It has been reported that, the mean length of MBV infected shrimp is significantly shorter than uninfected shrimp from the same pond (Flegel, 2006). The importance of MBV in shrimp culture is exemplified by problems related to infection that is usually encountered in hatchery and grow out operations and should be eliminated since it can slow the growth in the culture system (Flegel, 2006). The target organs of MBV are hepatopancreas and anterior midgut (Lightner *et al.,* 1983b). The presence of hypertrophied nuclei with single or multiple spherical occlusion bodies is the principal diagnostic feature of MBV infection (Lightner *et al.,* 1983b). Direct staining with

malachite green and conventional histopathology was used initially to detect MBV (Lightner and Redman, 1991) and is still the gold standard.

Hepatopancreatic parvo virus (HPV) was first reported by Lightner and Redman, (1985) in cultured populations of four different penaeid shrimp species from different culture facilities in China (*P. chinensis*), Singapore (*P. merguiensis)*, The Philippines (*P. monodon)* and Kuwait (*P. semisulcatus*). Later it was reported in cultivated *P. monodon* (HPVmon) (Flegel, 2006) from Thailand. Currently HPV is considered a member of the *P. aroviridae* (Bonami *et al.*, 1995) but its position within the family still remains uncertain. *Parvoviridae* can be divided into two subfamilies viz *Parvovirinae* and *Densovirinae*. Both of them are able to infect vertebrates and invertebrates. Apparently HPV shows more similarities within the autonomous parvoviruses than with the arthropod infecting parvovirus (Bonami *et al.*, 1995, Pantoja*etal.*, 1999). The host range of HPV encompasses both wild and cultured penaeid species (Lightner, 1996a; Spann *et al.*, 1997) and the freshwater prawn *Macrobrachium rosenbergii* (Anderson *et al.*, 1990). This virus infects several penaeid species and is widely distributed in many parts of the world including Asia, Africa, Australia and North and South America (Lightner and Redman, 1991; Lightner 1996a).Transmission of HPV is believed to be both vertical and horizontal (Lightner and Redman, 1991). High levels of HPV infections have been reported especially in early juvenile stages (Flegel *et al.,* 1995; Lightner, 1996a).

Yellow head virus (YHV) of the black tiger prawn *P. monodon* was first reported in Thailand in 1992, although it is known to have caused extensive losses on the eastern coast of the Gulf of Thailand as early as 1991 (Flegel*et al.*, 1995; Flegel, 2006). Histopathological studies have shown that, shrimp with YHV infection display a generalized multifocal to diffuse necrosis, with obvious nuclear pyknosis and karyorrhexis, basophilic, spherical, cytoplasmic inclusions occur in affected tissues, especially in haemocytes, lymphoid organs, cuticular epithelium, gills and connective tissue of several organs, including muscles, antennal glands, gonads, haematopoietic organs and nerve tracts (Lightner, 1996a).The yellow colour in the cephalothorax region results from the underlying yellow hepatopancreas visible through the translucent carapace in moribund shrimp (Chantanachookin *et al.,* 1993). Based on its general morphology and perinuclear site of accumulation, Boonyaratpalin *et al.* (1993) and Chantanachookin *et al.* (1993) reported that it

was baculo virus like. Baculo viruses have been previously reported to infect the penaeid shrimp species and like most DNA containing viruses, they were found to replicate and assemble in the nuclei of infected cells (Lightner, 1993).

2.2 Physical overview of virus affected shrimp

Typical signs of white spot disease is the presence of distinct white cuticular spot (Fig. 1) (0.5-3 mm in diameter) most apparent at the exoskeleton and epidermis of diseased shrimp about 2 days after onset. The white spots start at the carapace and 5th and 6th abdominal segments that later affect the entire body shell. The moribund shrimp display red discoloration and have loose cuticle. Affected shrimps manifest surface swimming and gathering at pond dikes with broken antennae. IHHNV infected shrimps show erratic swimming behavior, rising slowly to the water surface, hanging and rolling over until the ventral side is up. Eventually, the animal sinks to the bottom. Shrimps would eventually right themselves up, become weak and lose their appetite for food. They repeat the process of rising to the surface and sinking until they die usually within 4-12 h. The shrimp's manifests decreased preening and delayed molting. Acutely affected shrimps develop white opaque abdominal muscles, bluish to distinctly blue cuticular color often with mottled buff to tan pigment patches in the cuticular hypodermis and very soft cuticles.

MBV affected shrimps exhibit pale-bluish-gray to dark blue-black coloration, sluggish and inactive swimming movements, loss of appetite and retarded growth. An increased growth of benthic diatoms and filamentous bacteria may cause fouling on the exoskeleton/gills. Infected pond-reared shrimps at 45 days of culture (DOC) stocked at 4 to 100 per m2 manifested slow growth rates and pale yellow to reddish brown hepatopancreas. HPV inffected shrimps develop loss of appetite and retarded growth. Benthic diatoms, protozoans such as *Zoothamnium sp* and filamentous bacteria may cause fouling on the exoskeleton of infected shrimps. Occasionally, white opaque areas on the tail or abdominal muscles are observed and YHV affected shrimps show light yellowish, swollen cephalothorax (Fig. 2). The gills appear whitish, yellowish or brown.

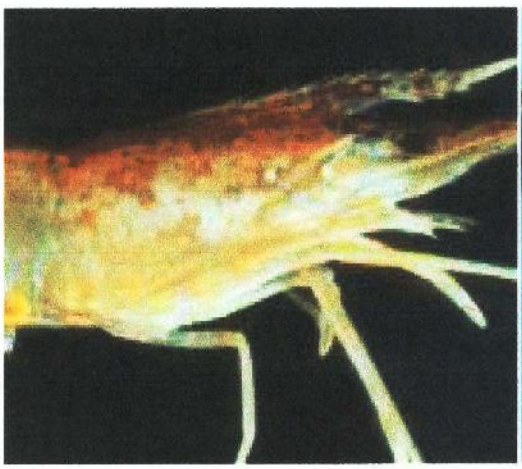

Figure 1: White spot virus affected shrimp **Figure 2:** Yellow head virus affected shrimp

2.3 PCR methods

DNA-based detection methods for detection of penaeid shrimp viruses are now used routinely in a number of laboratories around the world. Lotz (1997) reported that these include were probed for such diseases as white spot syndrome virus (WSSV), yellow head virus (YHV), infectious hypodermal and haematopoeitic necrosis virus (IHHNV) and monodon baculo virus (MBV) and haepatopancreatic parbo virus (HPV) which pose the greatest threat to world shrimp culture production. DNA probes have also been developed for an intracellular parasites and bacteria infecting shrimp. DNA-based techniques will have an important role to play in efforts to develop sustainable shrimp culture in Asia and elsewhere. Production facilities in Thailand are currently using PCR techniques to screen shrimp post-larvae for WSSV. Culturing such larvae in closed (biosecure) or semi-closed culture systems can prevent or minimize viral infections, leading to a viable shrimp industry. The development of specific pathogen- free shrimp stocks will also depend on the use of such techniques.

Chanratchakool *et al.* (1998) stated the routine use of DNA-based diagnostic techniques was hampered by a number of potential problems. The extreme sensitivity of these methods allows the detection of target DNA present at very low levels. However, positive results provide little quantitative assessment of the infection level, and do not indicate whether the pathogen is replicating or causing disease in the species tested. Thus, carrier status and viability of the

pathogen are not determined using DNA-probes. The extremely high specificity of these tests, coupled with the ability of many viruses to rapidly change in genetic structure, can result in failure to detect a virus that has altered its genetic profile. 4 Large differences in sensitivity are related to the PCR method used (e.g., 1-step PCR or 2-step PCR with nested primers). PCR methodologies are highly susceptible to contamination. Contamination during processing may result in false positives, particularly in 2-step PCR methods. PCR tests must be conducted in very well managed, clean laboratories. "False negatives" are easily caused by the selection of inappropriate host tissue sources for detection of the pathogen in question, incorrect choice of DNA extraction method, or low pathogen prevalence in the population sampled. DNA-based detection and diagnostic methods have the potential for widespread application of in aquaculture. As the technology is already being adopted rapidly in developing countries in Asia, there is an urgent need to address these issues and to develop an action plan for research and training activities that will facilitate more effective utilization

Chapter-3

Materials and Methods

3. Materials and Methods

3.1 Study area

In the experiment, wild shrimp broods were used for the analysis of 5 different viral pathogens e.g. WSSV, IHHNV, MBV, HPV and YHV. Samples were collected from five selected hatcheries of Kolatoli and Sonapara hatchery zone in Cox's Bazar (Fig. 3). Name of the selected hatcheries are given below:

1. Modern Hatchery
2. Zamzam Hatchery
3. Quality Hatchery
4. Niribili Hatchery
5. Balaka Hatchery

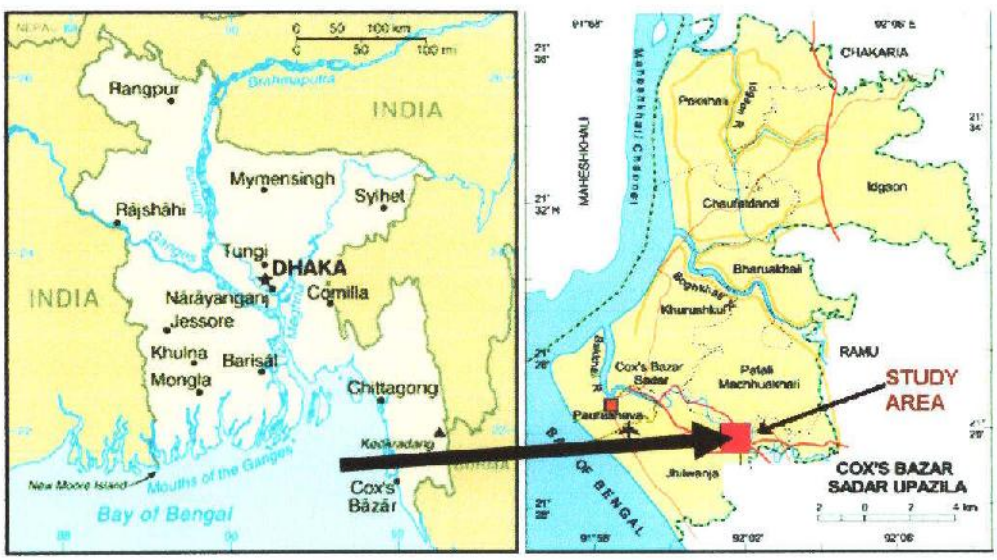

Figure 3: Location of the study area

3.2 Sample collection

In hatchery, broods were collected from nearby commercial fishing ground like south patches using mechanized fishing boat, which is locally known as "Mother Ship". All of the hatcheries in Cox's bazar collect the mother shrimp from Mother Ship and carried to the hatchery using oxygenated plastic bags. During mother collection some cautionary steps strictly followed to retain the quality of collected mother shrimp. After collection of broods, fully gravid female broods are stocked in fiber tank for spawning. Broods were collected from five commercial hatcheries during January- March, 2014. From each hatchery 20 female broods were selected for viral test in every month. Average length and weights of the selected broods were 23±3 cm and 140±10 gm, respectively. Samples were collected from different parts of the brood which recommended by IQ 2000 test protocol in reference with different viral test, like pleopod for WSSV and IHHNV, feces for MBV and HPV and gills for YHV. Sterilized scissors, forceps and toothpick were used to collect the sample. All collected samples were primarily preserved in 70% ethanol and finally carried out to the PCR lab of DoF for further analysis. Besides the sample collection, water temperature was recorded in every month during sampling time. Effect of temperature on viral pathogen intensity was observe by evaluating the percentage of virus affected brood in different months, where temperature varied monthly and an increasing trend of temperature changes was observed from January to March.

3.3DNA or RNA extraction

DNA was extracted for WSSV, IHHNV, MBV, HPV and RNA was extracted for YHV as per the IQ2000 detection and prevention system protocol.

3.3.1DNA isolation for WSSV, IHHNV, MBV and HPV

Two millimeter (20 mg) pleopods for WSSV and IHHNV or one centimeter fecal sample for MBV and HPV were taken in a 1.5ml eppendorf tube. Then the sample was homogenized with 600 µl DTAB solution provided in the IQ 2000 detection system kit. After 5 minutes of incubation at 75°C and a brief cooling, 700µl chloroform was added and transferred for

centrifuging at 12000xg for 5 minutes. Then 200 µl of upper liquid phase was transfered to another eppendorf tube with 100 µl CTAB and 900 µl ddH_2O and incubated at 75°C for 5 minutes. After cooling down, it was centrifuged at12000xg for 10 minutes. Then the supernatant was carefully decanted, the pellet was diossolved with 150 µl dissolving solution and incubated at 75°C for 5 minutes. Then the solution was cooled down to room temperature and centrifuged at12000xg for 5 minutes. After centrifuge, the clear solution was transferred to a fresh 1.5ml eppendorf tube with 300 µl 95% ethanol, centrifuged again at 1200xg for 5 minutes and washed the pellet with 200 µl 75% ethanol. DNA was precipitated in absolute ethanol. Finally, the pellet was dissolved in sterile distilled water, and used as a PCR template.

3.3.2 RNA isolation for YHV

For RNA extraction, half pieces (20 mg) of gill were placed into a 1.5ml eppendorf tube with 500 µl RNA extraction solution provided in the IQ2000 detection system kit. Then it was homogenized with disposable grinder and stand in room temperature for 5 minutes. After adding 100 µl of $CHCl_3$, samples were kept in room temperature for 3 minutes and then it was centrifuged at 12000xg for 15 minutes. After that 200 µl upper aqueous phase was transferred to another 1.5ml eppendorf tube with 200 µl isopropanol and centrifuged at 12000xg for 10 minutes. Then the isopropanol was removed and washed the pellet with 500 µl 75% ethanol. After washing, the pellet was spin downed at 7500xg for 5 minutes to recover RNA pellet. Then ethanol was decanted and the pellet was dried. Finally dry pellet was dissolved with 200 µl DEPC ddH_2O and used as a PCR template.

3.4 PCR process

IQ2000 detection and prevention kit were used to perform PCR with a view to identify the viruses. All PCR reactions were carried out in an Esco Healthcare swift mini prothermocycler, Model- SWT-MIP-0.2-2.

3.4.1 Amplification procedure of WSSV and IHHNV

For WSSV and IHHNV virus test, 2-step (nested) PCR protocol was performed. First reagent mixture was prepared by mixing 7.5 µl of first PCR premix with 0.5 µl of IQzyme polymerase

taq for every sample. Then 2 µl of extracted DNA sample was added with 8 µl of reagent mixture and performed first PCR program with an initial delay at 94°C for 2 min, followed by 15 cycles at 94°C for 20 s, 62°C for 20 s and 72°C for 30 s, with a final extension at 72°C for 30 s. For the nested PCR reaction, 14 µl of nested PCR premix and 1 µl of of IQzyme polymerase taq were added in every first PCR reaction product. Cycling conditions for the nested PCR reaction were similar to the first PCR reaction and total 30 cycles performed in this session. Finally PCR products were ready for gel electrophoresis.

3.4.2 Amplification procedure of MBV and HPV

For MBV and HPV virus detection, one step PCR protocol was performed. At first, reagent mixture was prepared by mixing 12.5 µl of PCR premix with 0.5 µl of IQzyme polymerase taq for every sample. Then 2 µl of extracted DNA sample was added with 13 µl of reagent mixture and performed PCR program followed by 35 cycles at 94°C for 20 s, 58°C for 20 s and 72°C for 30 s, with a final extension at 72°C for 30 s.

3.4.3 Amplification procedure of YHV

In case of YHV test, RT-PCR protocol was performed. First, reagent mixture was prepared by mixing 7 µl of first PCR premix with 0.5 µl of IQzyme polymerase taq and 0.5 µl of RT- PCR premix for every sample. Then 2 µl of extracted RNA sample was added with 8 µl of reagent mixture and performed first PCR program with an initial delay at 42°C for 30 min and at 94°C for 2 min, followed by 15 cycles at 94°C for 20 s, 62°C for 20 s and 72°C for 30 s, with a final extension at 72°C for 30 s. For the nested PCR reaction, 14 µl of nested PCR premix and 1 µl of of IQzyme polymerase taq were added in every first PCR reaction product and performed PCR program followed by 35 cycles at 94°C for 20 s, 58°C for 20 s and 72°C for 30 s, with a final extension at 72°C for 30 s. Finally samples were ready for gel electrophoresis.

3.5 Electrophoresis and Diagnosis

The principle component of gel electrophoresis was agarose powder, TBE buffer and EtBr. 2% agarose gel was prepared by adding 2gm agarose into a flask with 100 ml 1X TBE buffer and heat the mixture until it becomes hyaline without any gel particle. Then 5% EtBr solution was

mixed slowly and poured into the gel box. After coagulating agarose gel, 1X TBE buffer was added into the gel box until the buffer level is just submerged the gel. Then 6 µl each of the 'PCR product-loading dye mixture' was loaded into each well. DNA molecular weight marker was required for every electrophoresis because it was served as reference for PCR product size. When all the samples were loaded, the gel box was connected with power supply and switched on at 150V for 40 minutes. After completing electrophoresis, the gel was laid on a UV transilluminator to read the final result. The UV transilluminator was switched on, DNA banding pattern was visible. Then the resulting banding pattern was captured by using the digital camera. Every experiment had positive and negative standards of tested pathogen and a DNA marker which showed band at 848bp, 630bp and 333bp.

3.6 Statistical analysis

All statistical analysis was performed using SPSS (version 16) and Microsoft office version-7. ANCOVA (Analysis of Covariance) was performed to evaluate differences of prevalence among the hatchery and viral diseases or the effect of temperature at 95% level of significance. Post-hoc test (Tukey) was performed using ANOVA if significant differences were found.

Chapter-4

Result

4. Result

Recently shrimp industries of Bangladesh have faced some viral disease problem, which are reported to be introduced from wild broodstocks to hatchery (DoF, 2012). These viruses are White Spot Syndrome Virus (WSSV), Infectious Hypodermal hematopoetic Necrosis Virus ((IHHNV), Monodon Baculo Virus (MBV), Haeoatopancreatic Parbo Virus (HPV) and Yellow Head Virus (YHV). Since these viruses may have a significant disease threat to cultured shrimps, present studies were conducted to detect these viruses in wild broodstocks of shrimps from five commercial hatcheries during January to March, 2014 using polymerase chain reaction.

4.1 Determination of negative and positive samples

Polymerase chain reaction was performed to confirm the presence of 5 different viruses (WSSV, IHHNV, MBV, HPV and YHV) in broodstocks of *P. monodon* collected from commercial fishing ground of Bay of Bengal. TE buffer and DNA from WSSV, IHHNV, MBV, HPV and RNA from YHV positive sampleswere considered as negative and positive control respectively. The banding pattern and detecting process of WSSV, MBV and YHV prevalence are showed in fig. 4, 5 and 6 respectively. The severe WSSV positive samples were shown at 848bp, 630bp and 296bp, moderate positive samples were found at 630bp and 296bp, and very light positive samples were found at 848bp and 296bp (Fig. 4).The negative samples showed only one band at 848bp which was PCR product of housekeeping gene as an internal control.

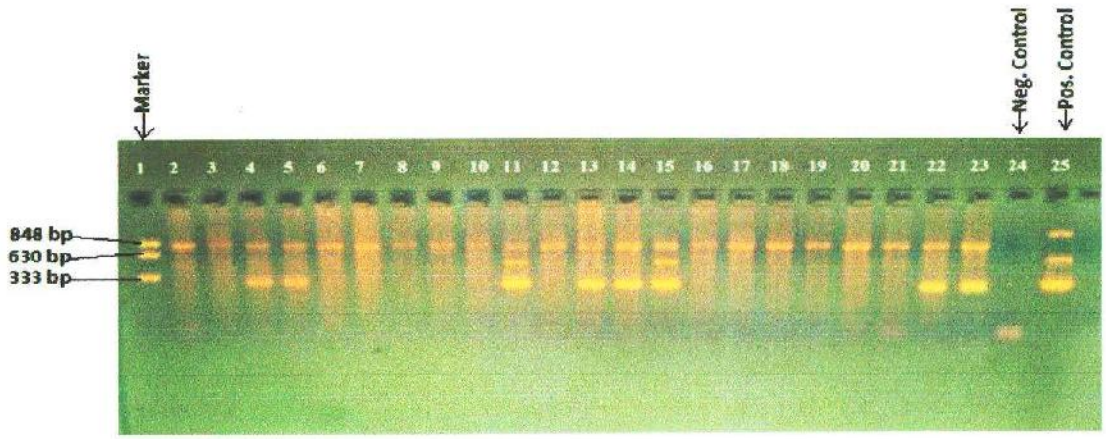

Figure 4: Gel electrophoresis analysis of White Spot Syndrome Virus. [Lane-1: DNA marker; Lane-2,3,7,8,9,10,12,16,17,18,19,20 and 21: WSSV negative sample; Lane-4,5,6,11,13,14,15,22 and 23: WSSV positive sample (Lane-4,5,13,14,22,23: very light positive and Lane-11,15: severe positive); Lane-24: WSSV negative standard and Lane-25: WSSV positive standard]

For MBV, the light positive samples formed only one band at 225bp and the moderate positive samples formed bands at 630bp and 225bp. The negative samples showed no band because fecal samples have slight possibility to get housekeeping gene. (Fig. 5)

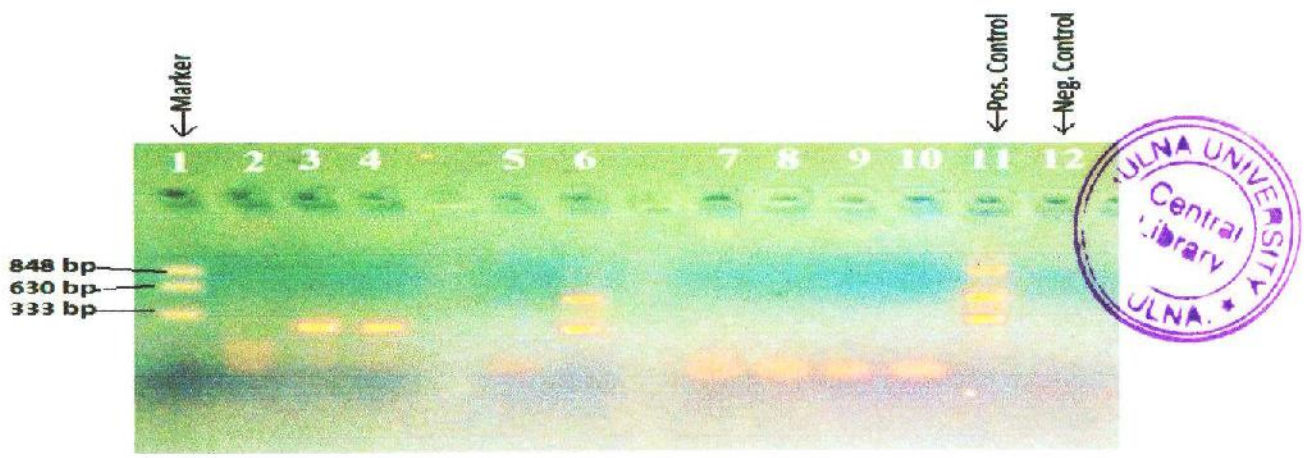

Figure 5: Gel electrophoresis analysis of MonodonBaculo Virus. [Lane-1: DNA marker; Lane-2, 5,7,8,9 and 10: MBV negative sample; Lane-3, 4 and 6: MBV positive sample (Lane-3,4: light positive; Lane-6: moderate positive); Lane-11: MBV positive standard and Lane-12: MBV negative standard]

The band at 680bp and 277bp indicates the presence of severe YHV positive samples and light YHV positive samples were showed band at only 277bp. The negative sample was formed only one band at 680bp which was PCR product of housekeeping gene as an internal control. (Fig. 6)

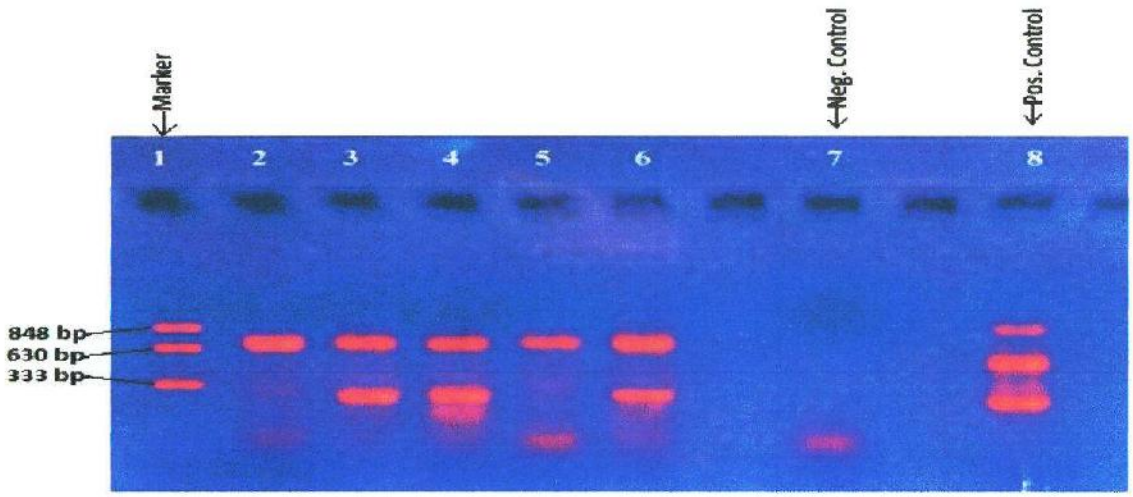

Figure 6: Gel electrophoresis analysis of Yellow Head Virus. [Lane-1: DNA marker, Lane-2 and 5: YHV negative sample, Lane-3, 4 and 6: moderate YHV positive sample, Lane-7: YHV negative standard, and Lane-8: YHV positive standard]

In the case of IHHNV, severe positive samples formed three bands at 848bp, 630bp and 340bp, very light positive samples formed two bands at 848bp and 340bp and moderate positive samples formed bands at 630bp and 340bp. The negative samples showed only one band at 848bp which was PCR product of housekeeping gene as an internal control

For HPV, the light positive samples displayed only one band at 339bp and the moderate positive samples displayed two bands at 630bp and 339bp. The negative sample showed no band because fecal samples have slight possibility to get housekeeping gene

4.2 Virus prevalence status in different hatchery

There was no significant difference on virus (WSSV, MBV, HPV, YHV) prevalence among the hatcheries except IHHNV (P>0.05) (Table 2, Table 1). The WSSV prevalence ranged from 20% (Modern, January) to 65% (Niribil, March), IHHNV prevalence ranged from 50% (Modern, January) to 95% (Zamzam, March), MBV prevalence ranged from 60% (Modern, January) to 90% (Modern and Quality, March;Niribil, February), HPV prevalence ranged from 20% (Zamzam, January; Modern, Januray; Modern, February) to 50% (Zamzam, March) and YHV prevalence ranged from 45% (Quality, Januray) to 75% (Quality, February; Modern, March) (Table 1)

Table 1: Monthly variation of different virus prevalence in different hatchery

Month	Hatchery name	% of WSSV prevalence	% of IHHNV prevalence	% of MBV prevalence	% of HPV prevalence	% of YHV prevalence
January	Balaka	30	65	70	25	55
	Quality	30	70	80	30	45
	Zamzam	45	80	70	20	65
	Modern	20	50	60	20	60
	Niribili	25	55	80	25	50
February	Balaka	40	75	80	30	65
	Quality	35	80	75	25	75
	Zamzam	40	90	85	30	50
	Modern	35	70	75	20	55
	Niribili	40	85	90	35	70
March	Balaka	50	90	85	40	70
	Quality	45	80	90	35	65
	Zamzam	60	95	80	50	65
	Modern	40	85	90	45	75
	Niribili	65	90	80	30	70

4.3 Temporal variation of virus prevalence

Temperature ranged from 26 °C to 28 °C during March. In this month highest 28 °C temperature was recorded from Niriblil and Bolaka where 26°C was recorded from Modern and Quality. In February, temperature ranged from 24 °C to 26 °C, where lowest value was recorded from Zamzam and highest value was recorded from Niribil. Temperature profile was found comparatively lower in January than March and February. Highest 23°C was recorded from Modern in this month and lowest 21 °C from Balaka. Average highest temperature was found in March (28±1) and lowest in January (22±0.70). (Fig. 7)

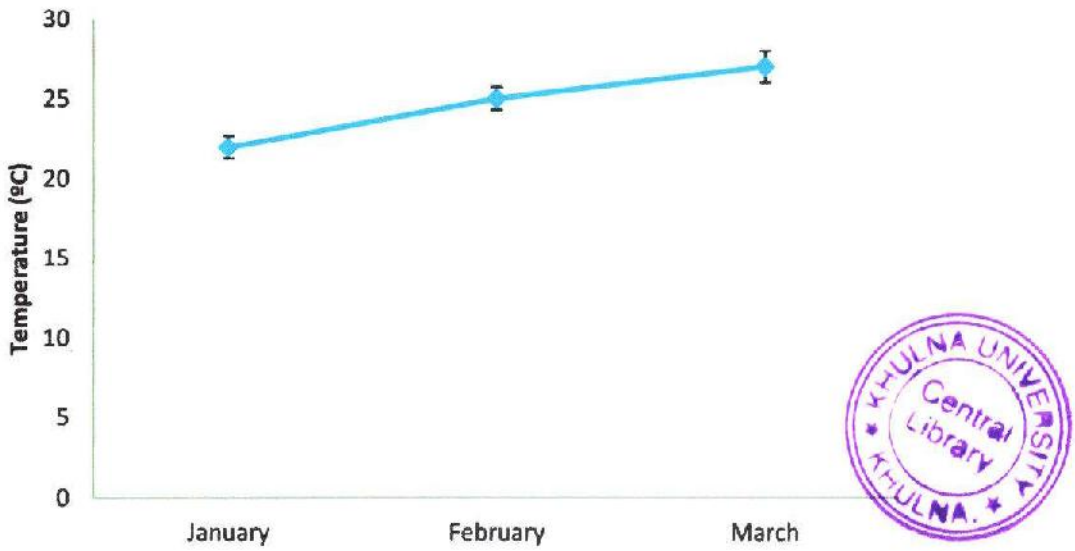

Figure 7: Temperature variation (Mean±SD) throughout this study

Analysis of covariance (ANCOVA) showed that the prevalence of WSSV and IHHNV varied significantly ($P<0.01$) with temperature changes from month to month and significantly highest prevalence was found in March. MBV and HPV showed significant ($p<0.05$) variance with temperature changes with highest prevalence in March rather than January and February. YHV didn't have any significant difference with the change of temperature. (Table 2)

Table 2: ANCOVA result of virus prevalence

Name of virus	P value (Hatchery)	P value (Temperature)
WSSV	0.244	0.003**
IHHNV	0.043*	0.000**
MBV	0.303	0.040*
HPV	0.405	0.022*
YHV	0.776	0.064

Here, **means 1% significant difference and * means 5% significant difference

Boxplot analysis stated that among the five viruses (HPV, IHHNV, MBV, WSSV, and YHV) IHHNV and MBV were found to cause the highest contamination rate in comparison with others (Fig. 8). And, HPV showed the lowest contamination rate among the virus tested (Fig. 8).

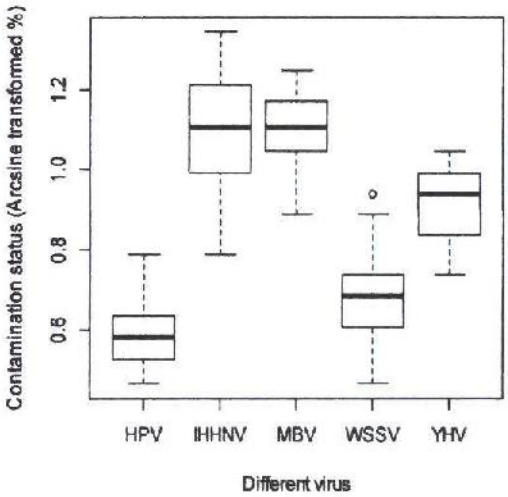

Figure 8: Boxplot of contamination status of different virus

Tukey post-hoc HSD test (Fig. 9) stated the similar results. There were no significant differences of contamination rate between MBV and IHHNV, and WSSV and HPV (P>0.05) in different hatcheries during the study period. However, MBV and IHHNV, YHV, and WSSV and HPV showed significantly (P<0.05) highest, moderate and lowest contamination rate respectively.

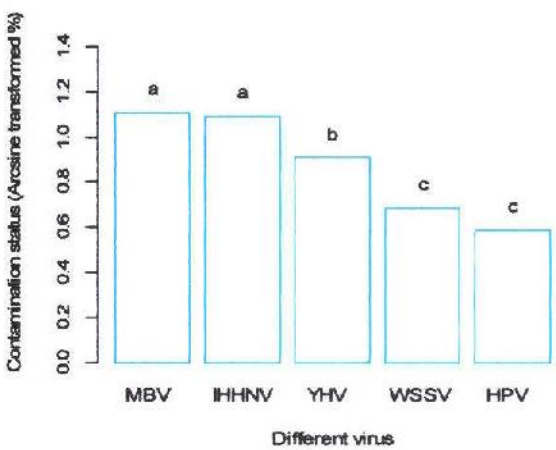

Figure 9: Trend of viral contamination rate,Tukey HSD test (multiple comparisons): different letter indicates significant difference (P<0.05)

Chapter-5

Discussion

5. Discussion

Viral diseases cause million dollar losses for shrimp industries in the world (Wyban*et al.*, 1992; Lightner, 1999a). In 1996, Bangladesh faced a huge amount of moneytary loss due to white spot disease. To detect viral diseases of Penaeidae shrimps, different methods are being used such as clinical signs, histopathology, molecular methods (PCR) and transmission electron microscopic (TEM). Viral acute infections are associated with mass death (100%). On the other hand, Bell and Lightner (1988); Lightner (1996a) stated that because of genetic mutations due to geographic isolation there may not be a suitable molecular method but IQ 2000 kit could detect these five viral diseases (WSD, IHHND, MBD, HPD and YHD) accompanied with histopathological changes created by pathogenic viruses. So in this study, PCR detection is discussed.

The samples were collected from five hatcheries of Cox's bazar after washing of broodstocks of *P. monodon* and tested by IQ2000 detection and prevention kite for each virus. For WSSV and IHHNV nested PCR, for MBV and HPV one step PCR and for YHV RT-PCR methods were followed.

From the result it was found that the percentage of prevalence of WSSV, IHHNV, MBV, HPV and YHV in brood was not same. The rate of infections varied from month to month and hatchery to hatchery. There was no significant difference of virus prevalence among the hatchery except IHHNV. However, virus prevalence rate significantly increased during January to March with the increase of temperature. WSSV and IHHNV were showed 1% significant variation ($p<0.01$) in prevalence rate with the change of temperature. IHHNV showed significant difference on its prevalence in different hatcheries, that possibly caused by sudden temperature variation in mother tanks. MBV and HPV also showed significant variation ($p<0.05$) on prevalence rate with the change of temperature (Table 2). March is the most temperate month (28 ±1°C) has had a highest significant contamination rate for all viruses (Fig. 7). This variation on prevalence rate was profoundly caused by change of temperature and water depth.

In this study, the IHHNV, MBV, and YHV was found comparatively higher than WSSV and HPV, this is also indicates that maximum natural broods in Cox's bazaar are highly impacted by IHHNV and MBV yet also by YHV rather than WSSV and HPV. Temperature is a risk factor. The highest WSSV (52%), IHHNV (88%), MBV (85%), HPV (40%) and YHV (69%) prevalence in broods was found in the month of March, which was significantly higher than the prevalence found in January 2014 (Table 2). It is unknown why the prevalence of WSSV infection was high during March. However, certain assumptions and inferences may be proffered. In March temperature become high, sudden strome and sudden rainfall may be found in the Bay of Bengal in Bangladesh. Withyachumnarnkul*et al.* (2003) stated that strong seawater currents forced the broodstock to aggregate in certain 'safe' areas, causing population congestion and therefore increasing the chance of horizontal transmission of WSSV into shrimp. Another possibility is that broodstock catchers prefer to trawl in shallow water (e.g. 10 to 20 m depth) during monsoon season because rough sea does not permit them to trawl in the deep sea (e.g. 50 to 70 m depth). Such inshore shrimp populations might contain large numbers of WSSV infected broodstock. Population dynamics (e.g. mortality, growth, reproduction and movement) of wild shrimp are regulated by environmental and ecological factors. Changes of environmental factors such as temperature, salinity, and dissolve oxygen may adversely affect wild shrimp population. According to the result, the percentage of viral infection to wild brood gradually increased from January to March with increasing temperature and salinity level. In January, the highest WSSV (45%), IHHNV (80%) and YHV (65%) prevalence in Zamzam hatchery, MBV (80%) prevalence in Quality and Niribili hatchery, and HPV (30%) prevalence in Quality hatchery were found. In the month of February 2014, highest WSSV (40%) prevalence in Balaka, Zamzam and Niribili hatchery, IHHNV (90%) prevalence in Zamzam hatchery, MBV (90%) and HPV (35%) prevalence in Niribili hatchery and YHV (75%) prevalence in Quality hatchery were found in broodstock of *Penaeus monodon*. PCR test result showed that during three months of study period the highest WSSV (65%) prevalence in Niribili hatchery, IHHNV (95%) and HPV (50%) prevalence in Zamzam hatchery, MBV (90%) prevalence in Quality and Modern hatchery and YHV (75%) prevalence in Modern hatchery occured in March. Iqbal *et al.*, (2011) reported similar trend of viral infection percentage from Cox's bazar area.

The average highest WSSV (48.33%), IHHNV (88.33%) and HPV (33.33%) prevalence was found in Zamzam hatchery. The possible reason might be the collection of broods from shallow water where water temperature, salinity and P^H varied from deep water area. However, Hoa (2009) notes that environmental parameters such as salinity, pH, temperature, ammonia and nitrite are known to be very stable and/or low in the deep sea, and that this reduces stress to adult tiger shrimp. When changes occur in environmental factors, their interactions may be extremely important. Exposure to a single factor at extreme condition may be tolerable; however, their combinations may be more adverse or even lethal. Under these conditions, wild shrimp exposure to virus may predispose the host to infection. Viruses and their hosts interact in a complex way within the marine environment. Snieszko (1974) described that infectious diseases of fishes occur when they are exposed to virulent pathogens under certain environmental stress conditions. So, virus may interact with shrimp depending upon environmental condition and physiology of the host. How do natural factors influence this interaction is essentially unknown, particularly because the antiviral immunity of the shrimp is poorly understood.The incidence of viral infection is lower among broods harvested from the deeps zone than those harvested from the shallow zone described by Debnath *et al.* (2014). *P. monodon* is the carier of IHHNV. WSSV and HPV are transmitted vertically from mother. It could not remove completely by washing with potassium permanganet, formalin and iodine. These viruses mainly hampered the farm production. The highest MBV prevalence was found in Niribili hatchery because they did not maintain washing protocol properly. It is possible to remove MBV completely by proper washing with potassium permanganet, formalin and iodine with considerable dose. So MBV may cause many problems in hatchery production. The highest YHV prevalence was also found in Niribili hatchery. Maximum time YHV is transmitted vertically from mother.

A critical aspect of this study was the diagnostic test to determine the rate of infection. PCR detects only a fragment of DNA or RNA. Whether the virus is viable or infectious cannot be known, and a non-viable virus present on the sample surface can also contribute to a PCR positive result. Due to lack of a continuous shrimp cell line, PCR has been the sole and routine diagnostic tool in detecting WSSV, IHHNV, MBV, HPV and YHV infection. To avoid contamination, we should be ensured the availability of virus free broods from nature using PCR test. Otherwise a virus negative brood bank should be established to reduce the contamination.

Chapter-6

Conclusion

6. Conclusion

First viral disease entered in our country from Thailand in 1994 and destroyed the whole harvest at that time. Some research institutions started to find out the methods to reduce the risk from these infections but the risk still remain. The maximum broodstocks of *P. monodon* in hatchery were affected by WSSV, IHHNV, MBV, HPV and YHV and their prevalence was highest in the month of March, when temperature was also high. The highest prevalence percentage of viruses was found in Zamzam hatchery and the percent of IHHNV, MBV and YHV prevalence was comparatively higher than WSSV and HPV. The present study showed that if hatchery follows proper washing protocol, some viruses can be removed but not all because some are vertically infected from mother in wild condition. For better production in farm level, every shrimp hatchery should use virus free broods for spawning.

Chapter-7

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Appendix

APPENDIX

Appendix-A

ANCOVA (Analysis of covariance) results to evaluate whether temperature affects the occurrence of the viruses.

Tests of Between-Subjects Effects

Dependent Variable: **WSSV**

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	7.181[a]	10	.718	184.378	.000
Hatchery	.038	5	.008	1.932	.244
Temp	.111	1	.111	28.571	.003
Hatchery * Temp	.020	4	.005	1.312	.379
Error	.019	5	.004		
Total	7.201	15			

a. R Squared = .997 (Adjusted R Squared = .992)

Tests of Between-Subjects Effects

Dependent Variable: **IHHNV**

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	18.166[a]	10	1.817	976.745	.000
Hatchery	.051	5	.010	5.432	.043
Temp	.201	1	.201	108.004	.000
Hatchery * Temp	.037	4	.009	4.986	.054
Error	.009	5	.002		
Total	18.175	15			

a. R Squared = .999 (Adjusted R Squared = .998)

Tests of Between-Subjects Effects

Dependent Variable: **MBV**

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	18.458[a]	10	1.846	199.061	.000
Hatchery	.075	5	.015	1.627	.303
Temp	.071	1	.071	7.642	.040
Hatchery * Temp	.046	4	.011	1.236	.402
Error	.046	5	.009		
Total	18.505	15			

a. R Squared = .997 (Adjusted R Squared = .992)

Tests of Between-Subjects Effects

Dependent Variable: HPV

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	5.208[a]	10	.521	83.795	.000
Hatchery	.039	5	.008	1.253	.405
Temp	.066	1	.066	10.684	.022
Hatchery * Temp	.038	4	.010	1.538	.320
Error	.031	5	.006		
Total	5.239	15			

a. R Squared = .994 (Adjusted R Squared = .982)

Tests of Between-Subjects Effects

Dependent Variable: **YHV**

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Model	12.563[a]	10	1.256	125.066	.000
Hatchery	.024	5	.005	.487	.776
Temp	.056	1	.056	5.623	.064
Hatchery * Temp	.021	4	.005	.520	.727
Error	.050	5	.010		
Total	12.613	15			

a. R Squared = .996 (Adjusted R Squared = .988)

Appendix-B

The primer sequences of viruses are-

For WSSV:

First PCR primer-

146F1 (5'-ACT-ACT-AAC-TTC-AGC-CTA-TCTAG-3') and
146R1(5'-TAA-TGC-GGG-TGT-AAT-GTT-CTT-ACG-A-3)'.

Nested PCR primer-

146F2 (5'-GTA-ACT-GCCCCT-TCC-ATC-TCC-A-3') and
146R2 (5'-TAC-GGC-AGC-TGC-TGC-ACC-TTG-T-3').

For IHHNV:

First PCR primer-

389F1 (5'-CGG-AAC-ACA-ACC-CGA-CTT-TA-3') and
389R1 (5'-GGC-CAA-GAC-CAA-AAT-ACG-AA-3).

Nested PCR primer-

389F2 (5'-GAC-AAC-ACA-AGC-CGA-CTT-AT-3') and
389R2 (5'-CAA-AAG-GAC-CCA-AAT-ACG-TT-3')

For MBV:

MBV1.4F (5'-CGA- TTC-CAT-ATC-GGC-CGA-ATA-3') and
MBV1.4R (5'-TTG-GCA-TGC-ACT-CCC-TGA-GAT-3')

For HPV:

7490F (5'-TGG-AGG-TGA-GAC-AGC-AGG-3') and
7852R (5'-CCA-ACT-GTC-CTT-CGC-TCT-3').

For YHV:

RT- PCR primer-

10F (5'-CCG-CTA-ATT-TCA-AAA-ACT-ACG-3') and
144R (5'-AAG-GTG-TTA-TGT-CGA-GGA-AGT-3').

Nested RT-PCR primer-

Y (5'-ACG-CTC-TGT-GAC-AAG-CAT-GAA-GTT-3')