Detection and molecular characterization of the Microsporidian parasites in cultured Shrimp

A thesis submitted to the Department of Fisheries, University of Dhaka

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Masters of Science (MS) in Fisheries

Submitted By

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Declaration

I hereby declare that the dissertation entitled **"Detection and molecular characterization of the Microsporidian parasites in cultured Shrimp"** submitted to the Department of Fisheries, University of Dhaka for the degree of Master of Science (MS) is based on self-investigation, carried out under the supervision of **Dr Mohammad Shamsur Rahman**, Department of Fisheries and **Dr. Md. Aminul Islam Bhuiyan**, Department of Zoology, University of Dhaka, Dhaka-1000, Bangladesh.

I also declare that this or any part of this work has not been submitted for any other degree anywhere. All sources of knowledge used have been duly acknowledged.

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Certificate

We certify that the research work embodied in this thesis entitled **"Detection and molecular characterization of the Microsporidian parasites in cultured Shrimp"** submitted by **Md. Munjur Hossain**,Roll number: 814, Session: 2015-16, Registration number: 2011-112-776, has been carried out under our supervision.

This is further to certify that it is an original work and suitable for the partial fulfilment of the Degree of Master of Science (MS) in Fisheries from the Department of Fisheries, University of Dhaka.

We wish every success in his life.

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Abstract

Microsporidian parasite has emerged as a serious pathogen reported to be associated with retarded growth in cultured shrimp in many of the shrimp growing countries in Asia. As a part of ongoing disease surveillance among the farmed shrimp, this study investigated black tiger shrimp (*Penaeus monodon)* culturing in the south-west region especially Satkhira and Bagherhat district of Bangladesh for the prevalence of microsporidian parasite using light and scanning electron microscopy, histopathology and polymerase chain reaction (PCR). The evolutionary history among parasiteswere inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.21208716 was shown. Evolutionary analyses were conducted in MEGA7.

Squash preparation of hepatopancreas and stomach showed large number of microsporidian spores under light microscopy. Spores under scanning electron microscope appeared oval shapes. Histology of infected animals showed severe degeneration of hepatopancreatic tubules. Early and late stage of microsporidian parasites in hepatopancreatic tubules were also observed in some cases. Morphological dissimilarities among the parasites also observed.

DNA extracted from hepatopancreas was subjected to PCR amplification using primers targeting microsporidian SSU rRNA gene. The PCR yielded an expected product of ~328 bp and the sequences showed 83% identity with the *Paranucleospora theridion* (GenBank no. FJ594971.1) reported from Vietnam, Thailand and China.

Further screening of field samples was carried out using EHP-specific primers. Out of 10 *P. monodon* samples tested, none found to be positive. So the prevalence of EHP was not estimated. To understand the microsporidian parasite *Enterocytozoon hepatopenaei* (EHP) prevalence, samples should be drawn from more shrimp farms with information about their management strategies.

This is the first report identifying microsporidian parasites in cultured shrimp farm along the South-west region especially Satkhira and Bagherhat district in Bangladesh.

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List of Symbols and Abbreviations

Symbols	Details
^{o}C	Drgree Celsius
µg	Microgram
µl	Microlitre
bp	Base pair
cm	Centimeter
DNA	Deoxyribonucleic acid
eg	For example (exempli gratia)
et al	And others (et alliori)
mL	Milliliter
mm	Millimeter
EHP	*Enterocytozoon hepatopenaei*
ng	Nanogram
NaCl	Sodium Chloride
PCR	Polymerase Chain Reaction
PL	Post Larvae
rpm	Rotations per minute
rRNA	Ribosomal Ribonucleic Acid
g	gram
µm	Micrometer
ML	Maximum Likelihood
NJ	Neighbor-joining
SEM	Scanning Electron Microscope
MEGA	Molecular Evolutionary Genetics Analysis
MSA	Multiple Sequence Alignment

Chapter 1

Introduction

1.1 Background

Bangladesh is enriched with a vast aquatic resource in the forms of both freshwater and marine water. Brackish water bodies are suitable for shellfish production. The subtropical country, Bangladesh has a favorable condition for shrimp culture. All water bodies are very productive in this country. This country has been blessed by 60 shrimp species (DoF, 2012). Bangladesh entered the global export market for shrimp in the early 1970s. It is the second largest foreign income earning product of Bangladesh. Rapid expansion of shrimp culture has been started in BD from the 1980 (Shabuj et al., 2015). The World Bank and the Asian Development Bank financed projects to develop shrimp aquaculture in the 1980s. Much of the emphasis was on construction of modern hatcheries. Private investors also initiated similar projects to increase capacity and to introduce modern technology that would increase average yields. The Food and Agriculture Organization of the United Nations (FAO) has also provided assistance to the shrimp and fishing industry in meeting fish safety and quality control standards based on the Hazard Analysis Critical Control Point (HACCP) approach (Cato et al., 2012). The contribution of coastal aquaculture, particularly black tiger shrimp (*Penaeus monodon*) culture to both rural and national economics have become a major source of export earnings and employment in the coastal areas of Bangladesh. Bangladesh has a huge area of coastal tidal land (Territorial water 2640 sq.miles) continental shelf 41040 sq.miles. Exclusive Economic zone 24,800 sq.miles and an extended coast line of about 710 km of which only 0.143 million hectares of land has been brought under brackish water shrimp aquaculture (Shabuj et al., 2015).

In 2010-11, 2011-12, 2012-13, 2013-14 and 2014-15 the country produced 239460, 252523, 228769, 223788 and 230244 metric tons of shrimp and prawn of all kinds (DoF, 2016). Shrimp culture is widely practiced in Khulna, Satkhira, Bagerhat, Cox's bazar due to entrance of tidal water throughout the year in this regions. Shrimp species harvested from the country are mainly black tiger shrimp (Bagda), brown shrimp (Horina), Indian white shrimp (Chaka) and giant freshwater shrimp (Golda) (Rahman and Hossain, 2009).

Among the fisheries sectors about 77% foreign exchange comes by shrimp exports. It is currently recognized as a potential long-term sustainable industry for many tropical countries. Though rapid expansion of shrimp culture has been started in Bangladesh from the 1980s but the production of shrimp 250 kg/hactre/year that is negligible comparing to the other countries. (Shabuj et al., 2015).

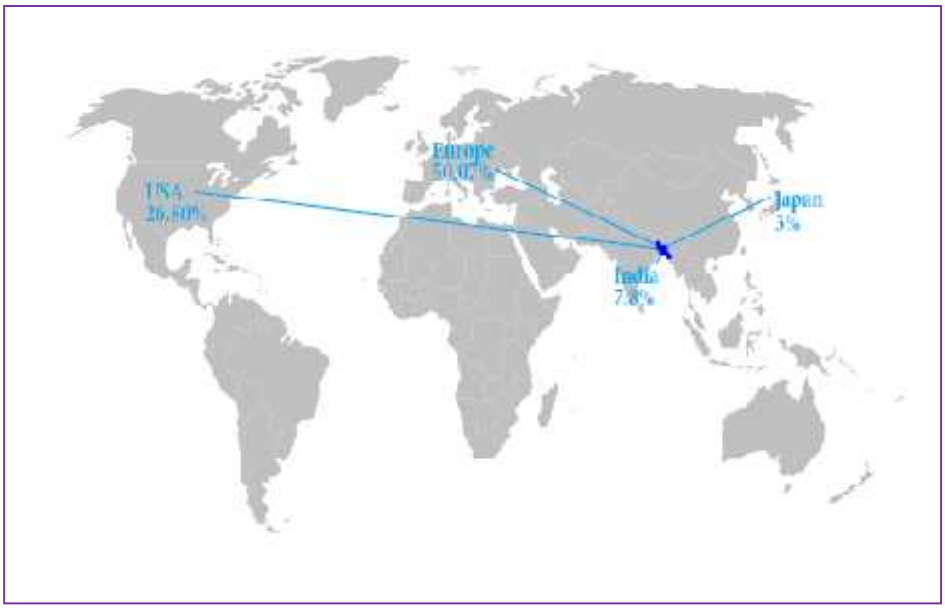


Fig. 1.1.Worldmap of shrimp export from Bangladesh
(Source: http://seatglobal.eu/wp-content/uploads/2013/04)

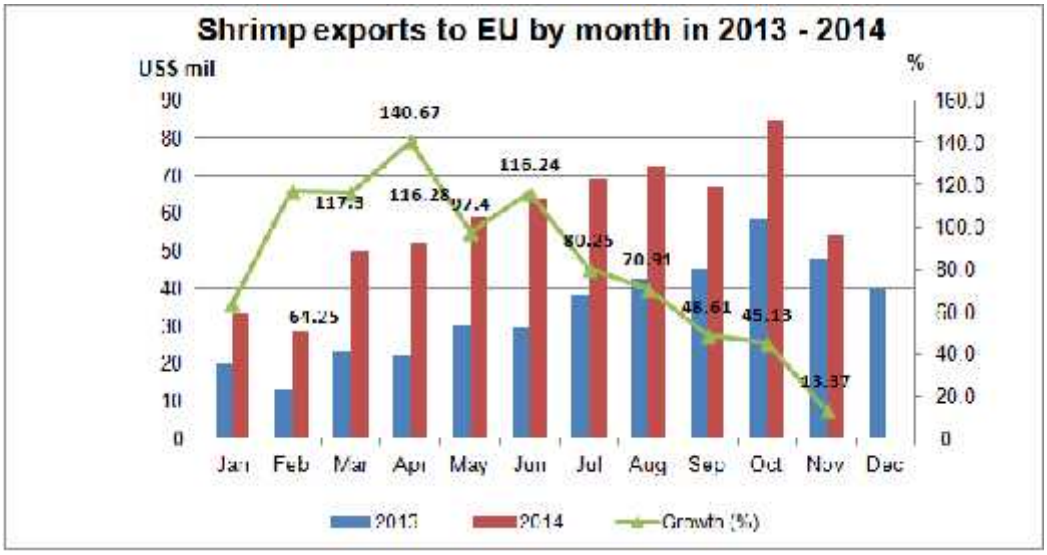


Fig. 1.2. Shrimp export from Bangladesh to EU in 2013-2014
(Source: http://seafood.vasep.com.vn/Uploads/image/Nguyen-Thu Trang/image/shrimp.png)

1.2 *Penaeus monodon*, the study species

Penaeus monodon, commonly known as the giant tiger prawn (FAO, 2010) or Asian tiger shrimp and also known by other common names, is a marine crustacean that is widely reared for food. *Penaeus monodon* was first described by Johan Christian Fabricius in 1798. That name was overlooked for a long time, however, until 1949, when Lipke Holthuis clarified to which species it referred. He also showed that *P. monodon* had to be the type species of the genus *Penaeus*(Holthuis, 1949).

Fig. 1.3. A photograph of *Penaeus monodon* (Source: http://gallery.bdfish.org/wp-content/gallery/*Penaeusmonodon*-appendage/00-*Penaeusmonodon*.jpg)

1.2.1 Taxonomic classification of black tiger shrimp

Phylum: Arthropoda

Subphylum: Crustacea

Class: Malacostraca

Order: Decapoda

Family: Penaeidae

Genus: *Penaeus*

Species: *Penaeus monodon* (Fabricius, 1798)

1.2.2 Life history of *Penaeus monodon*

The females of *Penaeus monodon* can reach about 33 cm (13 inch) long, but are typically 25–30 cm (10–12 inch) long and weight 200–320 g, males are slightly smaller of 20–25 cm (8–10 inch) long and weighing 100–170 g (FAO, 2010). The carapace and abdomen are transversely banded with alternative red and white. The antennae are grayish brown. Brown pereiopods and pleopods are present with fringing setae in red (Motoh, 1981). Its natural distribution is the Indo-Pacific, ranging from the eastern coast of Africa and the Arabian Peninsula, as far as Southeast Asia, the Pacific Ocean, and northern Australia (Holthuis, 1980). It is an invasive species in the northern waters of the Gulf of Mexico (Tresaugue and Matthew, 2011) and the Atlantic Ocean off the southern US (www.nmfs.noaa.gov. accessed on 2016). *Penaeus monodon* is the second-most widely cultured prawn species in the world, after whiteleg shrimp, *Litopenaeus vannamei* (FAO, 2010). In 2009, 770,000 tonnes were produced, with a total value of US$3,650,000,000 (FAO, 2010). In 2010, Greenpeace added *Penaeus monodon* to its seafood red list – "a list of fish that are commonly sold in supermarkets around the world, and which have a very high risk of being sourced from unsustainable fisheries". The reasons given by Greenpeace were "destruction of vast areas of mangroves in several countries, over-fishing of juvenile shrimp from the wild to supply farms, and significant human rights abuses.

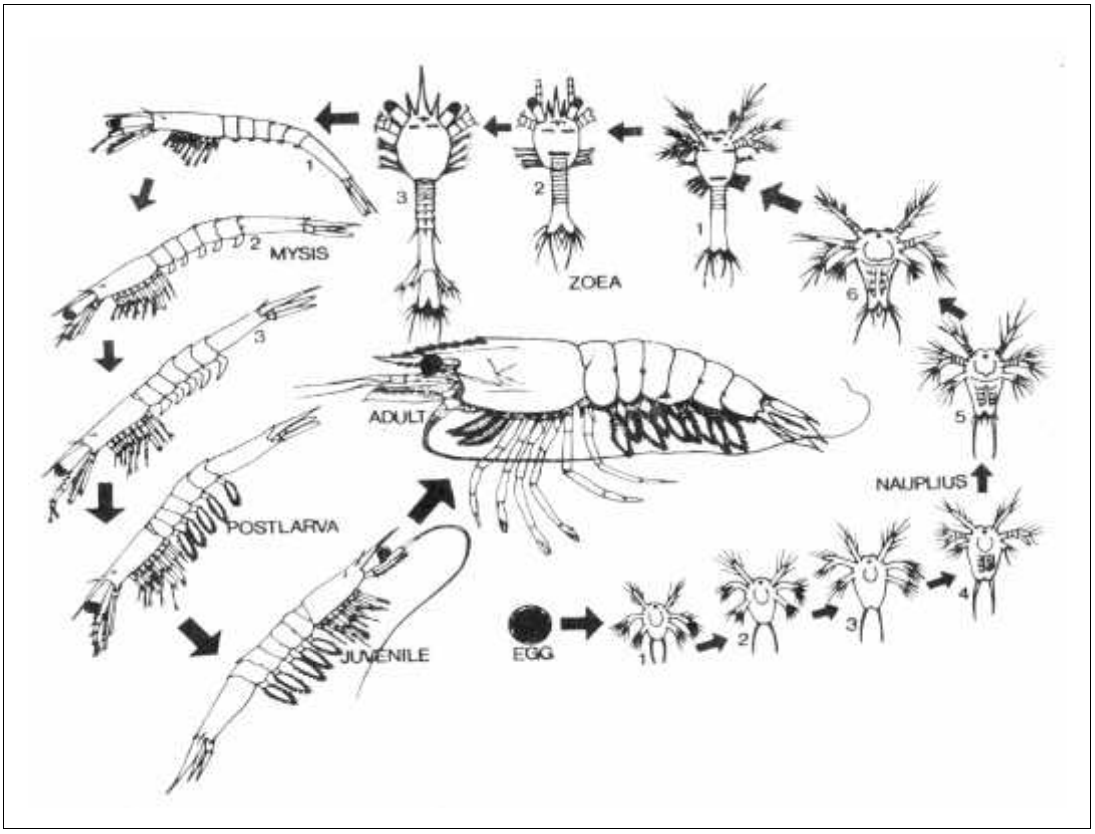


Fig. 1.4. Life cycle of *Penaeus monodon*

(Source: http://www.twwiki.com/uploads/wiki/b4/16/600451_2.png)

1.3 Shrimp diseases

Several diseases out break during culture period causes drastic loss to the farmers. According to most of the scientist, prevention is badly requirement to reduce the disease risk of shrimp than to take measures for medication after outbreaks. The intensification of shrimp farming has also produced a number of problems affecting the industry. These include environmental and physiological stress factors that are often related to disease and mortality; these factors are related to an increased susceptibility to infectious diseases.

Viral diseases have emerged during the past two decades as serious economic impediments to successful shrimp farming. While nearly 20 distinct viruses or groups of viruses are known to infect shrimp culture; White Spot Syndrome Virus (WSSV), Yellow Head Virus (YHV), Infectious Hypodermal and Hematopoietic Necrosis Virus (IHHNV), and Taura Syndrome Virus (TSV) pose a threat to the future of shrimp culture. Among all these, WSSV has become the biggest threat and huge economic loss in shrimp industry (Kesavan et al., 2016).

Microsporidians are obligate intracellular parasites known to infect a wide range of eukaryotic hosts. Development of the parasite generally occurs within the cytoplasm of the host cell via spore formation (Sporogony) and nuclear proliferation, though certain genera are known to undergo similar development within the host nucleoplasm. Symptoms of monodon baculovirus (MBV) and hepatopancreatic parvovirus (HPV) affecting shrimp showed presence of inclusion in hepatopancreatic cells sometimes looks similar to microsporidian (Tourtip et al., 2009). Several genera of microsporidia have been reported to infect crustacean hosts. These include *Agmasoma, Ameson, Nosema, Pleistophora, Tuzetia, Thelohania, Flabelliforma, Glugoides, Vavraia, Ordospora, Nadelspora* and *Enterospora*. From three species of penaeid shrimp along the west coast of Madagascar microsporidians has also identified by scanning electron microscopy (Toubiana et al., 2004).

Shrimp microsporidia are considered a potential threat for their populations, and also lead an economic impact on shrimp aquaculture. In Southeast Asia the mortality rate of shrimp cultivation have been increased due to the infection caused by microsporidium, *Enterocytozoon hepatopenaei* (EHP).

EHP outbreaks are occurring in China, Indonesia, Malaysia, Vietnam and Thailand. In black tiger shrimp, *Penaeus monodon* EHP was first characterized in Thailand in 2009 (Tourtip et al., 2009). As a part of ongoing disease surveillance among the farmed shrimp, Sritunyalucksanaet al. (2013) investigated *Penaeus (Litopenaeus) vannamei* cultured in the southeast coast of India for EHP infection using light and scanning electron microscopy, histopathology, polymerase chain reaction (PCR) and *in situ* hybridization. Although EHP does not appear to cause mortality but it has severe growth retardation effect in *P. vannamei* and *P. moodon* and is also suspected to infect *P. japonicas* (Hudson, et al., 2001; Tangprasittipap, et al., 2013).

Recently, shrimp farms in Asia and other areas have been reporting heavy infection with a microsporidian parasite, *Enterocytozoon hepatopenaei* (EHP) in cultured *Penaeus vannamei* impacting the production due to severe growth retardation (Newman, 2015). Rajendran et al. (2016) also have reported that *Enterocytozoon hepatopenaei* (EHP) has emerged as a serious pathogen reported to be associated with retarded growth in cultured shrimp in many of the shrimp growing countries in Asia.

1.4 *Enterocytozoon hepatopenaei* (EHP) – causative agent of retarded growth of shrimp

EHP is an abbreviation for *Enterocytozoon hepatopenaei*, a microsporidian which is a spore forming parasite. When a spore finds a suitable host in a shrimp, it hatches and the parasite begins to usurp some of physical growth of the shrimp. In general, it reduces the growth rate and increases the size variation in shrimp population (Sackton, 2015). It has ovoid spores and size of approximately 0.7–1 μm that shows 5–6 internal coils of the filament by transmission electron microscope (Suebsing et al., 2013). EHP is confined to the shrimp hepatopancreas and morphologically resembles an unnamed microsporidian previously reported in the hepatopancreas of *Penaeus japonicas* from Australia in 2001. It can be transmitted directly from shrimp to shrimp by the oral route (Tangprasittipap et al., 2013). The economic losses attributed to EHP infections have been rapidly growing, and EHP is now considered to be a critical threat to shrimp aquaculture (Tang et al., 2015). Very recently, PCR-positive samples have received for EHP from slow growing shrimp in India. Thus, EHP is an emerging problem that is under urgent need of control.

1.4.1 Taxonomic classification of *Enterocytozoon hepatopenaei*

Kingdom: Animalia

Phylum: Microspora

Class: Microsporea

Order: Microsporida

Family: Enterocytozoonidae

Genus: *Entercytozoon*

Species: *Enterocytozoon hepatopenaei*

1.4.2 Phylogenetic tree of *Enterocytozoon hepatopenaei*

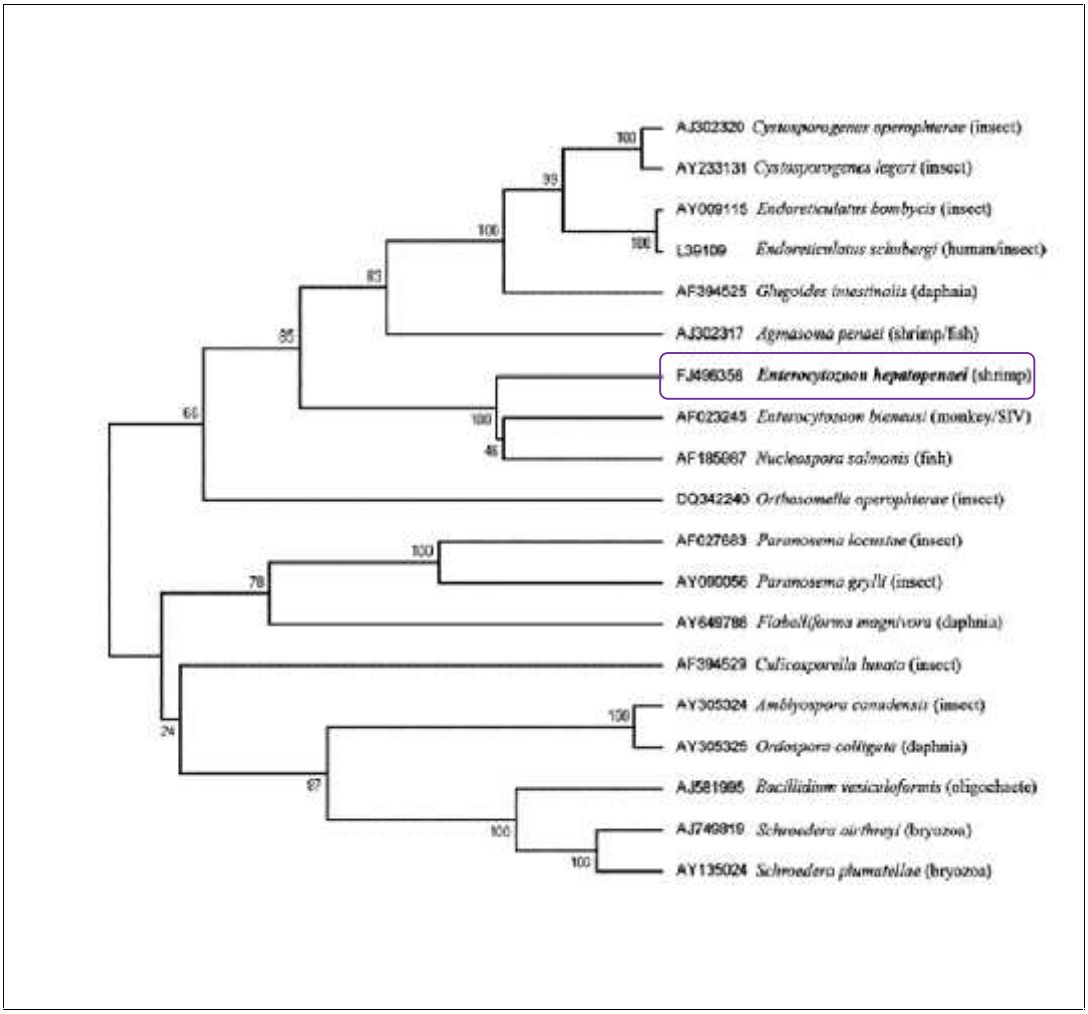


Fig. 1.5. Phylogenetic tree of *E. hepatopenaei* and selected microsporidians using CLUSTALW alignment of SSU rRNA gene sequences (Tourtip et al., 2009).

1.5 Histopathology

Histopathology is the microscopic examination of biological tissues to observe the appearance of diseased cells and tissues in very fine detail. It is the study of microscopic changes or abnormalities in tissues that are caused as a result of diseases. The word "histopathology" is derived from a combination of three Greek words: ‘histos’ meaning tissue, pathos meaning disease or suffering, and logos which refers to study. (http://www.ivyroses.com)

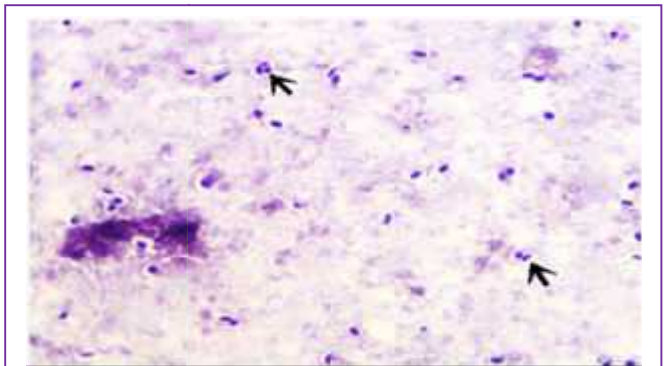


Fig. 1.6. A photomicrographs of smear preparation stained with haematoxylin–eosin (arrows indicate spores EHP). (Rajendran et al., 2016)

1.6 PCR Technique

Polymerase chain reaction (PCR) is a technique developed in 1983 by Kary Mullis (Bartlett et al., 2003) used in molecular biology to amplify a single copy or a few copies of a piece of DNA across several orders of magnitude, generating thousands to millions of copies of a particular DNA sequence. It is an easy and available tool to amplify a focused segment of DNA, useful for such purposes as the diagnosis and monitoring of genetic diseases, identification of criminals (in the field of forensics), and studying the function of a targeted segment of DNA (http://learn.genetics. utah.edu/content/labs/pcr).

PCR is now a common and often indispensable technique used in clinical laboratories and research laboratories for a variety of applications (Saiki et al., 1985). These include DNA cloning for sequencing, DNA-based phylogeny, or functional analysis of genes; the diagnosis of hereditary diseases; the identification of genetic fingerprints (used in forensic sciences and DNA paternity testing); and the detection of pathogens in nucleic acid tests for the diagnosis of infectious diseases

The method relies on thermal cycling, consisting of cycles of repeated heating and cooling of the reaction for DNA melting and enzymatic replication of the DNA. Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase for which the method is named after, are key components to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the

DNA template is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations. PCR is not generally considered to be a recombinant DNA method, as it does not involve cutting and pasting DNA, only amplification of existing sequences.

Almost all PCR applications employ a heat-stable DNA polymerase, such as Taq polymerase (an enzyme originally isolated from the bacterium *Thermus aquaticus*). This DNA polymerase enzymatically assembles a new DNA strand from DNA building-blocks, the nucleotides, by using single-stranded DNA as a template and DNA oligonucleotides (also called DNA primers), which are required for initiation of DNA synthesis. The vast majority of PCR methods use thermal cycling, i.e., alternately heating and cooling the PCR sample through a defined series of temperature steps.

A basic PCR set up requires several components and reagents. These components include:

- *DNA template* that contains the DNA region (target) to amplify
- Two *primers* that are complementary to the 3' (three prime) ends of each of the sense and anti-sense strand of the DNA target. Primers can be custom made in a laboratory that are complementary to the DNA segment to be amplified.
- *Taq polymerase* (as the DNA polymerase can attach to a DNA strand and elongate on its own. It should also be heat resistant, so that it can withstand the denaturation process.
- *De-oxynucleoside triphosphates* (dNTPs, sometimes called "deoxynucleotide triphosphates"; nucleotides containing triphosphate groups), the building-blocks from which the DNA polymerase synthesizes a new DNA strand.
- *Buffer solution*, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- *Bivalent cations*, magnesium or manganese ions; generally Mg^{2+} is used, but it can be used for PCR-mediated DNA mutagenesis, as higher Mn^{2+} concentration increases the error rate during DNA synthesis.
- *Monovalent cation* potassium ions.

In the first step, the two strands of the DNA double helix are physically separated at a high temperature in a process called DNA melting. In the second step, the temperature is

lowered and the two DNA strands become templates for DNA polymerase to selectively amplify the target DNA. The selectivity of PCR results from the use of primers that are complementary to the DNA region targeted for amplification under specific thermal cycling conditions.

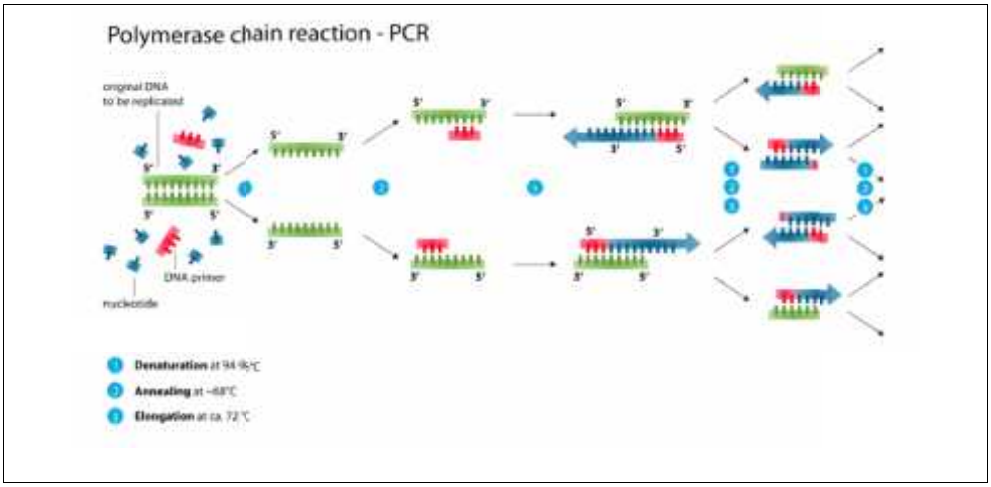


Fig. 1.7. Protocal of PCR reaction (Source: https://en.wikipedia.org)

To check whether the PCR generated the anticipated DNA fragment (also sometimes referred to as the amplimer or amplicon), agarose gel electrophoresis is employed for size separation of the PCR products. The size(s) of PCR products is determined by comparison with a DNA ladder (a molecular weight marker), which contains DNA fragments of known size, run on the gel alongside the PCR products.

- **Template DNA Extraction method**

From the EHP-infected penaeid shrimp, the hepatopancreas was removed and pooled (4–5 shrimp) into one sample.Total DNA was extracted from each sample using a High-pure DNA template preparation kit (Roche Bioscience) or a Maxwell-16 Cell LEV DNA purification kit (Promega) (Tang et al., 2015 and Tang et al., 2016). Total DNA was extracted from the hepatopancreas using a commercial tissue extraction kit (Qiagen, Germany). (Tourtip et al., 2009). In 2012, total DNA was extracted by DNeasy Blood and Tissue Kit (Qiagen) (Leiro et al., 2012). In 2015, DNA was extracted using Accuprep Genomic DNA Extraction Kit (BIONEER) (Chiyansuvata1 et al., 2015).

- **Primers**

Primer sets are needed for molecular characterization of any target region. Tourtip et al. (2009) were used MF1 (forward) 5′-CCGGAGAGGGAGCCTGAGA-3′ and MR1

(reverse) 5′-GACGGGCGGTGTGTACAAA-3′, relative to positions 242–260 and 1165–1183, respectively, of the small subunit (SSU) rRNA gene of *Enterocytozoon bieneusi* (Genbank accession No. AF024657) for identification of microsporidian parasite. Stentiford et al., (2013) were used the MF1 (forward) and MR1 (reverse) primer for identification of EHP. Rajendran et al. (2016) were also used the same primer sequences for identification of microsporidian parasite and used two sets of EHP-specific primers (ENF779-5′-CAGCAGGCGCGAAAATTGTCCA-3′ and ENR779-5′-AAGAGATATT GTATTGCGCTTGCTG-3′; ENF176-5′-CAACGCGGGAAAACTTACCA-3′and ENR 176-5′-ACCTGTTATTGCCTTCTCCCTCC-3′). Tang et al., (2015) were used the 18S rRNA and EHP-510 primer sequences. These were 18S-F (5′-CACCAGGTGAT TCTGCCTGA) and 18S-R (5′-TCTGAAATAGTGACGGGCGG) and EHP 510F (5′-GCCTGAGAGATGGCTCCCACGT) and EHP-510R (5′-GCGTACTATCCCCAGAGCCCGA). Tang et al. (2016) and Han et al. (2016) were used the EHP-510F and EHP-510R primer for the identification of the microsporidian parasite *Enterocytozoon hepatopenaei* (EHP).

1.7 Rationale

In recent years, aquaculture has become more and more important for Asia, particularly in Bangladesh. It represents the second largest export industry for Bangladesh after garments with 97% of the shrimp produced being exported (Kruijssen et. al., 2012) and contributing about 4% to national GDP (Haque et al., 2012) and employing approximately 1.2 million people for production, processing and marketing activities. Including their families, this sees approximately 4.8 million Bangladeshi people directly dependent on this sector for their livelihood (Paul et al., 2012). However, while the Bangladeshi shrimp industry grows, it has also drawn some controversy. Shrimp has emerged as an important item in the world seafood production. Asian countries like Taiwan, Indonesia, Thailand and India have emerged as global leaders in shrimp production. No other primary commodity enjoyed such spectacular growth in post-independence. Now-a-days, shrimp sector is under threat due to various disease attacks, especially microsporidian parasite cause's reduced growth, thereby decrease the total production. If we can detect the microsporidian parasite especially EHP and suggest the preventive measure the farmers will be able to manage their farm efficiently and increase the production.

So the proposed research work will be an attempt to develop ways to produce healthy or quality shrimp and hence boost up production of shrimp.

1.8 Research gap

The detection of microsporidian parasite in cultured shrimp has not done yet in Bangladesh. So this thesis work will be focused mostly on detection and molecular characterization of the microsporidian parasite in cultured shrimp collected from different regions of south-west Bangladesh, especially from Satkhira, Bagerhat district. Routine histology will be applied for the identification of microsporidian parasite and also for PCR to amplify the SSU rRNA gene from microsporidian parasite for molecular characterization.

1.9 Objectives

The overall objective of the research work was to determine prevalence and characterize the microsporidian parasite from hepatopancreas of cultured Shrimp using microscopic and molecular techniques

The specific objectives were-

1. To identify the microsporidian parasite from collected shrimp samples by microscopic observation and routine histological techniques.
2. To characterize the microsporidian parasite by using SSU rRNA gene sequencing
3. To identify the EHP by species specific gene sequencing
4. To determine prevalence and phylogenetic relationship among microsporidian parasite

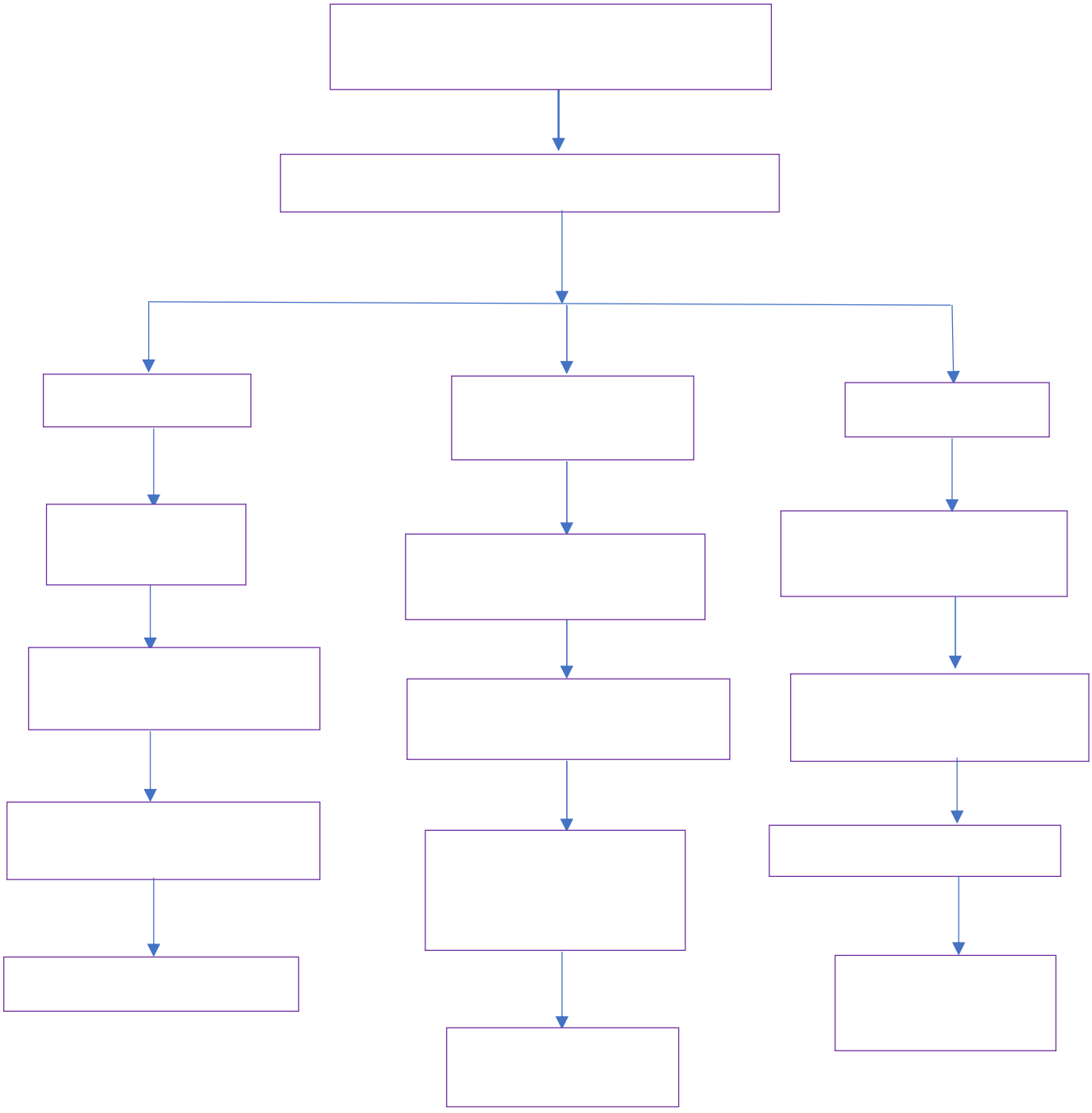
Chapter 2

Materials and Methods

2.1 Study design

For identification and molecular characterization of microsporidian parasite Histopathology, Scanning electron microscopy and PCR technique was followed.

2 (A) Flow chart of the study



2.2 Collection of shrimp samples

For the detection and molecular characterization of microsporidian parasite, 3-4 month ages of *Penaeus monodon* were collected from various shrimp farms in Satkhira sadar upazilla and Bagerhat (Morrelgang and Mongla upazilla) districts.

Satkhira Sadar is located at 22.7167°N 89.0750°E. It is bounded by Kalaroa Upazila on the north, Tala Upazila on the east, Debhata and Assasuni upazilas on the south and Basirhat and Baduria CD Blocks in North 24 Parganas district in West Bengal, India, on the west (Banglapedia, 2016).

Morrelganj Upazilla is located at 22.4500°N 89.8583°E. It is bounded by Bagerhat sadar and Kachua upazilas on the north, Sarankhola and Mathbaria upazilas on the south, Pirojpur sadar and Bhandaria upazilas on the east, Rampal and Mongla upazilas on the west. Main rivers: Baleshwar, Ghasiakhali, Panguchi and Bhola (Encyclopedia, 2012).

Mongla is located at 22.4833°N 89.6083°E.It is bounded by Rampal Upazila on the north, the Bay of Bengal on the south, Morrelganj and Sarankhola Upazilas on the east and Dacope Upazila on the west. The main rivers are Pasur and Mongla.It is the second biggest seaport of the country (Encyclopedia, 2012).

From the Satkhira sadar upazilla 32 samples were collected and 8 samples were collected from (4 from Morrelgang and 4 from Mongla upazilla) Bagherhat district. The samples were collected from April, 2016 to November, 2016.

2.3 Disease sign in shrimp samples

The typical signs of microsporidian parasite at shrimp facilities were slow growth rates of less than 0.15 grams per day, wide size variations reflecting a coefficient of variation greater than 0.2, and loose or soft shells. The age and size of the Juveniles were varied from one to four month in culture and from 1 to 10 g body weight. In the early of the day, the samples were collected from the gher.In a typical microsporidian parasite infection scenario, shrimp growth rates decline 60 to 70 days after stocking, resulting in poor feed-conversion rates and increasing sludge deposition on pond bottoms. This deteriorating environment further aggravates EHP infection levels, and growth rates decline further.

A. Geographic Map of Sadar Upazila, Satkhira

B. Representative gher of SadarUpazila, Satkhira

C. Geographic Map of Morrelgang Upazila, Bagherhat

D. Representative gher of Morrelgang Upazila, Bagherhat

E. Geographic Map of Mongla Upazila, Bagherhat

F. Representative gher of Mongla Upazila, Bagherhat

Plate 2.1 Geographic location of shrimp sample collection gher. Maps was reproduced from google map (https://en.wikipedia.org).

Table: 2.1 Information on the shrimp sample collected from farming ghers of South-west region

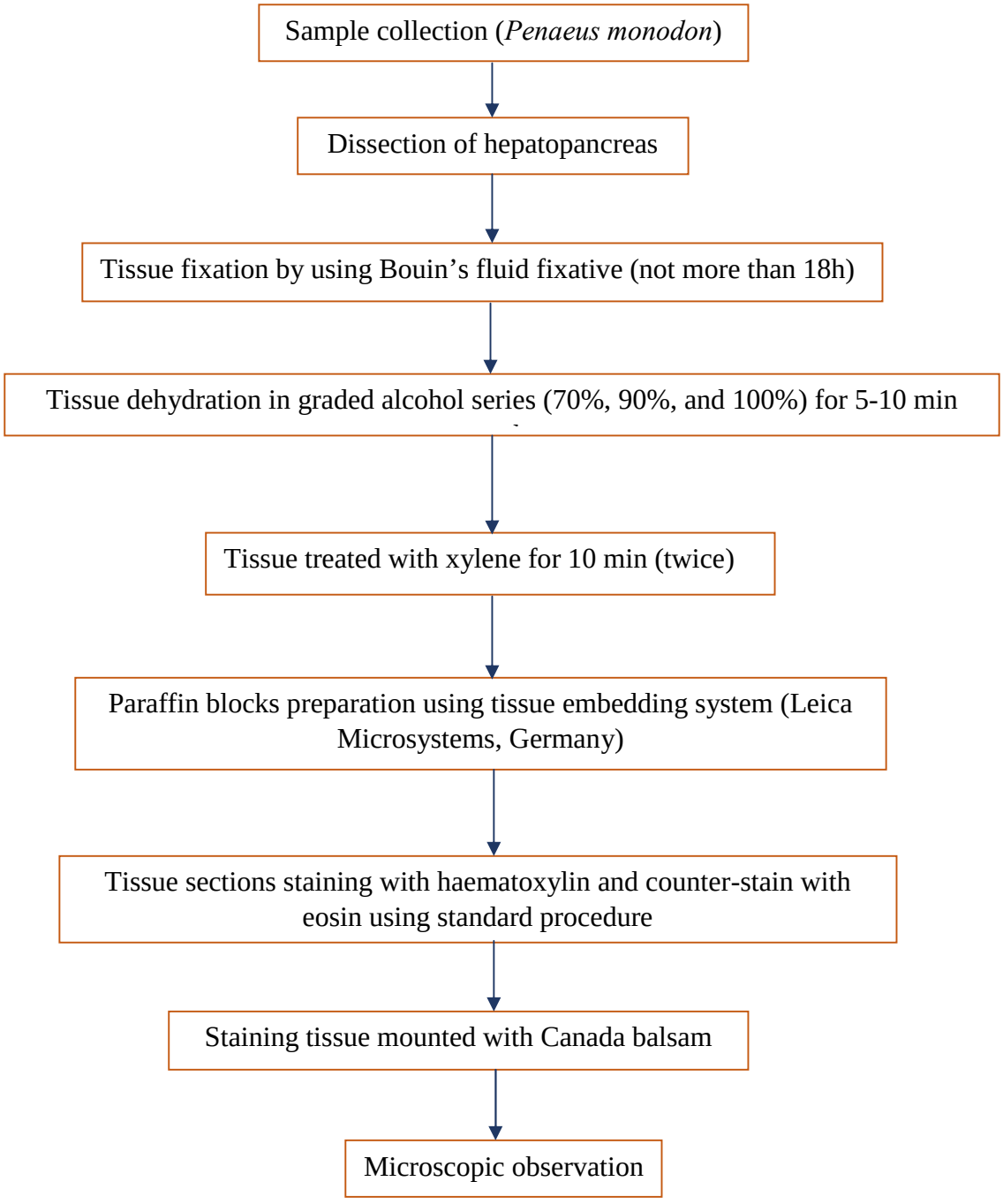
Sample ID	Sampling area	Sampling date	Owner of the gher	Area of the gher(bigha)	Name of the shrimp sp.	Age of the shrimp(month)	Dissection of organ	Organ for Histopathology / Molecular Diagnosis
1	Satkhira sadar	16-04-2016	Mosharaf	7	*Penaeus monodon*	3	Stomach	Histopathology
2	"	"	"	7	"	3	Muscle	"
3	"	"	"	7	"	3	Hepatopancreas	Histopathology and Molecular Diagnosis
4	"	"	Obydullah	3	"	3	Stomach	Histopathology
5	"	"	"	3	"	3	Muscle	"
6	"	"	"	3	"	3	Hepatopancreas	Histopathology and Molecular Diagnosis
7	"	"	Bablu	10	"	3	Stomach	Histopathology
8	"	"	"	10	"	3	Muscle	"
9	"	"	"	10	"	3	Hepatopancreas	Histopathology and Molecular Diagnosis
10	"	"	Yeakhan	4	"	2.5	Stomach	Histopathology
11	"	"	"	4	"	2.5	Muscle	"
12	"	"	"	4	"	2.5	Hepatopancreas	Histopathology and Molecular Diagnosis
13	"	"	Abdullah	5	"	3	Stomach	Histopathology
14	"	"	"	5	"	3	Muscle	"
15	"	"	"	5	"	3	Hepatopancreas	Histopathology and Molecular Diagnosis
16	"	20-05-2016	Abdul Hamid	4	"	3	"	"
17	"	"	Mosharaf	7	"	3	"	"
18	"	"	Ziad	50	"	3	"	"
19	"	"	Anarul	6	"	3	"	"
20	"	16-06-2016	Shawkat	18	"	3.5	"	"
21	"	"	Raju	20	"	3.5	"	"

22	"	"	Robiul	8	"	3.5	"	"
23	Morrelgang,Bagherhat	17-06-2016	Mizan	6	"	3	"	"
24	"	"	Abdul Malek	12	"	3.5	"	"
25	"	"	Shoharab	10	"	3	"	"
26	"	"	Tanvir	15	"	3	"	"
27	Mongla,Bagherhat	18-06-2016	Shahin	7	"	3	"	"
28	"	"	Ripon	6	"	3	"	"
29	"	"	Nazrul	9	"	3.5	"	"
30	"	"	Hafizul	5	"	3	"	"
31	Satkhira sadar	18-07-2016	Mosharaf	7	"	3	"	"
32	"	"	Khairul	5	"	3.5	"	"
33	"	"	Imdadul	4	"	3	"	"
34	"	20-09-2016	Obydullah	3	"	3	"	"
35	"	"	Ruhul Amin	25	"	3	"	"
36	"	"	Kamrul	8	"	3.5	"	"
37	"	"	Liton	4	"	3	"	"
38	"	18-11-2016	Mosharaf	7	"	3	"	"
39	"	"	Ziad	50	"	3.5	"	"
40	"	"	Shawkat	18	"	3	"	"

2.4 Histopathology

Microscopic changes or abnormalities in tissues that are caused as a result of diseases were studied using the techniques of histopathology. After collection of samples for microscopic observation, slide was prepared by histopathological protocol.

2 (B) Protocol for the Histopathology



2.4.1 Sample preparation for histology

After the collection of shrimp, at first the hepatopancreas was dissected from the shrimp body by the help of the forcep. Then the dissected hepatopancreas was transferred into the vial.

2.4.2 Steps followed in histology

(a) Fixation

The hepatopancreas was fixed in Bouin's fluid for 18 hours, but not more than 18 hours. Ideally sample was remained in fixative for long enough for the fixative to penetrate into every part of the tissue and then for an additional period to allow the chemical reactions of fixation to reach equilibrium (fixation time).

Aqueous Bouin's Fluid:

- Saturated Aqueous Solution of Picric Acid 75ml
- 40% formaldehyde
- 5% Acetic Acid

(b) Sample resection

After fixing the sample, the hepatopancreas was required further dissection to select appropriate areas for examination.

(c) Dehydration (upgrade)

The hepatopancreas was left in alcohol in the following way. (Ethyl Alcohol)

A typical dehydration sequence for hepatopancreas

1. 70% ethanol 45 min
2. 90% ethanol 5-10 min
3. 100% ethanol 5-10 min
4. 100% ethanol 15 min

(The tissue was broken down when deep yellow color was present. If the yellow color was not gone a few crystals of Helium Carbonate was put and then again was put in 70% alcohol solution the tissue was kept for 2-3 hours again).

At this point all but a tiny residue of tightly bound (molecular) water was removed from the sample.

(d) Clearing in Xylenes

The tissue was transferred in xylene for about 10 to 15 minutes. It was removed all the alcohol from the tissue. (Not more than 15mins, otherwise the tissue will break down).

(e) Impregnation

The Xylene wax solution was prepared by adding wax in xylene for 10-15 mins for proper saturation.

(f) Embedding

The sample was embedded in melted wax for 30-45 mins. The tissue was kept with paraffin wax in the incubator at 65^{o}c. In paraffin the tissue was become roasted.

(g) Block making

Paper boat was made and treated with glycerin. Melted wax poured in boat, which was kept in the water tray. The wax was stirred with a hot needle to remove all the air bubbles in the melted wax, during pouring in the paper boat. Gradually, the tissue embedded in the wax was put in the boat in a longitudinal parallel position. Care was taken so that no air bubbles remain attached with the tissue and in the wax. It was then cooled. A block was thus made.

(h) Trimming

The block was made perfectly square for attachment to the holder.

(i) Section cutting

The square block was suitably attached to the holder, with some wax. It was cooled for good attached. The holder was now dipped in the microtomy machine. The razor of the machine was set at 45^{o} angle. The microtome was drived a knife across the surface of the paraffin cube and produced a series of thin sections of very precise thickness. The thickness of the sections was preset, and a thickness between 5 - 10 µm was optimal for viewing with a light microscope. The sections was then mounted on individual microscope slides.

(j) Affixing

By Mayer's albumin, the ribbon of tissue was attached on the slide.

Mayer's Albumin

- Fresh egg' white 50ml
- Glycerin 50ml
- Sodium Salicylate 5ml

In 20cc Distilled water 3-5 drop of Mayer's albumin was shaken to make it diluted and used.

(k) Stretching

Mayer's albumin was put in the slide. The ribbon of the tissue was arranged in rows putting the slide on the moderator not stretching table and tissue was allowed to dry. (Stretching could not be done in hot water. 40^{o}c was sufficient to stretch tissue).

(l) Deparaffing

The slide with the tissue was kept in xylene for 5-10 mins to remove the wax.

(m) Hydration (Downgrade)

The slide with the tissue was now transferred to the following grades of alcohol:

1.100% ethanol 10 min

2. 90% ethanol 5min

3. 70% ethanol 5min

The distilled water was used to wash the slide to remove the alcohol. (If the tissue was transferred directly from the xylene to water, it would certainly have broken down).

(n) Staining

The slide with the tissue was now dipped into the haematoxylene and washed in running water for half an hour by which it was oxidized to blue color. Nucleus was stained by this method.

(o) Differentiation (by acid water)

Then it was counter stained by means of eosin which colored the cytoplasm. The slide was kept in eosin for about 5 mins until it was stained well.

(p) Dehydration (upgrade)

Now, the slide was kept in the media of alcohol in the following ways

70% ethanol	3-5 min
90% ethanol	5-10 min
100% ethanol	10-15 min

(q) Clearing

The stained tissue was cleared in xylene for 5-10 minutes.

(r) Mounting

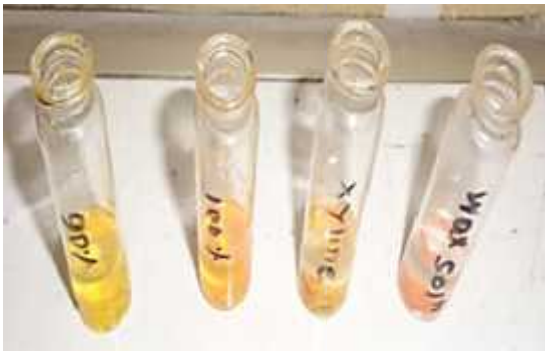
Finally the tissue was mounted by means of cover slip with Canada balsam. Then the slide was observed under microscope.

A. Infected shrimp

B. Infected hepatopancreas

C. Hepatopancreas in Bouins fluid

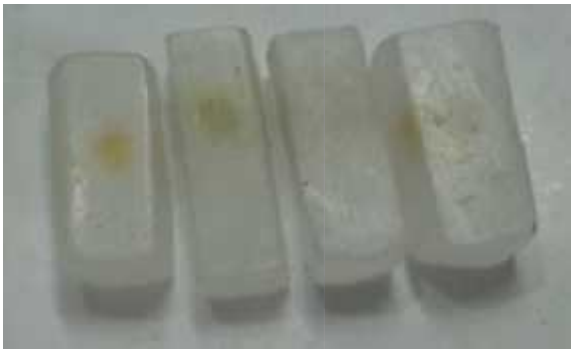
D. Cutting of hepatopancreas into small pieces



E. Dehydration upgrade (90%, 100%), Clearing in Xyline and Impregnation in Wax solution

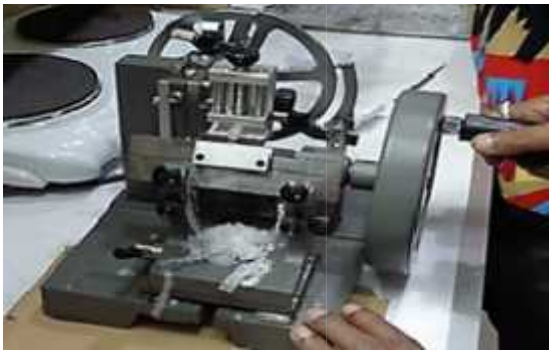
F. Block with melted wax

G. Tissue transfer into block



H. Trimming block

Plate 2.2 Histopathological procedure of shrimp hepatopancreas to check microsporidian parasite (continue……)



I. Section cutting by Microtomy machine

J. Stretching in incubator

K. Deparaffing in Xylene

L. Hydration downgrade (100%, 90%, 70% alcohol)

M. Staining with Haematoxylene and washed in running water

N. Counter staining with Eosin and Dehydration upgrade (70%, 90%, and 100%)

O. Mounting (cover slip) with Canada balsam

P. Microscopic observation of prepared slide

Plate 2.3 Histopathological procedure of shrimp hepatopancreas to check microsporidian parasite

2.5Scanning Electronic Microscope (SEM)

In the scanning electronic microscope, fixation is the first and most important step for optimum preservation of biological samples. The preparation of biological sample for SEM was discussed below

(a) Fixatives and buffers

For fixating the biological sample (hepatopancreas), the fixative paraformaldehyde was used which was usually mixed with phosphate buffer (Davidson's fixative), or with a low concentration of glutaraldehyde together with phosphate buffer.

(b) Washing and dehydrating procedures

The following procedure was a general outline for processing samples following fixation. Generally a 5 mL vial or something similar was worked well for carrying out these steps. Before started the following chemical were needed-

- Fresh buffer solution (with no fixative added)
- A series of ethanol concentrations in distilled water for dehydration – for example 50%, 60%, 70%, 80%, 90% and 100% ethanol.

Dehydration Procedure

- The sample was rinsed with fresh buffer (no fixative added) in the fume hood – repeat three times.
- Buffer solution was replaced with the lowest concentration ethanol solution in dehydration series 70%, and left for 15-20 minutes.
- This process was continued until the sample was in 100% ethanol, then repeated the 100% ethanol step.
- Samples was now dehydrated and ready for drying.

(c) Drying after dehydration

The sample was processed via 'critical point drying' (CPD).

- Here, the sample that was dehydrated to the point of 100% ethanol (as outlined in the procedure above), was then placed in the chamber of a CPD apparatus with enough 100% ethanol to completely covered it.

- The chamber was then sealed and cooled, as valves were opened to let liquid CO2 in and vent ethanol out, until liquid CO2 was completely replaced the ethanol.
- The chamber was then sealed again and slowly heated. When the chamber pressure exceeds 1072 psi and the temperature exceeds 31°C, a critical point was achieved whereby the liquid and gas phases of CO_2 was in equilibrium.
- The CO2 was then slowly drained from the chamber and the sample as a gas, thereby avoiding the effects of surface tension that occurred as water changes from liquid to gas.
- An alternative to critical point drying was the use of a chemical drying agent, Hexamethyldisilizane (HMDS).

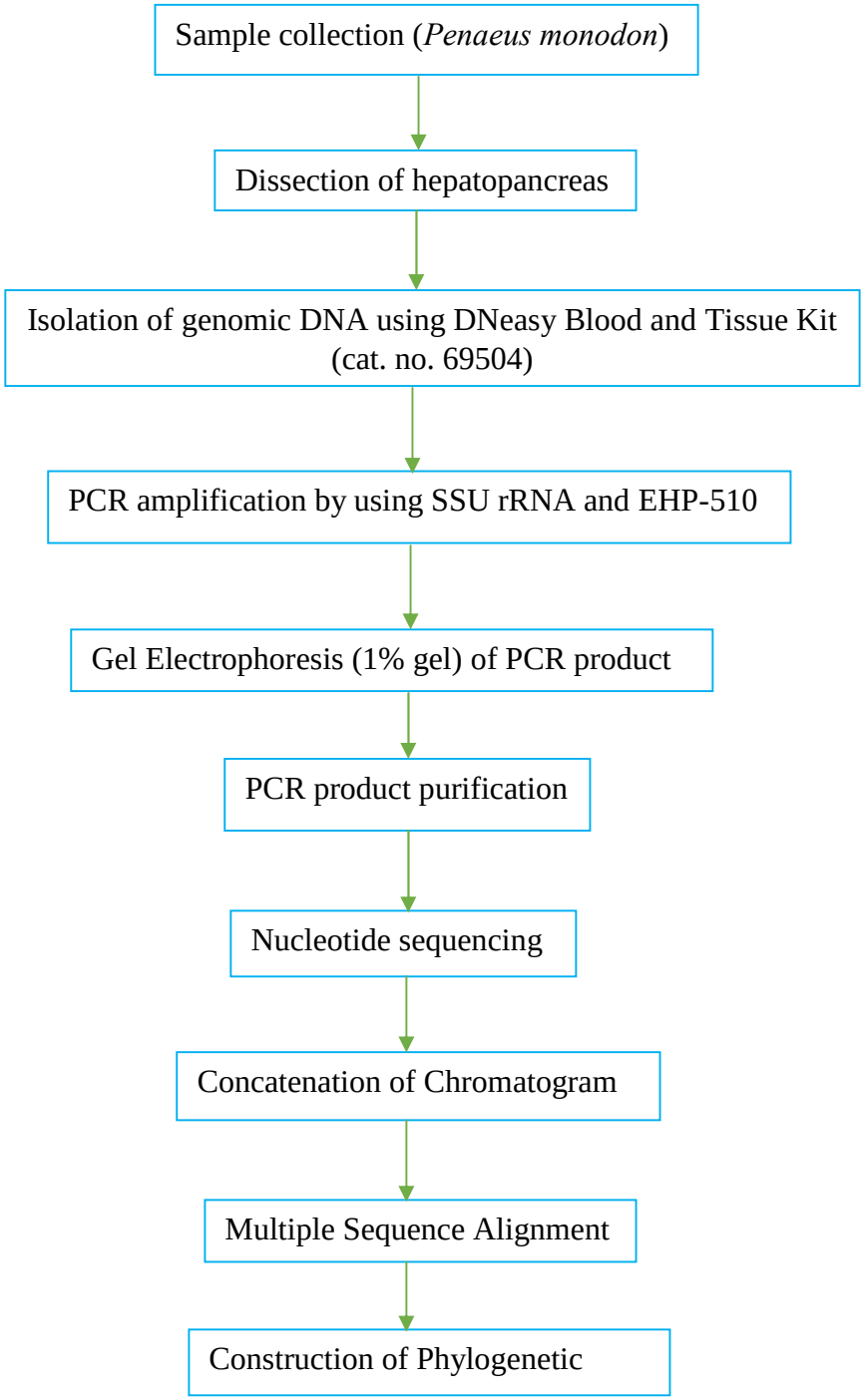
This technique was proven to work with most biological sample types, it was easy and it negated the need for expensive and time consuming CPD equipment. The procedure was simple:

1. The sample was transferred from 100% ethanol into a 1:2 solution of HMDS: 100% ethanol and left for 20 minutes
2. Sample was transferred to a fresh solution of 2:1 HMDS:ethanol for 20 minutes.
3. Sample was transferred into 100% HMDS for 20 minutes, and repeated this step.
4. When the sample was submerged in the final 100% HMDS solution leave covered or capped loosely in a fume hood overnight
5. All HMDS steps needed to be carried out in the fume hood wearing the necessary personal protection gear as it was highly toxic
6. The HMDS would evaporate, leaving the sample ready for sputter coating and imaging in the SEM.

2.6 Molecular techniques used for the identification of EHP

Molecular techniques were used for the identification and characterization of the microsporidian parasite shown in Flow Chart 2 (C).

2 (C) Protocol for Molecular identification of microsporidian parasite



2.6.1 DNA Extraction

There are two types of DNA Extraction Procedure for the DNeasy Blood and Tissue Kit (Qiagen, Germany). Thesetwo types of DNA Extraction Procedure was followed for DNA Extraction.

2.6.1.1 DNA Extraction Procedure — using a mortar and pestle

1. At first around 50-70 mg of hepatopancreas was grinded in liquid nitrogen using a mortar and pestle. Then the powder was placed in a 2 ml microcentrifuge tube. (To prevent cross-contamination, the mortar and pestle between samples were cleanedthoroughly).

2. 180 µl Buffer ATL was added.

3. 20 µl proteinase K was added and mixed thoroughly by vortexing and incubated at 56°C until the hepatopancreas were completely lysed. During incubation to disperse the sample vortexing were performed occasionally and placed in a thermomixer. Lysis was completed in 1–3 h.

4. After incubation, the sample was vortexing for 15 s and was added 200 µl Buffer AL to the sample, and mixed thoroughly by vortexing. Then 200 µl ethanol (96–100%) was added and mixed again thoroughly by vortexing. (It is essential that the sample, Buffer AL, and ethanol were mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution. Buffer AL and ethanol were be premixed and added together in one step to save time when processing multiple samples).

5. The mixture from step 4 (including any precipitate) was placed into the DNeasy Mini spin column in a 2 ml collection tube. Then it was centrifuged at ≥6000 x g (8000 rpm) for 1 min. After centrifuge, the flow-through and collection tube was discarded.

6. The DNeasy Mini spin column was placed in a new 2 ml collection tube and added 500 µl Buffer AW1, and was centrifuged for 1 min at ≥6000 x g (8000 rpm). After centrifuge, the flow-through and collection tube was discarded

7. The DNeasy Mini spin column was placed in a new 2 ml collection tube and added 500 µl Buffer AW2, and was centrifuged for 3 min at 20,000 x g (14,000 rpm) to dry the

DNeasy membrane. After centrifuge, the flow-through and collection tube was

discarded. (It is important to dry the membrane of the DNeasy Mini spin column, since

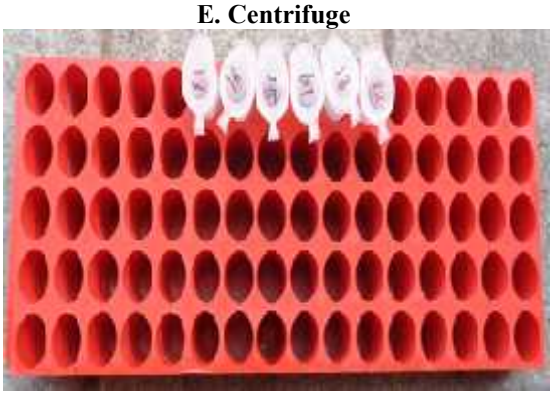
A. Instruments for DNA Extraction

B. Weight Balancer

C. Vortex Machine

D. Incubator

E. Centrifuge



F. Chemicals for DNA Extraction

G. Extracted DNA in Tube

H. DNA Quantification

Plate 2.4 Instruments and Chemicals for DNA Extraction (Using Mortar and pestle)

residual ethanol might be interfered with subsequent reactions. This centrifugation step was ensured that no residual ethanol would be carried over during the following elution).

8. The DNeasy Mini spin column was placed in a clean 2 ml microcentrifuge tube and 200 µl Buffer AE was added directly onto the DNeasy membrane. Then it was incubated at room temperature for 1 min, and then centrifuged for 1 min at ≥6000 x g (8000 rpm) to elute.

2.6.1.2 DNA Extraction Procedure — using an electric homogenizer

1. At first around 50-70 mg of hepatopancreas was placed in a 2 ml microcentrifuge tube.

2. 180 µl PBS, pH 7.2 (50 mM potassium phosphate, 150 mM NaCl) was added andhomogenized the sample using an equivalent electric homogenizer.

3. 20 µl proteinase K and200 µl Buffer AL (without added ethanol) were added and were mixed thoroughly by vortexing and incubated at 56°C for 10 min.

4. After incubation 200 µl ethanol (96–100%) was added and mixed again thoroughly by vortexing.

5. The mixture from step 4 (including any precipitate) was placed into the DNeasy Mini spin column in a 2 ml collection tube. Then it was centrifuged at ≥6000 x g (8000 rpm) for 1 min. After centrifuge, the flow-through and collection tube was discarded.

6. The DNeasy Mini spin column was placed in a new 2 ml collection tube and added 500 µl Buffer AW1, and was centrifuged for 1 min at ≥6000 x g (8000 rpm). After centrifuge, the flow-through and collection tube was discarded

Figure 2.1 A photograph of electric homogenizer used for DNA extraction

7. The DNeasy Mini spin column was placed in a new 2 ml collection tube and added 500 µl Buffer AW2, and was centrifuged for 3 min at 20,000 x g (14,000 rpm) to dry the DNeasy membrane. After centrifuge, the flow-through and collection tube was discarded. (It is important to dry the membrane of the DNeasy Mini spin column, since residual ethanol might be interfered with subsequent reactions. This centrifugation step was ensured that no residual ethanol would be carried over during the following elution).

8. The DNeasy Mini spin column was placed in a clean 2 ml microcentrifuge tube and 200 µl Buffer AE was added directly onto the DNeasy membrane. Then it was incubated at room temperature for 1 min, and then centrifuged for 1 min at ≥6000 x g (8000 rpm) to elute.

2.6.2 Purity of DNA

Absorbance of purified DNA by Nano Drop spectrophotometer (Nano Drop 2000 UV-Vis spectrophotometer, Thermo Fisher Scentific Inc., USA) was checked at 260 nm to determine the purity of DNA of shrimp parasite which was around 1.8. Ratios of less than 1.8 indicate that the preparation was contaminated, either with protein, RNA or with phenol.

2.6.3Polymerase chain reaction

Amplified of washed DNA samples were performed by Polymerase Chain Reaction (PCR) for further analysis.

a) Preparation of reaction Mixture

The reaction mixture for PCR was prepared by mixing the specific volume of the components in an appropriate sized tube provided in the following table

- 12.5µl of Hot start colorless Master Mix containing – dNTPs, Buffer, $MgCl_2$, Taq Polymerase (Cat: M7432, Origin: Promega, USA) without template DNA was prepared and aliquoted into PCR tubes.
- Specified template was added into a properly labeled PCR tube. The PCR tube containing reaction mixture and template DNA was capped properly followed by vortex and centrifuge briefly to mix the mixture gently and collect all components to the bottom of the tube respectably.
- The total mixture was then recollected, sealed and placed in a thermal cycler and the cycling was started immediately. PCR amplification was done in an oil free thermal cycler (Applied Bio systems 2720 Thermal cycle).

Table: 2.2 Components and Volume of Reaction Mixture for PCR (10 reactions)

Reaction components	Volume (µL)	Total Volume (µL)
Master mix	12.5	12.5×10 = 125
Forward primer	1	1× 10 =10
Reverse primer	1	1× 10 = 10
Template DNA	1	1× 10 = 10
Nuclease free water	9.5	9.5 × 10 = 95
Total reaction volume	25	250

For the partial amplification of gene, the following primer pairs were used in PCR showed in table 2.3.

Table: 2.3 Primers and their sequence used in PCR

Primer	Type	Sequences
SSU rRNA	Forward	CAGCAGCCGCGGTAATTCC
SSU rRNA	Reverse	CCCGTGTTGAGTCAAATTAAGC
EHP-510	Forward	5′-GCCTGAGAGATGGCTCCCACGT
EHP-510	Reverse	5′-GCGTACTATC CCCAGAGCCCGA

b) PCR conditions

The reaction mixtures containing PCR tubes were preheated at 95°c for 5 minutes in the thermal cycle to ensure the denaturation of all DNA templates. The PCR reaction was then continued according to the following table

Table: 2.4 Amplification cycle, temperature and required time for PCR (SSU rRNA Primer)

Number of cycle	Step	Temperature	Time

1	Pre Heat	95°c	5 min
32cycles	Denaturation Annealing Extension	95°c 53°c 72°c	30 sec 30 sec 1 min
1	Final Extension	72°c	5 min

After completion of cycling, the reactions were held at 4°c.

Table: 2.5 Amplification cycle, temperature and required time for PCR (EHP-510 Primer)

Number of cycle	Step	Temperature	Time
1	Pre Heat	94°c	3 min
35 cycles	Denaturation Annealing Extension	94°c 60°c 72°c	30 sec 30 sec 30 sec
1	Final Extension	72°c	5 min

After completion of cycling, the reactions were held at 4°c.

Fig. 2.1.A photograph PCR machine used for DNA amplification

2.6.4 Gel Electrophoresis of the amplified products and documentation

The successful amplification of the desired genes was visualized by resolving the PCR products in 1% agarose gel depending on the size of amplicon.

- The gel was prepared using 0.32g 1% Agarose powder (V3125, Promega, USA) and 40 ml 1X TAE buffer (V4251, Promega, USA)
- The mixer was heated in a hot plate and magnetic stirrer (VS-130HS, Vision scientific Inc., LTD, Korea) for about 3 minutes to dissolve the agarose
- The boiled mixture was allowed to cool to about 45°C and 2µl of Ethidium Bromide was added.The gel was poured onto gel casing present with well former (comb) and allowed to set on a flat surface for about 15 minutes.
- After solidification of the gel, the comb was removed and buffer (1X TAE) was poured into tank to submerge the solidified gel.
- Samples were prepared by mixing 5µl PCR product with 1µl loading dye and each 6µl prepared PCR product was loaded into the wells formed in the gel.
- Electrophoresis was conducted in 1X TAE buffer at 100 volts for 60 minutes.
- 1 kb DNA ladder was also electrophoresed along the side of the amplified sample DNA.
- DNA bands were observed and photographed by Alphalmager MINI gel-documentation system

Fig.2.2. A Photograph of gel electrophoresis of PCR product at 120V for 60min

2.6.5 Purification of PCR products

The PCR products were resolved by agarose gel electrophoresis to confirm successful amplification of the desired sequence. The PCR products of specific genes were purified

with the Wizard PCR SV gel and PCR Clean-up System Kit (Promega, USA) according to the manufacturer's instruction prior to sequencing. The steps of purification are given below in brief

(a) Processing of PCR products

An equal volume of Membrane Binding Solution was added to the PCR amplification. Binding of DNA. A SV Minicolumn was inserted into collection tube. The prepared PCR product was transferred to the Minicolumn assembly and incubated at room temperature for 1 minute. The preparation was centrifuged at 16000X g for 1 minute using the centrifuge (Sigma, USA). The flow through was discarded and the Minicolumn was reinserted into collection tube.

(b) Washing

700µl Membrane Wash Solution was added into the Minicolumn and centrifuge at 16000Xg for 1 minute. The flow through was discarded and the Mini column was reinserted into Collection Tube. The previous step was repeated with 500µl Membrane Wash Solution and centrifuged at 16000 X g for 5minutes. The Collection Tube was emptied and the column assembly was re-centrifuged for 1 minute with the micro-centrifuge lid open to allow evaporation of any residual ethanol.

(c) Elution

The Minicolumn was carefully transferred to a clean 1.5 mL micro centrifuge tube. 50 µL Nuclease-Free Water was added to the Minicolumn and incubated at room temperature for 1minute followed by centrifugation at 16000 X g for 1 minute. The Minicolumn was discarded and DNA was stored at 4°C or -20°C.

2.7.6 SSU rRNA sequencing

The PCR products were sent to First Base Laboratories Snd Bhd (Malaysia) where cycle sequencing was performed using BigDye Terminator v 3.1 Cycle Sequencing Kit according to manufacturer's instruction and extension product was purified followed by capillary electrophoresis using ABI Genetic Analyzer (Applied Biosystem, USA). Bidirectional sequences were done for all the 10 representative samples. The basic sequencing protocol involved amplification of target sequence by PCR and purification

of desired amplicon followed by cycle sequencing reaction, cycle sequencing product purification and capillary electrophoresis.

2.6.7 Bioinformatics tools

Different bioinformatics tools were used to analyze the sequences. These tools are given below

(a) Finch TV version 1.4

To view DNA sequence Geospizas Finch TV version 1.4 was used. And it leads the way with raw data views, BLAST searching and the ability to reverse complement sequences and traces.

(b) Nucleotide BLAST

BLAST for Basic Local Alignment Search Tool was used for comparing primary sequence information. The statistical significance of matches was used to infer functional and evolutionary relationships between sequences as well as helps to identify members of gene families.

(c) CLUSTALW

CLUSTALW a program which was used for multiple sequence alignment (MSA). This helps to find out the similarity, dissimilarity or identify between different sequences.

(d) Molecular Evolutionary Genetics Analysis (MEGA) version 7.0 software

MEGA was used for the comparative analysis of molecular sequence data was used for reconstructing the evolutionary histories of species and inferring the nature and extent of selective forces shaping the evolution of genes and species.

Chapter 3

Results

3.1 Gross external sign for microsporidian parasite infection

The clinical signs associated with the microsporidian parasite infection in farmedshrimp are not specific, generally associated with stunted growth and increased mortality. Based on the data obtained from farms in Satkhira sadar upazilla and Bagherhat district, after stocking infected postlarvae into the shrimp farms, the shrimp grew at a normal rate during the first 25 days, after that, the shrimp health started to deteriorate; the infected shrimp exhibited reduction in feed consumption (50–70%) and discolored hepatopancreas. The growth (i.e. gain in mass, shrimp were weighted weekly) of the infected shrimp was only 10–40% that ofthe non-infected shrimp. Mortality attributed to microsporidian parasite infection wasvariable, with farm reports of a daily mortality of approximately 1–2%. Severe infections by microsporidian parasite can increase the susceptibility for other bacterial infections. We have found, in some cases, microsporidian parasite infections wereaccompanied by opportunistic infections of *Vibrio* spp. (named septic hepatopancreatic necrosis, SHPN), and this could result in increased mortalities.

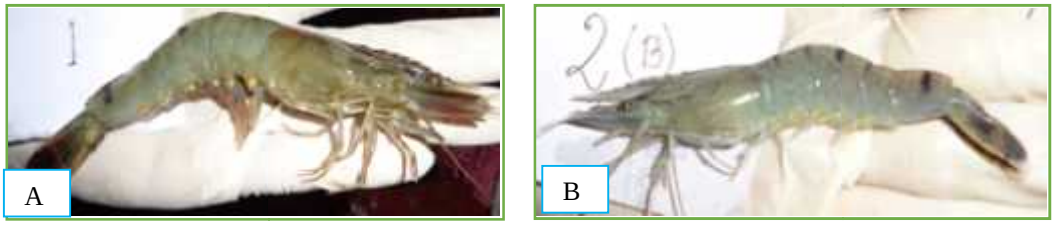


Fig. 3.1. Photograph of *Penaeus monodon.* **(A)** Normal shrimp **(B)** Slow growth shrimp

3.2 Histopathology

In this study histological examination of the infected shrimp´s hepatopancreas and stomach showed the presence of the microsporidian parasite. The hepatopancreas and stomach samples were prepared by the smear method (Bell and Lightner, 1988) and viewed by light microcopy (Euromex Holland).

From the collected 40 samples, 21 samples were prepared for histopathology observation. Out of 21 samples, 13 samples from Satkhira sadar and 8 samples from Bagerhat district were processed and 16 samples showed positive results for the prevalence microsporidian spores. The overall prevalence was (16/21) approximately 77%.

From 13 samples at Satkhira sadar, 12 samples showed positive for microsporidian spores and from 8 samples at Bagerhat district (Morrelgang 2 and Mongla 2) 4 samples showed positive results. So, the prevalence from Satkhira sadar samples was showed 93% and from Morrelgang samples 50% and from Mongla samples 50%.

In hepatopancreas samples, the mirosporidian spores were found only in the cytoplasm of hepatopancreatic tubule epithelial cells. The stomach samples was also prepared and showed positive results.

3.2.1 Observation of samples of Satkhira sadar upazilla, Satkhira

During the period of April to November 2016, the samples were collected from Satkhira sadar upazilla. The collected sample ID were- 1 to 22 and 31 to 40.

From these collected samples, 1, 3, 6, 9, 12, 15, 19, 20, 21, 32, 33, 37 and 39 were prepared for histological observation.

3.2.1.1 Sample – 1, 3

In 16 April 2016, the sample-1 (stomach) and sample-3 (hepatopancreas) were collected from 3 month ages of shrimp. The owner of the shrimp gher was Mosharaf Hossain and the area of the gher was 7 bigha. The prepared stomach and hepatopancreatic tissue slide were observed under microscope. The following figures showed the presence of microsporidian parasite.

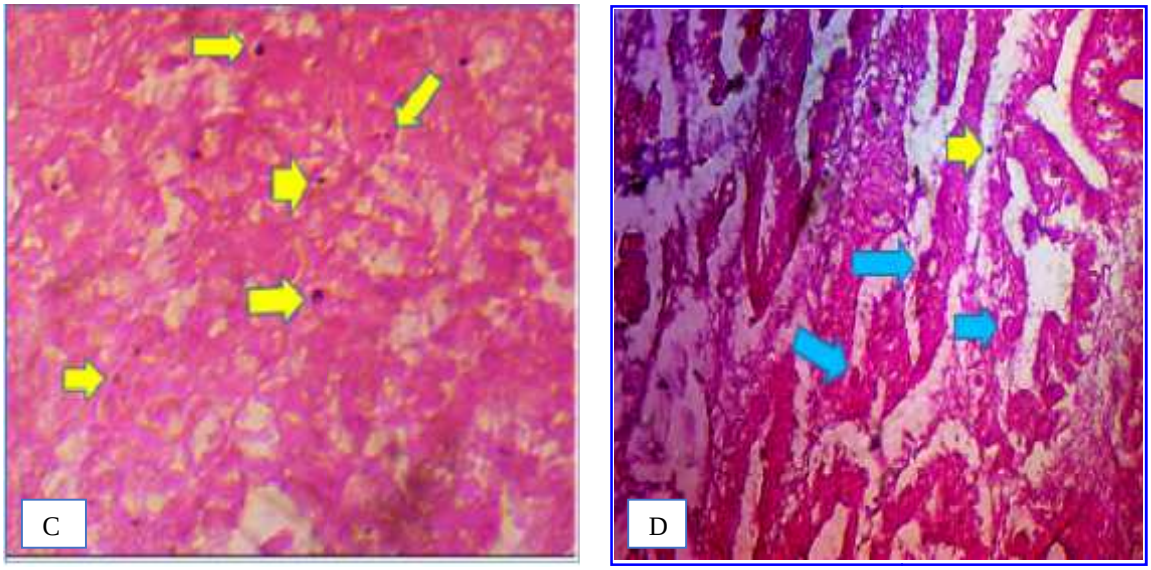


Fig. 3.2. H&E (Mayer-Bennet haematoxylin and eosin-phloxine) histology of microsporidian parasite in the infected shrimp. **(C)** H&E staining of stomach tissue of the *Penaeus monodon.* Yellow color arrows indicated the microsporidian parasite; **(D)** H&E staining of hepatopancreatic tissue of the *P. monodon.* Yellow color arrow indicated the microsporidian parasite and light blue color arrows represented the plasmodium stage of microsporidian parasite.

3.2.1.2 Sample – 6, 9

In 16 April 2016, the sample-6 (hepatopancreas) and sample-9 (hepatopancreas) were collected from 3 month ages of shrimp. The owner of the shrimp gher were Obydullah and Bablu and the area of the gher were 3 and 10 bigha. The prepared hepatopancreatic

tissue slide were observed under microscope. The following figures showed the presence of microsporidian parasite.

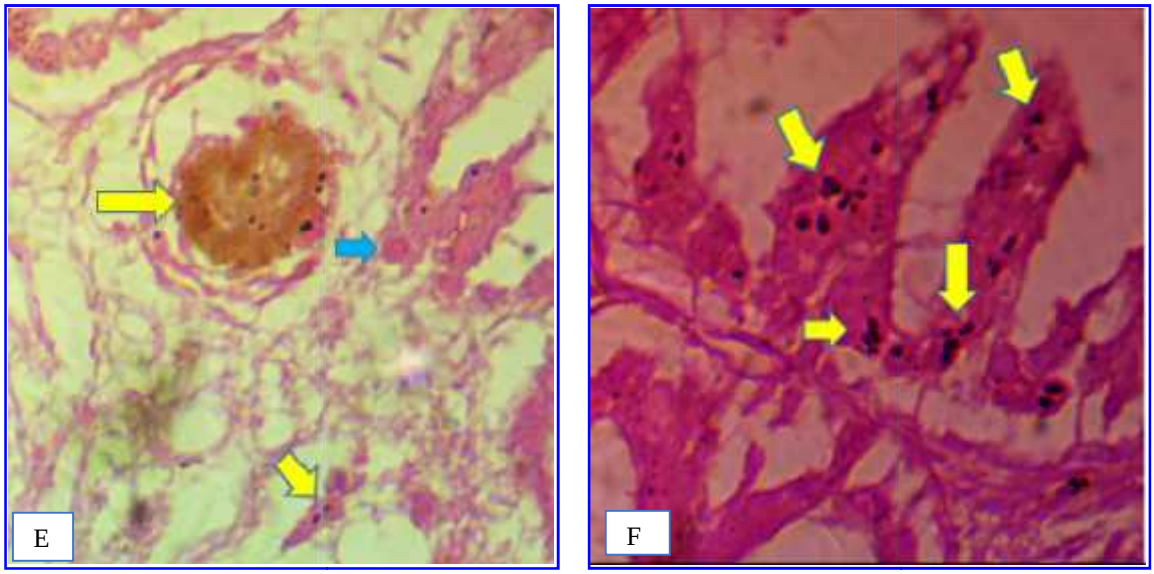


Fig. 3.3. Light microscopic observations on the spores of the microsporidian parasite recovered from *P. monodon*. **(E)** Smear preparation of infected hepatopancreas stained with haematoxylin–eosin. Yellow color arrows indicated the microsporidian parasite and light blue color arrow represented the plasmodium stage of microsporidian parasite; **(F)** Smear preparation of infected hepatopancreas stained with haematoxylin–eosin. Yellow color arrows indicated the microsporidian parasite.

3.2.1.3 Sample – 12, 15

In 16 April 2016, the sample-12 (hepatopancreas) and sample-15 (hepatopancreas) were collected from 2.5 and 3 month ages shrimp. The owner of the shrimp gher were Yeakhan and Abdullah and the area of the gher were 4 and 5 bigha. The prepared hepatopancreatic tissue slide were observed under microscope. The following figures showed the presence of microsporidian parasite.

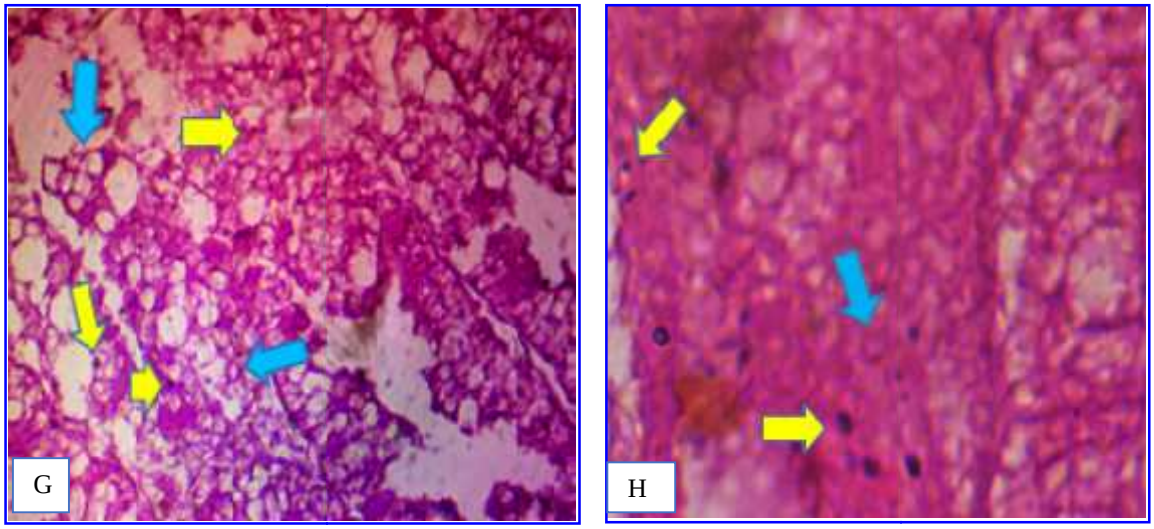


Fig. 3.4. Histological sections of hepatopancreas of *P. monodon*infectedwith microsporidian parasite. **(G)** H&E staining of hepatopancreatic tissue of the *P. monodon.* Yellow color arrows indicated the microsporidian parasite and light blue color arrows represented the plasmodium stage of microsporidian parasite; **(H)**Smear preparation of infected hepatopancreas stained with haematoxylin–eosin. Yellow color arrows indicated the microsporidian parasite and light blue color arrow represented the plasmodium stage of microsporidian parasite.

3.2.1.4 Sample – 19, 20

In 20 May 2016 and 16 June 2016, the sample-19 (hepatopancreas) and sample-20 (hepatopancreas) were collected from 3 and 3.5 month ages of shrimp. The owner of the shrimp gher were Anarul and Shawkat and the area of the gher were 6 and 18 bigha. The prepared hepatopancreatic tissue slide were observed under microscope. The following figures showed the presence of microsporidian parasite.

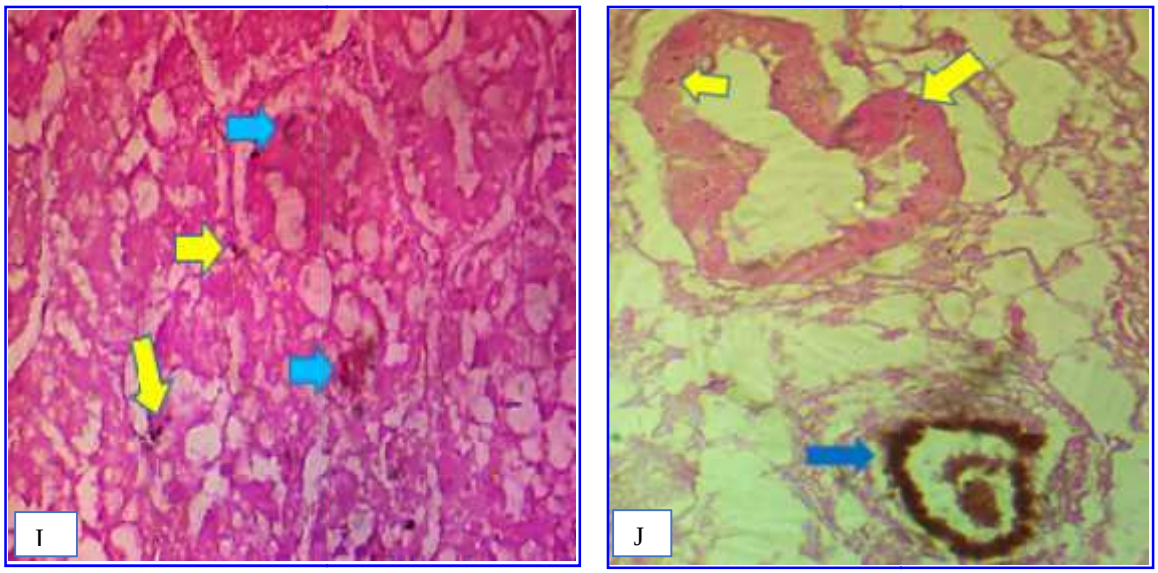


Fig. 3.5. H&E (Mayer-Bennet haematoxylin and eosin-phloxine) histology of microsporidian parasite in the infected shrimp. **(I)** H&E staining of hepatopancreatic tissue of the *Penaeus monodon.* Yellow color arrows indicated the microsporidian parasite and light blue color arrows represented the plasmodium stage of microsporidian parasite; **(J)** H&E staining of hepatopancreatic tissue of the *Penaeus monodon.* Yellow color arrows indicated the microsporidian parasite and light blue color arrow represented the plasmodium stage of microsporidian parasite.

3.2.1.5 Sample – 21, 32

In 16 June 2016 and 18 July 2016, the sample-21 (hepatopancreas) and sample-32 (hepatopancreas) were collected from 3.5 month ages of shrimp. The owner of the shrimp gher were Raju and Khairul and the area of the gher were 20 and 5 bigha. The prepared hepatopancreatic tissue slide were observed under microscope. The following figures showed the presence of microsporidian parasite.

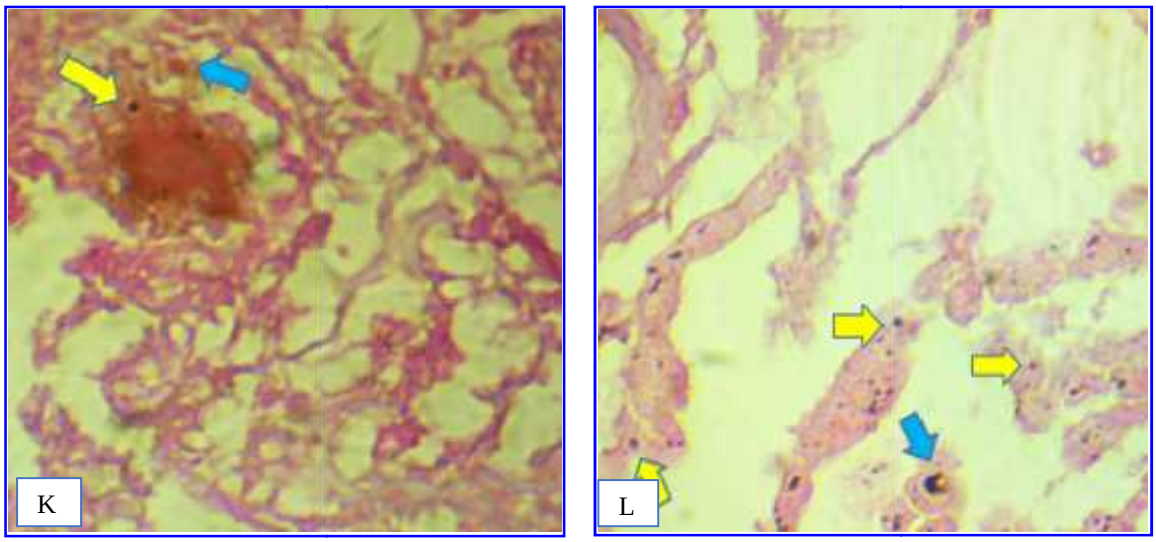


Fig. 3.6. Photographs of microsporidian parasitein the hepatopancreatic tissue of *P. monodon*. **(K)** H&E-stained smear of hepatopancreatic tissue. Yellow color arrow indicated the microsporidian parasite and light blue color arrow represented the late plasmodium stage of microsporidian parasite; **(L)**Smear preparation of infected hepatopancreas stained with haematoxylin–eosin. Yellow color arrows indicated the microsporidian parasite and light blue color arrow represented the late plasmodium stage of microsporidian parasite.

3.2.1.6 Sample – 33

In 18 July 2016, the sample-33 (hepatopancreas) was collected from 3 month ages of shrimp. The owner of the shrimp gher was Imdadul and the area of the gher was 4 bigha. The prepared hepatopancreatic tissue slide were observed under microscope. The following figure showed the presence of microsporidian parasite.

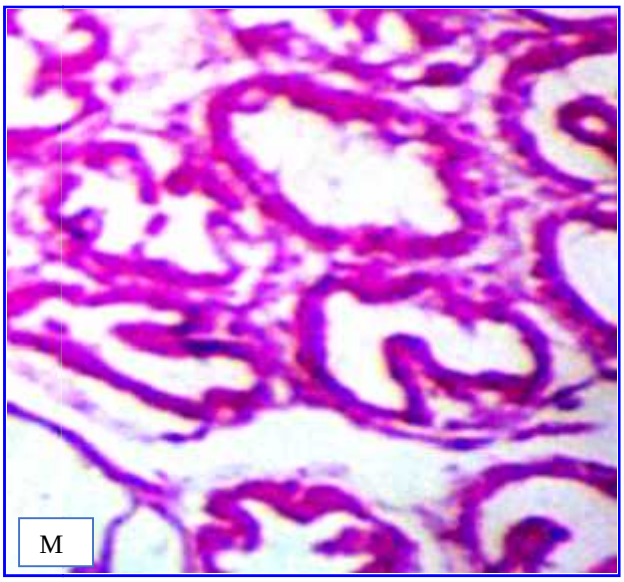


Fig. 3.7. Histological section of hepatopancreas of *P. monodon.* **(M)**The histological slide showed no sign of microsporidian parasite.

3.2.1.7 Sample – 37, 39

In 20 september 2016 and 18 november 2016, the sample-37 (hepatopancreas) and sample-39 (hepatopancreas) were collected from 3 and 3.5 month ages of shrimp. The owner of the shrimp gher were Liton and Ziad and the area of the gher were 4 and 50 bigha. The prepared hepatopancreatic tissue slide were observed under microscope. The following figures showed the presence of microsporidian parasite.

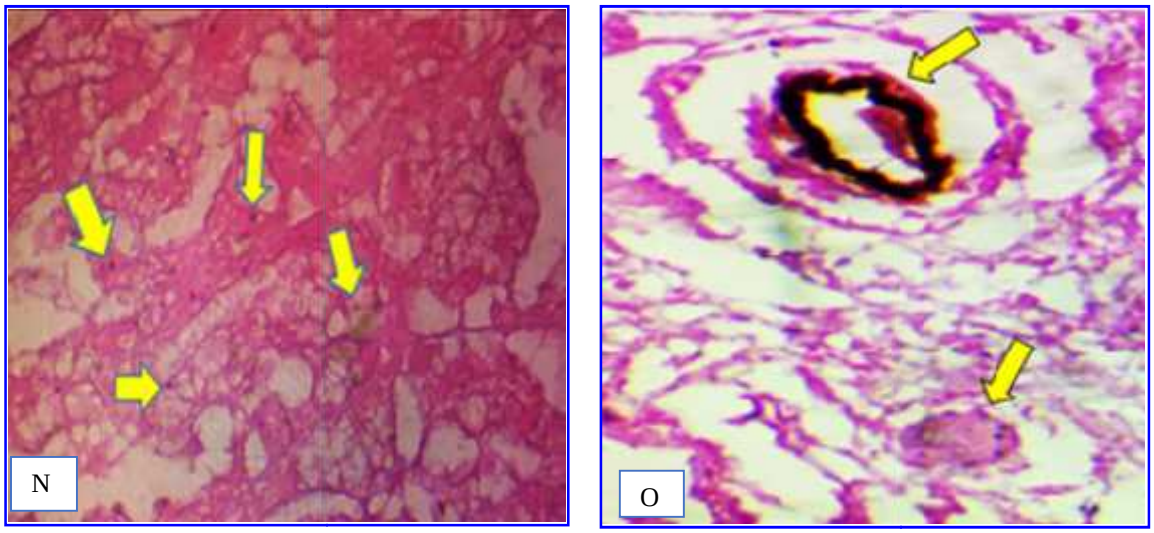


Fig. 3.8. Light microscopic observations on the spores of the microsporidian parasite

recovered from *P. monodon*. **(N)** Smear preparation of infected hepatopancreas stained with haematoxylin–eosin. Yellow color arrows indicated the microsporidian parasite; **(O)** Smear preparation of infected hepatopancreas stained with haematoxylin–eosin. Yellow color arrows represented the microsporidian parasite.

3.2.2Observation of samples of Morrelgang upazilla, Bagherhat

In 17-06-2016, the samples were collected from Morrelgang upazilla. The collected sample numbers were- 23, 24, 25, 26

From these collected samples, all samples were prepared for histological observation.

3.2.2.1 Sample – 23, 24

In 17 June 2016, the sample-23 (hepatopancreas) and sample-24 (hepatopancreas) were collected from 3 and 3.5 month ages of shrimp. The owner of the shrimp gher were Mizan and Abdul Malek and the area of the gher were 6 and 12 bigha. The prepared hepatopancreatic tissue slide were observed under microscope. The following figures were observed.

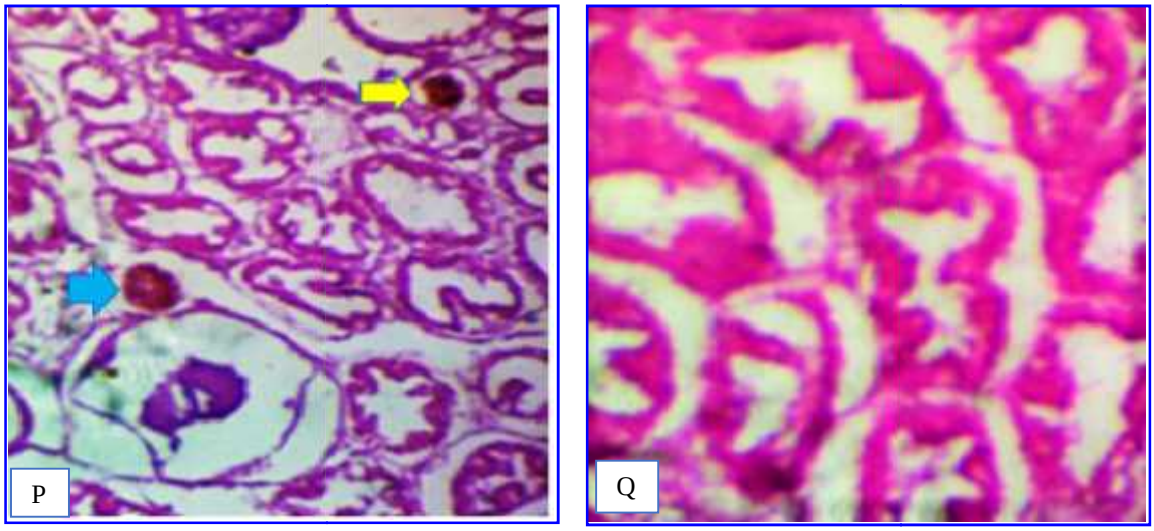


Fig. 3.9. Histological sections of hepatopancreas of *P. monodon.* **(P)**H&E-stained smear of hepatopancreatic tissue. Yellow color arrow indicated the microsporidian parasite and light blue color arrow represented the late plasmodium stage of microsporidian parasite; **(Q)** The histological slide showed no sign of microsporidian parasite.

3.2.2.2 Sample – 25, 26

In 17 June 2016, the sample-25 (hepatopancreas) and sample-26 (hepatopancreas) were collected from 3.5 and 3 month ages of shrimp. The owner of the shrimp gher were Shoharab and Tanvir and the area of the gher were 10 and 15 bigha. The prepared hepatopancreatic tissue slide were observed under microscope. The following figures were observed.

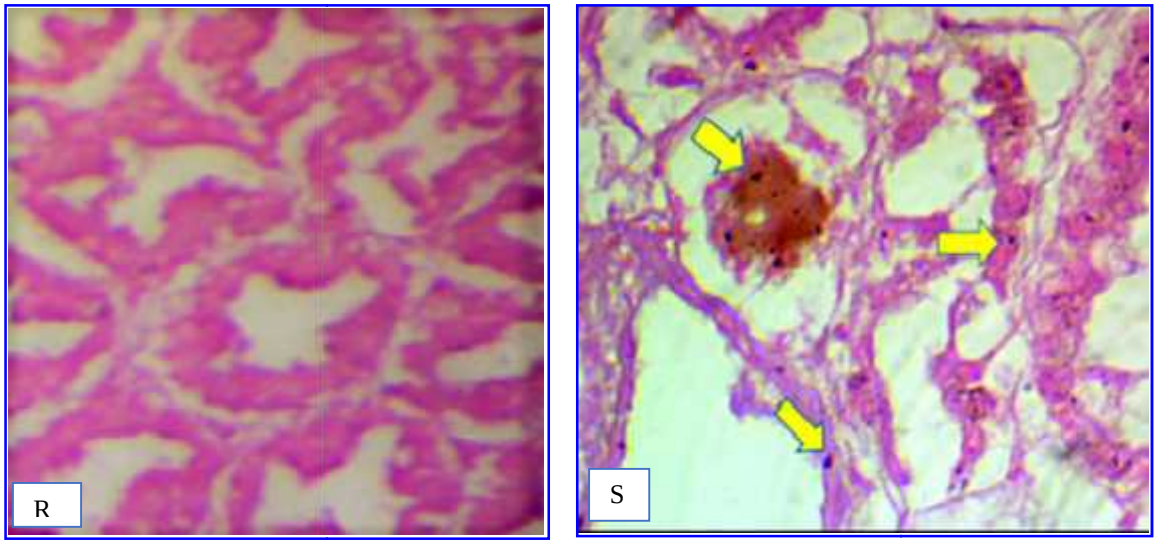


Fig. 3.10. Histological sections of hepatopancreas of *P. monodon.* **(R)** The histological slide showed no sign of microsporidian parasite; **(S)**Smear preparation of infected hepatopancreas stained with haematoxylin–eosin. Yellow color arrows represented the microsporidian parasite.

3.2.3Observation of samples of Mongla upazilla, Bagherhat

In 18-06-2016, the samples were collected from Mongla upazilla. The collected sample numbers were- 27, 28, 29, 30

From these collected samples, all samples were prepared for histological observation.

3.2.3.1 Sample – 27, 28

In 18 June 2016, the sample-27 (hepatopancreas) and sample-28 (hepatopancreas) were collected from 3 month ages of shrimp. The owner of the shrimp gher were Shahin and Ripon and the area of the gher were 7 and 6 bigha. The prepared hepatopancreatic tissue slide were observed under microscope. The following figures were observed.

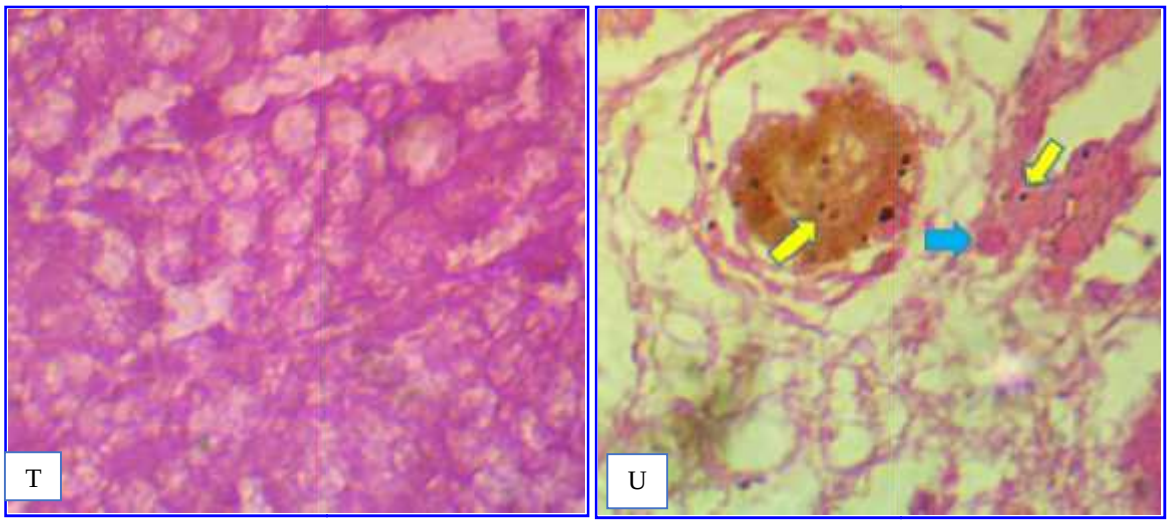


Fig. 3.11. Histological sections of hepatopancreas of *P. monodon.* **(T)** The histological slide showed no sign of microsporidian parasite; **(U)**Smear preparation of infected hepatopancreas stained with haematoxylin–eosin. Yellow color arrows represented the microsporidian parasite and light blue color arrow represented the plasmodium stage of microsporidian parasite.

3.2.3.2 Sample – 29, 30

In 18 June 2016, the sample-29 (hepatopancreas) and sample-30 (hepatopancreas) were collected from 3.5 and 3 month ages of shrimp. The owner of the shrimp gher were Nazrul and Hafizul and the area of the gher were 9 and 5 bigha. The prepared hepatopancreatic tissue slide were observed under microscope. The following figure was observed.

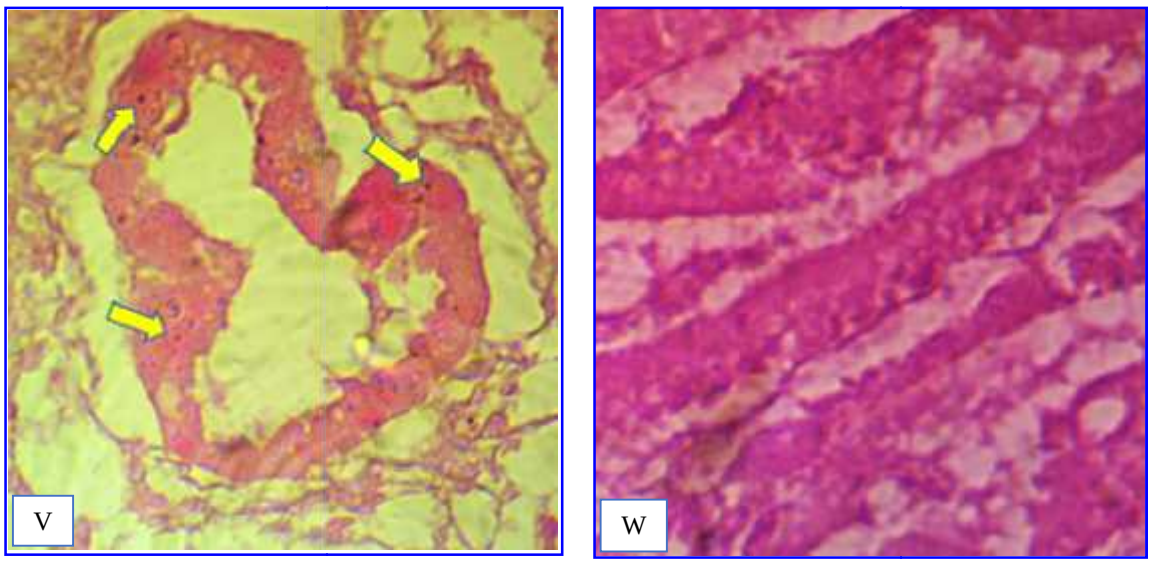


Fig. 3.12. H&E staining of hepatopancreatic tissue of the *Penaeus monodon.***(V)**Smear preparation of infected hepatopancreas stained with haematoxylin–eosin. Yellow color arrows represented the microsporidian parasite; **(W)** The histological slide showed no sign of microsporidian parasite.

3.3 Scanning Electronic Microscopy (SEM) of shrimp samples

After preparation of the slide for light microscope, 6 samples were also prepared for observing under Scanning Electronic Microscope. The samples ID were – 3, 9, 20, 23, 28 and 37.

The scanning electronic microscope was used for the detection of the position of the microsporidian parasite. The microscopic observation under scanning electronic microscope was done in the CARS (Center for Advanced Research in Science) at University of Dhaka.

3.3.1 Sample - 3, 9

In 16 April 2016, the sample-3(hepatopancreas) and sample-9 (hepatopancreas) were collected from 3 month ages of shrimp. The owner of the shrimp gher were Mosharaf Hossain and Bablu and the area of the gher were 7 and 10 bigha. The prepared hepatopancreatic tissue slide were observed under scanning electronic microscope. The following figures showed the presence of microsporidian parasite.

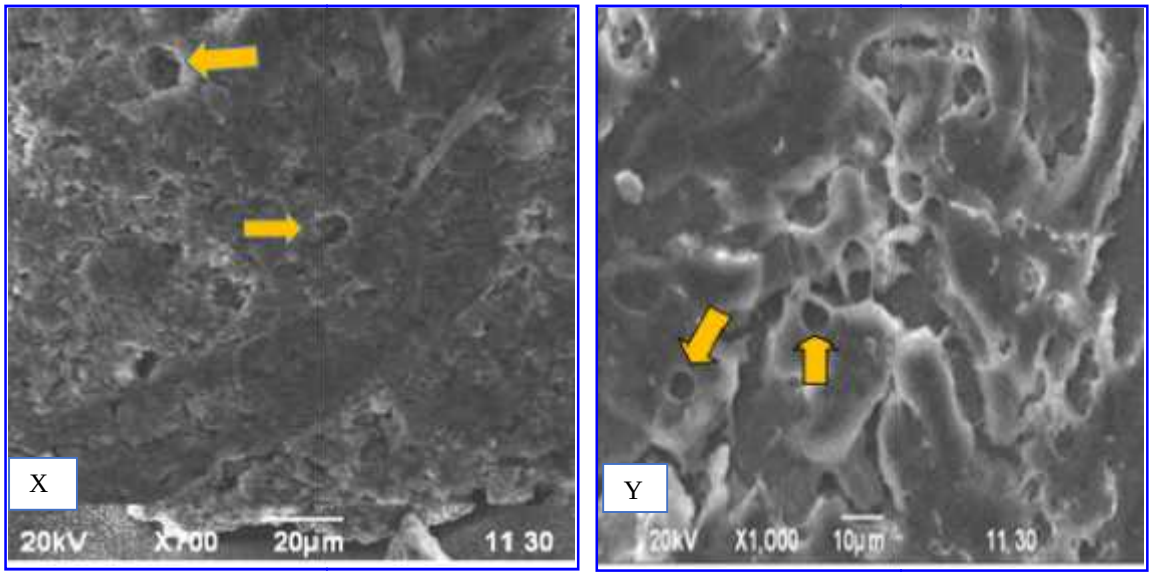


Fig. 3.13. Scanning electron microscopic (SEM) observation of spores of microsporidian parasiteinhepatopancreatic tissue of *P. monodon* **(X)** Surface of the

hepatopancreas showed the microsporidian parasite at 700 magnification; **(Y)** Surface of the hepatopancreas showed the microsporidian parasite at 1000 magnification.

3.3.2 Sample - 20, 23

In 16 June 2016 and 17 June 2016, the sample-20(hepatopancreas) and sample-23 (hepatopancreas) were collected from 3.5 and 3 month ages of shrimp. The owner of the shrimp gher were Shawkat and Mizan and the area of the gher were 18 and 6 bigha. The prepared hepatopancreatic tissue slide were observed under scanning electronic microscope. The following figures showed the presence of microsporidian parasite.

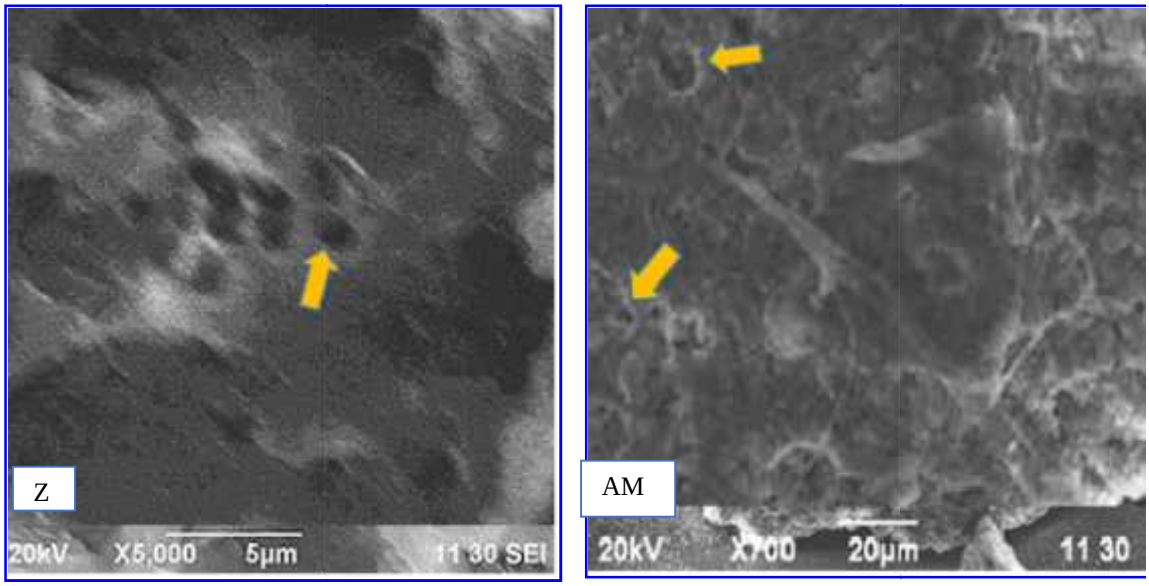


Fig. 3.14. Scanning electron microscopic observation of spores of microsporidian parasiteinhepatopancreatic tissue of *P. monodon* **(Z)** Surface of the hepatopancreas showed the microsporidian parasite at 5000 magnification; **(AM)** Surface of the hepatopancreas showed the microsporidian parasite at 700 magnification.

3.3.3 Sample - 28, 37

In 18 June 2016 and 20 september 2016, the sample-28(hepatopancreas) and sample-37 (hepatopancreas) were collected from 3 month ages of shrimp. The owner of the shrimp gher were Ripon and Liton and the area of the gher were 6 and 4 bigha. The prepared hepatopancreatic tissue slide were observed under scanning electronic microscope. The following figures showed the presence of microsporidian parasite.

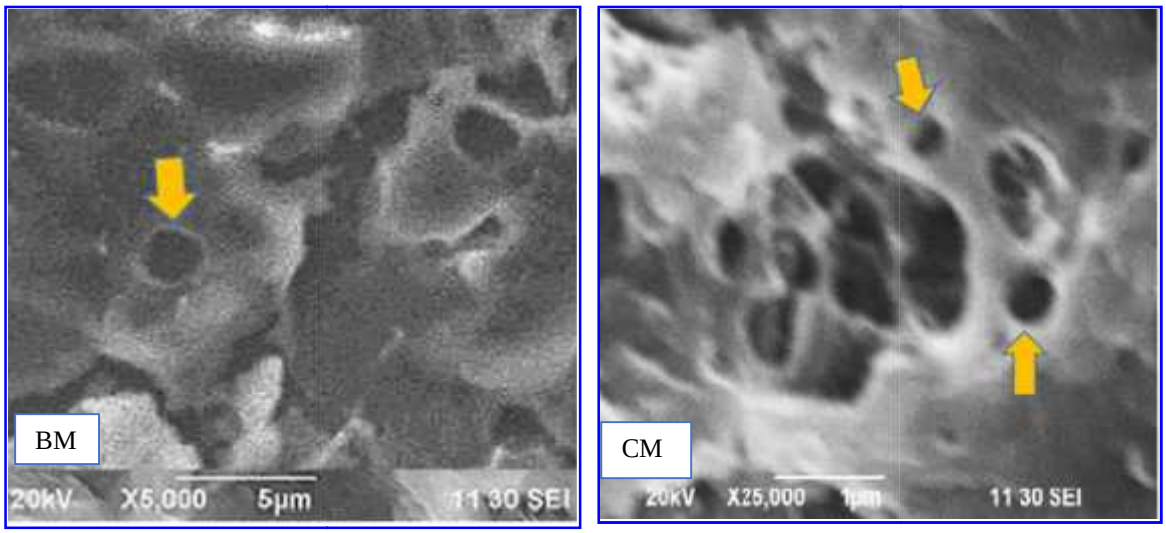


Fig 3.15. Scanning electron microscopic observation of spores of microsporidian parasiteinhepatopancreatic tissue of *P. monodon* **(BM)** Surface of the hepatopancreas showed the microsporidian parasite at 5000 magnification; **(CM)** Surface of the hepatopancreas showed the microsporidian parasite at 25000 magnification.

3.4 Molecular identification of microsporidian parasite

For molecular identification of the microsporidian parasite, SSU rRNA primer was used for ten different samples. Out of ten samples, five samples were showed positive band.

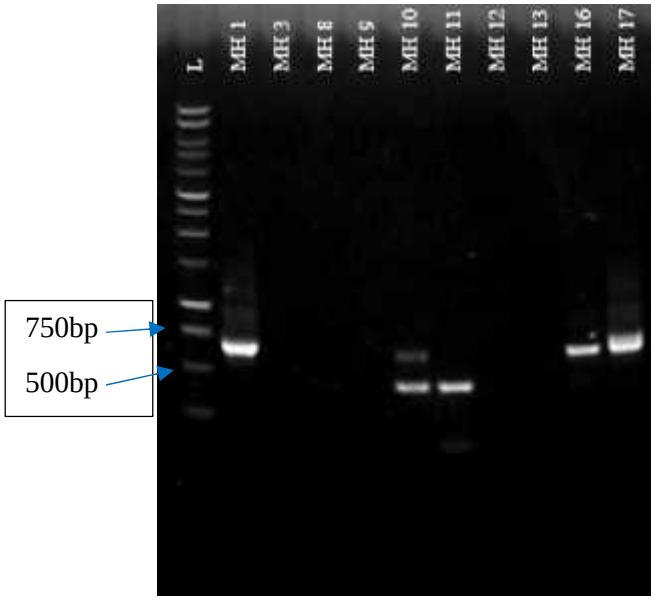


Fig. 3.16. Agarose gel (1%) electrophoresis of SSU rRNA primer generated PCR products for 10 different samples and L denotes DNA ladder of 1kb marker.

3.4.1 SSU rRNA sequence based identification

The final concentration and purity of the 5 samples measured before SSU rRNA sequencing. The identification of the SSU rRNA gene sequences of the five representative samples through nucleotide BLAST of NCBI is summarized in table 3.1. Form the table 3.1, it is clear that out of five samples, two samples (MH10 and MH11) matched with the microsporidian parasite. The table also provides information about scoring (maximum score, total score, query score, E value and percentage of identification) of the identified sequences with NCBI Gene Bank Accession number of the matched sequences.

Table: 3.1 SSU rRNA sequence (328bp) based identification of 4 representative samples.

Species ID	Identified species	Max score	Total score	Query cover	E value	Identity	Accession
MH1	*Penaeus semisulcatus* voucher KC1269 18S ribosomal RNA gene, partial sequence	832	832	100%	0.0	99%	DQ079766.1
MH10	*Paranucleospora theridion* isolate RB040612-11Pt small subunit ribosomal RNA gene, partial sequence	276	276	98%	3e-70	82%	KR187185.1
MH11	*Paranucleospora theridion* isolate RB040612-11Pt small subunit ribosomal RNA gene, partial sequence	274	274	100%	1e-69	81%	KR187185.1
MH17	*Aspergillus parasiticus* strain JAPC7 small subunit ribosomal RNA gene, partial sequence	327	327	94%	6e-86	90%	KY307854.1

3.4.2 Multiple Sequence Alignment

In figure 3.17 while compared the observed sequences of the two species MH10 and MH11 denote 58 polymorphic sites among them. Therefore, the dissimilarity was 17.7% (58/328 = 0.1768). MH10 was showed closely related to MH11.

```
                    10        20        30        40        50        60
                     |         |         |         |         |         |
HM8008472     AAAGTCCGTAGTCGTTGATGCAATTAAAAGGTGTAGATCAAGTGCACCAAGTTTGTTAGT
FJ5949761     AAAGTCCGTAGTCGTTGATGCAATTAAAAGGTGTAGATCAAGTGCACCAAGTTTGTTAGT
FJ5949711     AAAGTCCGTAGTCGTTGATGCAATTAAAAGGTGTAGATCAAGTGCACCAAGTTTGTTAGT
FJ5949871     AAAGTCCGTAGTCGTTGATGCAATTAAAAGGTGTAGATCAAGTGCACCAAGTTTGTTAGT
FJ5949881     AAAGTCCGTAGTCGTTGATGCAATTAAAAGGTGTAGATCAAGTGCACCAAGTTTGTTAGT
KR1871831     AAAGTCCGTAGTCGTTGATGCAATTAAAAGGTGTAGATCAAGTGCACCAAGTTTGTTAGT
AJ4313662     AAAGTCCGTAGTCGTTGATGCAATTAAAAGGTGTAGATCAAGTGCACCAAGTTTGTTAGT
KR1871851     AAAGTCCGTAGTCGTTGATGCAATTAAAAGGTGTAGATCAAGTGCACCAAGTTTGTTAGT
MH10xx0       AGAGTCCGTAGTTGTGGATACAATGAAAAGGTGATGATCAAGAACACTCAGTTTGTTAGT
MH11xx1       AGAGTCCGTAGTTGTGGATACAATGAAAAGGTGATGATCAAGAACACTCAGTTTGTTAGT
* ********** ** *** **** ********  *******  ***  ***********
Prim.cons.    AAAGTCCGTAGTCGTTGATGCAATTAAAAGGTGTAGATCAAGTGCACCAAGTTTGTTAGT

                   70        80        90       100       110       120
                    |         |         |         |         |         |
HM8008472     TGTAGCGGAGCGGACAGGGAGCATGGTATAGGTTGGCGAAGAATGAAATCTCAAGACCCA
FJ5949761     TGTGGCGGAGCGGACAGGGAGCATGGTATAGGTTGGCGAAGAATGAAATCTCAAGACCCA
FJ5949711     TGTGGCGGAGCGGACAGGGAGCATGGTATAGGTTGGCGAAGAATGAAATCTCAAGACCCA
FJ5949871     TGTGGCGGAGCGGACAGGGAGCATGGTATAGGTTGGCGAAGAATGAAATCTCAAGACCCA
FJ5949881     TGTGGCGGAGCGGACAGGGAGCATGGTATAGGTTGGCGAAGAATGAAATCTCAAGACCCA
KR1871831     TGTGGCGGAGCGGACAGGGAGCATGGTATAGGTTGGCGAAGAATGAAATCTCAAGACCCA
AJ4313662     TGTGGCGGAGCGGACAGGGAGCATGGTATAGGTTGGCGAAGAATGAAATCTCAAGACCCA
KR1871851     TGTGGCGGAGCGGACAGGGAGCATGGTATAGGTTGGCGAAGAATGAAATCTCAAGACCCA
MH10xx0       CGTGGCAGAGTGGACAGGGAATACAGTATAGGCTGGCGAAGAATGAAATCTCAAAACCCA
MH11xx1       CGTGGCAGAGTGGACAGGGAATACAGTATAGGCTGGCGAAGAATGAAATCTCAAAACCCA
 ** ** *** **********  *  ******* ********************** *****
Prim.cons.    TGTGGCGGAGCGGACAGGGAGCATGGTATAGGTTGGCGAAGAATGAAATCTCAAGACCCA

                  130       140       150       160       170       180
                    |         |         |         |         |         |
HM8008472     ACCTGGACCAACTGAGGCGAAAGCGGTGCTCTTGTACGCGTCTGTGGATCAAGGACGAAG
FJ5949761     ACCTGGACCAACTGAGGCGAAAGCGGTGCTCTTGTACGCGTCTGTGGATCAAGGACGAAG
FJ5949711     ACCTGGACCAACTGAGGCGAAAGCGGTGCTCTTGTACGCGTCTGTGGATCAAGGACGAAG
FJ5949871     ACCTGGACCAACTGAGGCGAAAGCGGTGCTCTTGTACGCGTCTGTGGATCAAGGACGAAG
FJ5949881     ACCTGGACCAACTGAGGCGAAAGCGGTGCTCTTGTACGCGTCTGTGGATCAAGGACGAAG
KR1871831     ACCTGGACCAACTGAGGCGAAAGCGGTGCTCTTGTACGCGTCTGTGGATCAAGGACGAAG
AJ4313662     ACCTGGACCAACTGAGGCGAAAGCGGTGCTCTTGTACGCGTCTGTGGATCAAGGACGAAG
KR1871851     ACCTGGACCAACTGAGGCGAAAGCGGTGCTCTTGTACGCGTCTGTGGATCAAGGACGAAG
MH10xx0       GCTTGGACTAACGAAGGCGAAGGCAGTATTCTTGTACAGATCTGAGGATCAAGGACGAAG
MH11xx1       GCTTGGACTAACGAAGGCGAAGGCAGTATTCTTGTACAGATCTGAGGATCAAGGACGAAG
 * ***** ***  ******* ** **  ********   **** ***************
Prim.cons.    ACCTGGACCAACTGAGGCGAAAGCGGTGCTCTTGTACGCGTCTGTGGATCAAGGACGAAG
```

```
                 190       200       210       220       230       240
                    |         |         |         |         |         |
HM8008472    GCTAGAGGATCGAAAGTGATTAGACACCGCTGTAGTTCTAGCAGTAAACTATGCCGACAT
FJ5949761    GCTAGAGGATCGAAAGTGATTAGACACCGCTGTAGTTCTAGCAGTAAACTATGCCGACAT
FJ5949711    GCTAGAGGATCGAAAGTGATTAGACACCGCTGTAGTTCTAGCAGTAAACTATGCCGACAT
FJ5949871    GCTAGAGGATCGAAAGTGATTAGACACCGCTGTAGTTCTAGCAGTAAACTATGCCGACAT
FJ5949881    GCTAGAGGATCGAAAGTGATTAGACACCGCTGTAGTTCTAGCAGTAAACTATGCCGACAT
KR1871831    GCTAGAGGATCGAAAGTGATTAGACACCGCTGTAGTTCTAGCAGTAAACTATGCCGACAT
AJ4313662    GCTAGAGGATCGAAAGTGATTAGACACCGCTGTAGTTCTAGCAGTAAACTATGCCGACAT
KR1871851    GCTAGAGGATCGAAAGTGATTAGACACCGCTGTAGTTCTAGCAGTAAACTATGCCGACAT
MH10xx0      GCTAGAGGATCGAAGGTGATTAGAGACCACTGTAGTTCTAGCAGTAAACTATGCCAACAT
MH11xx1      GCTAGAGGATCGAAGGTGATTAGAGACCACTGTAGTTCTAGCAGTAAACTATGCCAACAT
************** ********* *** ************************** ****
Prim.cons.   GCTAGAGGATCGAAAGTGATTAGACACCGCTGTAGTTCTAGCAGTAAACTATGCCGACAT

                    250       260       270       280       290       300
                      |         |         |         |         |         |
HM8008472    TCTCTGTTGTTATGACGGGGAGGGGAGAAATCTTAGTTTTCGGGCTCTGGGGATAGTACG
FJ5949761    TCTCTGTTGTTGAGACGGGGAGGGGAGAAATCTTAGTTTTCGGGCTCTGGGGATAGTACG
FJ5949711    TCTCTGTTGTTGAGACGGGGAGGGGAGAAATCTTAGTTTTCGGGCTCTGGGGATAGTACG
FJ5949871    TCTCTGTTGTTGAGACGGGGAGGGGAGAAATCTTAGTTTTCGGGCTCTGGGGATAGTACG
FJ5949881    TCTCTGTTGTTGAGACGGGGAGGGGAGAAATCTTAGTTTTCGGGCTCTGGGGATAGTACG
KR1871831    TCTCTGTTGTTGAGACGGGGAGGGGAGAAATCTTAGTTTTCGGGCTCTGGGGATAGTACG
AJ4313662    TCTCTGTTGTTGAGACGGGGAGGGGAGAAATCTTAGTTTTCGGGCTCTGGGGATAGTACG
KR1871851    TCTCTGTTGTTGAGACGGGGAGGGGAGAAATCTTAGTTTTCGGGCTCTGGGGATAGTACG
MH10xx0      TCTCTATCATTGAGATGGGGAAGGGAGAAATCTTAGTTTTTGGGCCACGGGGATAGTACG
MH11xx1      TCTCTATCAGTGAGATGGGGAAGGGAGAAATCTTAGTTTTTGGGCCACGGGGATAGTACG
***** *   *  ** ***** ****************** ****   ************
Prim.cons.   TCTCTGTTGTTGAGACGGGGAGGGGAGAAATCTTAGTTTTCGGGCTCTGGGGATAGTACG

                    310       320
                      |         |
HM8008472    CTTGCAAGAGTGAAACTTAAAGCGAAAT
FJ5949761    CTTGCAAGAGTGAAACTTAAAGCGAAAT
FJ5949711    CTTGCAAGAGTGAAACTTAAAGCGAAAT
FJ5949871    CTTGCAAGAGTGAAACTTAAAGCGAAAT
FJ5949881    CTTGCAAGAGTGAAACTTAAAGCGAAAT
KR1871831    CTTGCAAGAGTGAAACTTAAAGCGAAAT
AJ4313662    CTTGCAAGAGTGAAACTTAAAGCGAAAT
KR1871851    CTTGCAAGAGTGAAACTTAAAGCGAAAT
MH10xx0      TTCGCAAGGAAGAAACTTAAACTGAGAT
MH11xx1      TTCGCAAGGAAGAAACTTAAACTGAGAT
 * *****   **********  ** **
Prim.cons.   CTTGCAAGAGTGAAACTTAAAGCGAAAT
```

Fig 3.17. Multiple sequence alignment of SSU rRNA gene fragments of the closely related two species where black among the red indicates polymorphic sites. (CLUSTALW view, alignment width 60 bases).

3.4.3 PairwiseSequence Alignment

In figure 3.18 while compared the observed sequences of the two species MH10 and MH11 denote 1 polymorphic sites among them. Therefore, the dissimilarity was 0.30% (1/328 = 0.0030).

```
                    10         20         30         40         50         60
                     |          |          |          |          |          |
MH10xx0      AGAGTCCGTAGTTGTGGATACAATGAAAAGGTGATGATCAAGAACACTCAGTTTGTTAGT
MH11xx1      AGAGTCCGTAGTTGTGGATACAATGAAAAGGTGATGATCAAGAACACTCAGTTTGTTAGT
************************************************************
Prim.cons.   AGAGTCCGTAGTTGTGGATACAATGAAAAGGTGATGATCAAGAACACTCAGTTTGTTAGT

                    70         80         90        100        110        120
                     |          |          |          |          |          |
MH10xx0      CGTGGCAGAGTGGACAGGGAATACAGTATAGGCTGGCGAAGAATGAAATCTCAAAACCCA
MH11xx1      CGTGGCAGAGTGGACAGGGAATACAGTATAGGCTGGCGAAGAATGAAATCTCAAAACCCA
************************************************************
Prim.cons.   CGTGGCAGAGTGGACAGGGAATACAGTATAGGCTGGCGAAGAATGAAATCTCAAAACCCA

                   130        140        150        160        170        180
                     |          |          |          |          |          |
MH10xx0      GCTTGGACTAACGAAGGCGAAGGCAGTATTCTTGTACAGATCTGAGGATCAAGGACGAAG
MH11xx1      GCTTGGACTAACGAAGGCGAAGGCAGTATTCTTGTACAGATCTGAGGATCAAGGACGAAG
************************************************************
Prim.cons.   GCTTGGACTAACGAAGGCGAAGGCAGTATTCTTGTACAGATCTGAGGATCAAGGACGAAG

                   190        200        210        220        230        240
                     |          |          |          |          |          |
MH10xx0      GCTAGAGGATCGAAGGTGATTAGAGACCACTGTAGTTCTAGCAGTAAACTATGCCAACAT
MH11xx1      GCTAGAGGATCGAAGGTGATTAGAGACCACTGTAGTTCTAGCAGTAAACTATGCCAACAT
************************************************************
Prim.cons.   GCTAGAGGATCGAAGGTGATTAGAGACCACTGTAGTTCTAGCAGTAAACTATGCCAACAT

                   250        260        270        280        290        300
                     |          |          |          |          |          |
MH10xx0      TCTCTATCATTGAGATGGGGAAGGGAGAAATCTTAGTTTTTGGGCCACGGGGATAGTACG
MH11xx1      TCTCTATCAGTGAGATGGGGAAGGGAGAAATCTTAGTTTTTGGGCCACGGGGATAGTACG
********* **************************************************
Prim.cons.   TCTCTATCA2TGAGATGGGGAAGGGAGAAATCTTAGTTTTTGGGCCACGGGGATAGTACG

                   310        320
                     |          |
MH10xx0      TTCGCAAGGAAGAAACTTAAACTGAGAT
MH11xx1      TTCGCAAGGAAGAAACTTAAACTGAGAT
****************************
Prim.cons.   TTCGCAAGGAAGAAACTTAAACTGAGAT
```

Fig. 3.18. Pairwise sequence alignment of SSU rRNA gene fragments of the MH10 and MH11 where black among the red indicates polymorphic sites. (CLUSTALW view, alignment width 60 bases).

3.4.4 Phylogenetic analysis

Phylogenetic analysis (Figure 3.19) based on the partial SSU rRNA gene sequences of the representative two samples using neighbor-joining confirming the taxonomic position

of the MH10 and MH11. From the tree it is also clear that MH10 and MH11 is closely related with the microsporidian parasite *Paranucleospora theridion.*

Phylogeny Test

Test of Phylogeny: Bootstrap method

No. of Bootstrap Replications: 1000

Substitutions Type: Nucleotide

Model/Method: Maximum Likelihood method

Rates among sites: Uniform rates

ML Heuristic Method: Nearest-neighbor-Interchange (NNI)

No. of sites: 328

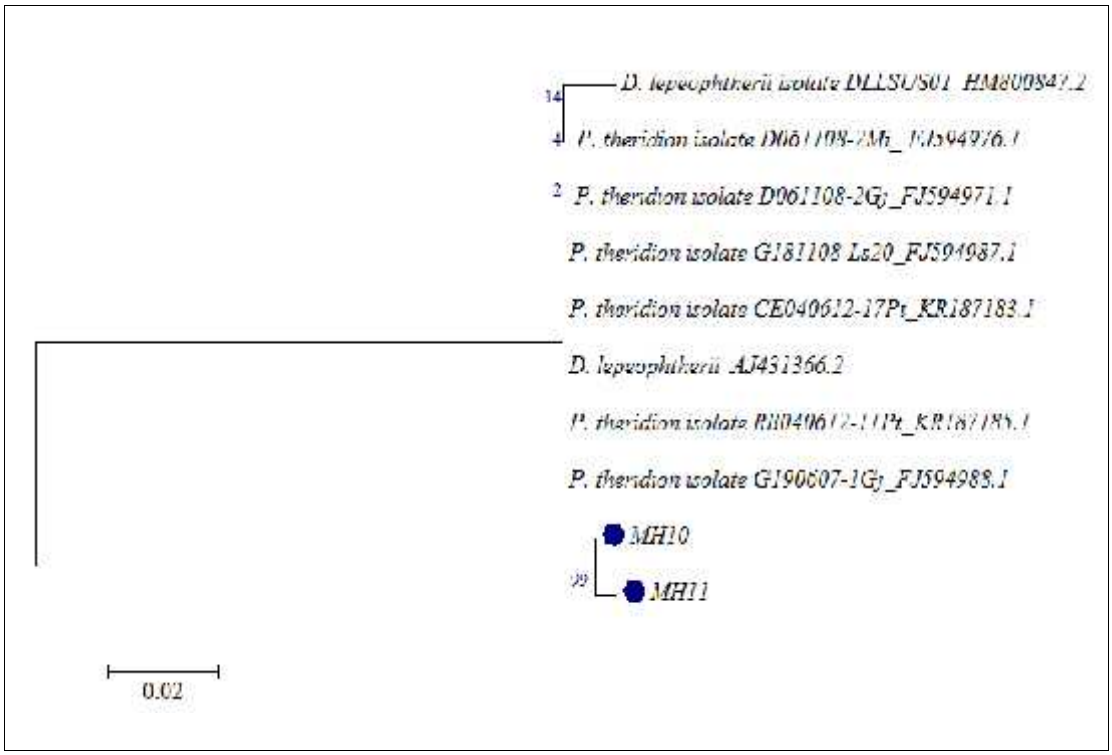


Fig. 3.19.Molecular Phylogenetic analysis by Maximum Likelihood method. The Neighbor-Joining phylogenetic tree based on partial SSU rRNA gene sequences. The evolutionary distances were computed using the Maximum Composite Likelihood method. Numbers in tree are bootstrap values. Blue circle indicates position of the studied species.

3.4.5 Identification of the *Enterocytozoon hepatopenaei* by EHP-510 primer

The same samples were used for the detection of the EHP that were used for SSU rRNA primer. But no samples showed the positive results for EHP.

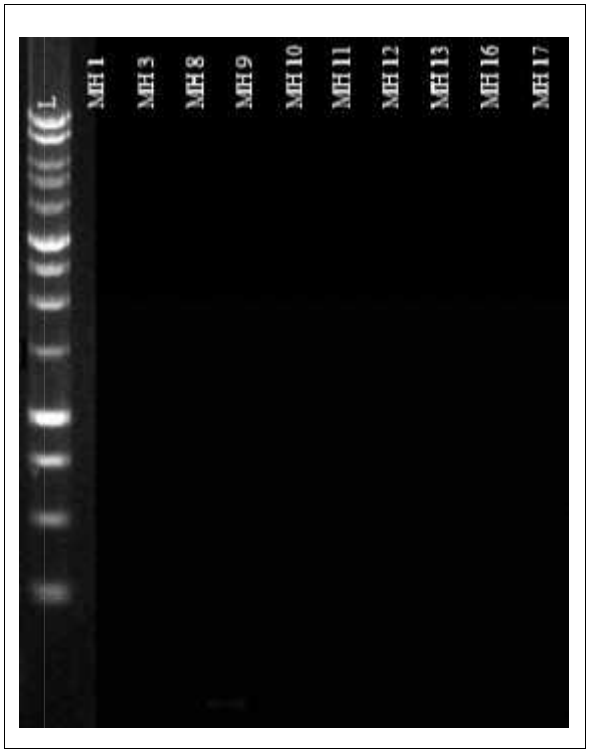


Fig. 3.20. Agarose gel (1%) electrophoresis of EHP-510 primer generated for 10 different samples and L denotes DNA ladder of 1kb marker.

Table: 3.2 Overview of the results for the identification of the microsporidian parasite in cultured shrimp samples collected from South-west farming region of Bangladesh

Sample ID	Sampling Area	Area of the gher(bigha)	Dissection of organ	Histology		Molecular Diagnosis	
				Light microscopy	SEM	SSU rRNA	EHP-510
1	Satkhira sadar	7	Stomach	√ (+)			
2	"	7	Muscle				
3	"	7	Hepatopancreas	√ (+)	√ (+)	√ (+)	√ (-)
4	"	3	Stomach				
5	"	3	Muscle				
6	"	3	Hepatopancreas	√ (+)			
7	"	10	Stomach				
8	"	10	Muscle				
9	"	10	Hepatopancreas	√ (+)	√ (+)	√ (-)	√ (-)
10	"	4	Stomach				
11	"	4	Muscle				
12	"	4	Hepatopancreas	√ (+)			
13	"	5	Stomach				
14	"	5	Muscle				
15	"	5	Hepatopancreas	√ (+)		√ (-)	√ (-)
16	"	4	"				
17	"	7	"				
18	"	50	"				
19	"	6	"	√ (+)			

20	"	18	"	√ (+)	√ (+)	√ (+)	√ (-)
21	"	20	"	√ (+)			
22	"	8	"				
23	Morrelgang,Bagherhat	6	"	√ (+)	√ (+)	√ (-)	√ (-)
24	"	12	"	√ (-)			
25	"	10	"	√ (-)			
26	"	15	"	√ (+)		√ (-)	√ (-)
27	Mongla,Bagherhat	7	"	√ (-)			
28	"	6	"	√ (+)	√ (+)	√ (+)	√ (-)
29	"	9	"	√ (+)			
30	"	5	"	√ (-)			
31	Satkhira sadar	7	"				
32	"	5	"	√ (+)		√ (+)	√ (-)
33	"	4	"	√ (-)			
34	"	3	"				
35	"	25	"				
36	"	8	"				
37	"	4	"	√ (+)	√ (+)	√ (-)	√ (-)
38	"	7	"				
39	"	50	"	√ (+)		√ (+)	√ (-)
40	"	18	"				

Chapter4

Discussion

Microsporidian parasites are a dangerous form of pathogen found around the world. These are spore forming unicellular parasites that can affect aquaculture severely.The phylum Microsporidia consists of obligate intracellular parasites thought to be derived from the Fungi (Edling et al., 1996; James, 2006). They are known to parasitise most of the major phyla of invertebrates and five classes of vertebrates (Canning and Vavra, 2000). The higher level taxonomy of the phylum, largely based upon morphological characteristics of parasite life stages has been increasingly challenged by the utilisation of nucleic acid-based approaches to phylogeny.

Microsporidia are important parasites of invertebrates.Many microsporidian species are transmitted horizontally to new hosts, others are transmitted vertically (i.e., female-to-offspring transmission) or by a combination of the two modes. Such variation in the mode of transmission affects the evolution of virulence, which is generally reduced in vertically transmitted microsporidia (Dunn and Smith, 2001).

The most common symptoms among microsporidia affected shrimp are discoloration in muscles due to spores. Of the 187 genera described to date (Vavra and Lukes, 2013) almost half are known to infect aquatic organisms. Excluding genera which infect aquatic life stages of insects, approximately 20 genera infect fish, 50 genera infect aquatic arthropods, and at least 21 genera infect aquatic non-arthropod invertebrates, protists, and hyper parasites of aquatic hosts. Three genera of Microsporidia (*Agmasoma*, *Pleistophora* and *Ameson*) reported in penaeid shrimp(Anderson et al. 1989; Lightner 1996) only *Agmasomapenaei* is fairly common in wild Thai shrimp, producingopaque white patches in infected shrimp tissuesleading to the common names ‘white back shrimp’,‘milk shrimp’, ‘cotton shrimp’ or ‘roe shrimp’ (Flegel et al. 1992; Pasharawipas et al., 1994).Very recently, new microsporidian parasites were identified such as *E. hepatopenaei,E. bieneusi, Nosema,Paranucleospora theridion* etc. from cultured shrimp that affect the growth of shrimp.

Historic ability to detect microsporidian infections based upon external clinical signs has been augmented by application of pathological and molecular diagnostic approaches, allowing for detection of cryptic and subclinical infections (Jones et al., 2012).

Histological examination for shrimpmicrosporidian parasite on the tissuedistribution in naturally and experimentally microsporidiosis-infected revealed the presenceof large number of plasmodia in hepatopancreas,very few in gut epithelial cells and none in otherorgans. The hepatopancreas ofshrimp is supposed to be homologous to liver andpancreas of vertebrates and is responsible formetabolic activities of the shrimp body.

Our results agree with the report of Tangprasittipapet al., (2013) who described the occurrenceof microsporidian parasite in the hepatopancreaswhich is only organ which reacted to microsporidian probe.

Histological examination also revealed the presenceof plasmodium within a vacuole as observed byTourtip et al., (2009) in the hepatopancreatic tubular epithelial cells of microsporidian infected *P. monodon*.Development of plasmodium within a vacuole isone of the important characteristics of microsporidian parasite *E. hepatopenaei*,whereas several microsporidians do not possessvacuole and develop directly in the cytoplasmof host cells, and some microsporidians such as*Encephalitozoon* spp., *Glugoides intestinalis*and *Paranucleospora theridion*possessa parasitophorous vacuole at early stage ofinfection in the host cytoplasm for protection (Cali and Kolter 1993; Cali and Takvorian 1999; Vavra and Larsson 1999).

Shrimp samples were collectedfrom shrimp farms located at Satkhira district (Satkhira sadar upazilla) and Bagherhat district (Morrelgang and Mongla upazilla). The localization of plasmodium and morphology of the spores observed inthe present study were similar to the microsporidian parasite *Paranucleospora theridion.*

According to the Fish Site,microsporidian parasite *Enterocytozoon hepatopenaei* (EHP) is different. It only infects the tubules of the hepatopancreas in shrimp, which damages the ability of this critical organ to gain nutrition from feed.From Indonesia, high mortality of juvenile *P. vannamei* in hatcheries and juvenile shrimp less than one month old in grow out ponds has occurred in association with hapolosproidian infections of the hepatopancreas. Prevalence in affected ponds was as high as 30% at around one month after stocking and decreased thereafter to less than 5% at harvest due to progressive

mortality (overall mortality 60% to 90%). The losses have been estimated at approximately US$5 million. The gross signs of disease and histopathology caused by the parasite resembled those previously described from the Americas (Nunan et al., 2007), and sequences of PCR-amplified rRNA sequences shared 96% identity with those form the American report. Given the 4% difference in sequence identity, it is uncertain whether the parasite is a variant of the previously described American species or a new species. Nor is it certain whether the infections originated from imported shrimp stocks or by transmission from local carriers. The presenceof *E. hepatopenaei* in the hepatopancreascauses damage to the hepatopancreatic tissue andmay result in elevating level of AST and ALT inthe haemolymph of *E. hepatopenaei*-infectedshrimp. Similar observation was also made bymany workers in shrimp and fish under conditionsof shrimp. High level of AST and ALT wasobserved in the blood of fish and haemolymph ofshrimp exposed to ammonia (Jeney et al. 1992; Vedel et al., 1998; Pan et al. 2003; Fouzi et al.2012). Battison (2006) observed elevated level ofAST and ALT in Aerococcus viridans-infected lobsters,and this elevation was due to tissue damagein hepatopancreatic connective tissue caused bythe pathogen.

Clinical signs specific to*E. hepatopenaei* infection were not observed inshrimp except stunted growth as observed by previousworkers (Tourtip et al., 2009; Tangprasittipapet al., 2013; Tang et al., 2015; Rajendranet al. 2016). Ha et al., (2010) reported the associationof the white faeces disease with onemicrosporidian parasite as observed by Flegel(2012) in farm-reared *L. vannamei* in Vietnamand Thailand.Hence, the specific clinical sign was stunted growth which furtherconfirmed by histology and PCR for EHP infection,but not with the white faeces syndrome. In these present study, we also tried to identify the *E. hepatopenaei*(EHP) by EHP specific primer. But none have showed positive results.

Ding et al. (2016) reported a microsporidianparasite localizing the cytoplasm of the epithelialcells of hepatopancreas of Chinese mitten crab,*Eriocheir sinensis*, as observed in the present study and sequence analysis of SSU rRNA revealed that the parasite had 83% sequence identityto that of*Paranucleospora theridion*. An intracytoplasmicmicrosporidian parasite with spore size of1.9 × 1.4 µm was observed in hepatopancreas of*P. monodon*, but a detail study on taxonomy wasnot carried out (Anderson et al., 1989).

Light microscopic observation on white faecal matter revealed thatthe samples collected from different farms contained large number of microsporidian spores. Association of microsporidian with white faeces syndrome (WFS) had been reported previously Ha et al. (2010), and Flegel (2012) has indicated that severe infection with a microsporidian morphologically similar to *E. hepatopenaei*was associated with WFS of *P. vannamei*. On the contrary, later, Tangprasittipap et al. (2013) reported that EHP is not the cause of WFS and, further, according to Sriurairatana et al. (2014) the syndrome arises due to transformation, sloughing and aggregation of hepatopancreatic microvilli into vermiform bodies. Interestingly, recently, Vibrio bacteria and six species of fungi have been isolated fromshrimp naturally infected with white faeces syndrome (Chaweepack et al., 2015).Although the cause of the aggregated, transformed microvilli (ATM) associated with the WFS is yet to be identified, it has been reported that increase in the prevalence of ATM coincided with the increased prevalence of acute hepatopancreatic necrosis disease (AHPND) and EHP (Sriurairatana et al., 2014). As per the available information, investigationon WFS in India revealed no association with Vibrio or fungi andthe major pathogen recovered from the shrimp farms has been only EHP.

Microsporidian parasites of penaeid shrimp have been reported from numerous biogeographic regions. *Agmasoma penaei* appears to be a parasite of *Penaeus monodon* and *Fenneropenaeus merguiensis* (also known as *P. merguiensis*) from farms in Thailand (Flegel et al. 1992), and *P. notialis* and *P. monodon* in Senegal (Clotilde-Ba and Toguebaye 1994, 2000). Microsporidians of the genus *Thelohania* are parasites of *F. merguiensis* in tropical Australia (Owens and Hall-Mendelin 1990), of *Pandalus jordani* in the United States (Olson and Lannan 1984), and of *P. semisulcatus* in Mandapam in India (Thomas 1976). The genus *Pleistophora* has been reported as parasitising *Pandalus jordani* in the United States (Olson and Lannan 1984), *Litopenaeus stylirostris* (also known as *Penaeus stylirostris*) in Mexico (Alarcon-Gonzales 1990), and *Crangon franciscorum, C. nigricauda* and *C. stylirostris* in Yaquina Bay in Oregon (Breed and Olson 1977). Other species of microsporidians have been observed: *Ameson nelsoni* parasitising *Parapenaeus longirostris* in the Mediterranean (Campillo and Comps, 1977),*Inodosporus* sp. in *Palaemon serratus* (Decapode) along the Atlantic coast of France (Azevedo, 1991), and other non-identified microsporidian forms parasitic on *F. indicus* from the south of India (Ramasamy and Pandian 1985), *Pandalus borealis* along the coast of Labrador (Parsons and Khan, 1986) and *P. monodon* in Malaysia (Anderson

et al. 1989). A new species, *Tuzetia weidneri*, has also been described as a parasite on *Litopenaeus setiferus* and *Farfantepenaeus aztecus* (Canning et al. 2002). Fresh *Agmasoma penaei* (Sprague, 1950) spores from *Penaeus monodon* in Senegal (Clotilde-Ba and Toguebaye 2000) were described as measuring 3.9 × 2.1 µm, which is larger than the spores we found in *P. monodon* along the west coast of Madagascar.

However, we could not compare our results with those in other studies because they used fresh samples, whereas all samples examined for the present study were frozen. The fact that frozen samples could be the reason of the spores did not follow a normal distribution. However, a comparative study of results using standard SEM could be used to correct for artefact size differences. Image quality could also be improved by decreasing pressure up to the charging-effect limit.

Microsporidians can seriously impact fish farms, such as those events which have occurred with seabream in farms on the Languedoc coast of France (Mathieu-Daude et al. 1992). The microsporidians in shrimp from the South west region of Bangladesh, therefore require further study of their taxonomy, epizootiology and distribution, to accurately assess their potential impact on both wild penaeid populations, as well as the pathogenic risk to human health.

The morphology of the microsporidian spores observed in penaeid shrimp along the west coast of Madagascar (Toubiana et al., 2004). In this study, we observed morphological dissimilarities among the parasites. For identifying these reasons, molecular approaches were used.

The first report has published on ultrastructural features and a partialSSU rRNA gene sequence of a microsporidian exclusively infecting hepatopancreatic tubule epithelial cells of the black tiger shrimp *P. monodon* (Tourtip et al., 2009). Its unique ultrastructural features linked it to the family Enterocytozoonidae. Based on its distinction from the microsporidian genera *Nucleospora* and *Enterospora*in the family and on its similarity to the single species in the genus *Enterocytozoon*,Moreover,the ultrastructural examination of the sporesrevealed the presence of posterior vacuole, 5–6coils of polar filaments arranged in two rows andnature of wall of spore in the present study asobserved by previous workers (Wasson and Peper2000; Visvesvara 2002; Didier 2005; Tourtip et al., 2009). It is widely understood that EHP does not cause mortality but heavily limits growth.

Later, the occurrence of thisparasite was reported in pond-reared *L. vannamei*in Vietnam, China, Indonesia and Malaysia, and*P. stylirostris* in Brunei (Ha et al., 2010; Tang et al., 2015). The disease outbreak due to microsporidian parasite *E. hepatopenaei*spread to India also, and its occurrencewas reported in farm-reared *L. vannamei* (Rajendran et al., 2016). Unlike phenotypic identification, which can be affected by the presence or absence of non-housekeeping genes or by variability in expression of characters, SSU rRNA sequencing provides accurate identification of parasites with atypical phnotypic characteristics.

The reasons behind the use of SSU rRNA in parasite identification in this study are numerous. The gene has a suitable length of about 1200 bp which is large enough, with sufficient interspecific polymorphisms to provide distinguishing and statistically valid measurements. Besides, SSU rRNA gene is universally distributed in all parasite, thus relationships can be measured among them. The whole sequence of SSU rRNA is highly conserved (greengenes.lbl.gov) and functionally constant through evolutionary history.

Multiple sequence alignment has been done for the microsporidian parasite*Paranucleospora theridion* (MH10 and MH11) as they are more closely related to each other than they are with the other eight species. This approach has been used to identify the polymorphic sites in the base pair of the subjected species. The position of bacteria in the alignment represents their similarity that´s why two species clustered first and then the microsporidian parasite earn their places. However, this positioning is based on the amount of polymorphic sites in the species.

The constructed phylogenetic tree, involved a total of 10 (2 of our samples + 8 downloaded from NCBI GenBank) nucleotide sequences, supports the output of the multiple sequence alignment. To robust the positioning of species and to ascertain about their taxonomic position 7 more related reference species were downloaded from NCBI GenBank.We have found that the parasite shares 83% SSU rRNA gene sequence identity with that of *Paranucleospora theridion* isolates at GenBank (FJ594987.1)

The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.21208716 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The

tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 10 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 328 positions in the final dataset, which is in compliance with the recommended ideal guidelines for use of SSU rRNA gene sequencing for parasite identification. Evolutionary analyses were conducted in MEGA7 (Kumer et al., 2016).

There is a need to develop control measures to address the WFSproblems in farmed shrimp populations. Fumagillin inhibits reproduction of the microsporidia spores, so it has been widely used in apiculture to control microsporidium, *Nosema* sp. (Bailey, 1953). However, based on the shrimp bioassay and PCR analyses, it was found to be non-effective for suppressing EHP infection in shrimp. Some shrimp farmers are employing methods that they have come up with on their own. For example, we have found through discussions with local producers, those in Indonesia, farmers have two main strategies: (1) attempting to reduce Vibrio spp. populations in the ponds through the frequent addition of probiotics such as Bacillus spp. or Lactobacillus spp. to the water; and (2) attempting to reduce pathogens in the shrimp digestive system through the use of feed additives. The feed additives being tried include: garlic, in the forms of freshly crushed or processed powders (10–30 g/kg feed); Allicin (a major active component of garlic); vitamin C (2 g/kg feed); and antiprotozoals, such as metronidazole. We recommend the removal of white feces from the affected shrimp ponds, the white feces contain large quantities of EHP and as they can break down and sink to the pond bottom. The associated EHP can be ingested by shrimp, results in re-infection, and ultimately will increase the severity of the infection.

Chapter 5

Conclusion and Recommendations

5.1 Conclusion

This study describes the presence of microsporidian parasites in the *Penaeus monodon*and helps to comment about prevalence of the microsporidian parasite in shrimp farms by microscopic observation and molecular identification.

Microsporidian parasites are a dangerous form of pathogen found around the world. It affects fish and crustacean health and retards growth. It is expected that more recent threats for *P. monodon*such as *Enterocytozoon*sp. and a haplosporidian. So, the presence of the microsporidian parasites indicates a possibility of future outbreak of microsporidiosis and other diseases.

The results of this study also denote the microsporidian parasite is present in the south west region of the Bangladesh especially in Satkhira and Bagherhat district. The absence of microsporidian parasite *Enterocytozoon hepatopenaei* (EHP) in shrimp collected farms is not unquestionable and demands further research on this aspect.

Indeed, the species specific identification of microsporidian parasite done in this study will help the farm owners to improve their management and surveillance system through taking the specific actions for the specific disturbance.

5.2 Recommendations

- To understand the microsporidian parasite *Enterocytozoon hepatopenaei*(EHP) prevalence, samples should be drawn from more shrimp farms with information about their management strategies.
- During the current study, the occurrence of microsporidian parasite in shrimp was investigated, irrespective of season. So, further study should be conducted on seasonal occurrences in shrimp.
- The water management system of the farm should be improved and the owner should rethink about hygienic facilities of the shrimp that are affected by microsporidiosis.

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(References followed the style of the Journal “Aquaculture”)

Appendices

Appendix-

Laboratory reagents

Reagents, which were used in carrying out different methods together with their sources, are mentioned below:

Polymerase chain reaction (PCR) reagents

Master Mix Go Taq® (2X)
Nuclease Free Water
Forward Primer
Reverse Primer
Template DNA
Reaction Buffer (10x)

Gel loading buffer

Ingredients	Amount (g/L)
Sucrose	6.7
Bromophenol blue	0.04

Stored at 4°C

PBS

Ingredients	Amount (g/L)
NaCl (Sigma)	8.56
Na_2HPO_4 (Sigma)	1.18
K_2HPO_4 (Sigma)	0.23
KCl (Sigma)	0.20
Distilled water	1.0 litre

pH was adjusted to 7.5.

10% NaCl solution

Ingredients for 100mL

Peptone	1 g
NaCl	10 g
Distilled water	100ml
pH adjusted	7.4

Ethidium bromide solution

1.0g of ethidium bromide was dissolved in distilled water to a final volume of 100ml.

The container was wrapped in aluminium foil and stored at 4°C.

Molecular weight marker

Tris-EDTA buffer

Gel loading dye (10X)

WX 174 RF DNA Hae fragment (Gibco, BRL)

Alsever´s solution

Glucose (sigma) 2.5g

NaCl (sigma) 0.42

Tris Na-acetate (sigma) 0.8g

Citric acid (sigma) 0.055g

Total volume was made up to 100ml

Appendix-

Table: 2.1 Information on the shrimp sample collected from farming ghers of South-west region

Sample ID	Sampling area	Sampling date	Owner of the gher	Area of the gher (bigha)	Name of the shrimp sp.	Age of the shrimp (month)	Dissection of organ	Organ for Histopathology / Molecular Diagnosis
1	Satkhira sadar	16-04-2016	Mosharaf	7	*Penaeus monodon*	3	Stomach	Histopathology
2	"	"	"	7	"	3	Muscle	"
3	"	"	"	7	"	3	Hepatopancreas	Histopathology and Molecular Diagnosis
4	"	"	Obydullah	3	"	3	Stomach	Histopathology
5	"	"	"	3	"	3	Muscle	"
6	"	"	"	3	"	3	Hepatopancreas	Histopathology and Molecular Diagnosis
7	"	"	Bablu	10	"	3	Stomach	Histopathology
8	"	"	"	10	"	3	Muscle	"
9	"	"	"	10	"	3	Hepatopancreas	Histopathology and Molecular Diagnosis
10	"	"	Yeakhan	4	"	2.5	Stomach	Histopathology
11	"	"	"	4	"	2.5	Muscle	"
12	"	"	"	4	"	2.5	Hepatopancreas	Histopathology and Molecular Diagnosis
13	"	"	Abdullah	5	"	3	Stomach	Histopathology
14	"	"	"	5	"	3	Muscle	"
15	"	"	"	5	"	3	Hepatopancreas	Histopathology and Molecular Diagnosis
16	"	20-05-2016	Abdul Hamid	4	"	3	"	"
17	"	"	Mosharaf	7	"	3	"	"
18	"	"	Ziad	50	"	3	"	"
19	"	"	Anarul	6	"	3	"	"

20	"	16-06-2016	Shawkat	18	"	3.5	"	"
21	"	"	Raju	20	"	3.5	"	"
22	"	"	Robiul	8	"	3.5	"	"
23	Morrelgang,Bagherhat	17-06-2016	Mizan	6	"	3	"	"
24	"	"	Abdul Malek	12	"	3.5	"	"
25	"	"	Shoharab	10	"	3	"	"
26	"	"	Tanvir	15	"	3	"	"
27	Mongla,Bagherhat	18-06-2016	Shahin	7	"	3	"	"
28	"	"	Ripon	6	"	3	"	"
29	"	"	Nazrul	9	"	3.5	"	"
30	"	"	Hafizul	5	"	3	"	"
31	Satkhira sadar	18-07-2016	Mosharaf	7	"	3	"	"
32	"	"	Khairul	5	"	3.5	"	"
33	"	"	Imdadul	4	"	3	"	"
34	"	20-09-2016	Obydullah	3	"	3	"	"
35	"	"	Ruhul Amin	25	"	3	"	"
36	"	"	Kamrul	8	"	3.5	"	"
37	"	"	Liton	4	"	3	"	"
38	"	18-11-2016	Mosharaf	7	"	3	"	"
39	"	"	Ziad	50	"	3.5	"	"
40	"	"	Shawkat	18	"	3	"	"

Table: 3.2 Overview of the results for the identification of the microsporidian parasite in cultured shrimp samples collected from South-west farming region of Bangladesh

Sample ID	Sampling Area	Area of the gher(bigha)	Dissection of organ	Histology		Molecular Diagnosis	
				Light microscopy	SEM	SSU rRNA	EHP-510
1	Satkhira sadar	7	Stomach	√ (+)			
2	"	7	Muscle				
3	"	7	Hepatopancreas	√ (+)	√ (+)	√ (+)	√ (-)
4	"	3	Stomach				
5	"	3	Muscle				
6	"	3	Hepatopancreas	√ (+)			
7	"	10	Stomach				
8	"	10	Muscle				
9	"	10	Hepatopancreas	√ (+)	√ (+)	√ (-)	√ (-)
10	"	4	Stomach				
11	"	4	Muscle				
12	"	4	Hepatopancreas	√ (+)			
13	"	5	Stomach				
14	"	5	Muscle				
15	"	5	Hepatopancreas	√ (+)		√ (-)	√ (-)
16	"	4	"				
17	"	7	"				
18	"	50	"				
19	"	6	"	√ (+)			
20	"	18	"	√ (+)	√ (+)	√ (+)	√ (-)
21	"	20	"	√ (+)			

22	"	8	"				
23	Morrelgang,Bagherhat	6	"	√ (+)	√ (+)	√ (-)	√ (-)
24	"	12	"	√ (-)			
25	"	10	"	√ (-)			
26	"	15	"	√ (+)		√ (-)	√ (-)
27	Mongla,Bagherhat	7	"	√ (-)			
28	"	6	"	√ (+)	√ (+)	√ (+)	√ (-)
29	"	9	"	√ (+)			
30	"	5	"	√ (-)			
31	Satkhira sadar	7	"				
32	"	5	"	√ (+)		√ (+)	√ (-)
33	"	4	"	√ (-)			
34	"	3	"				
35	"	25	"				
36	"	8	"				
37	"	4	"	√ (+)	√ (+)	√ (-)	√ (-)
38	"	7	"				
39	"	50	"	√ (+)		√ (+)	√ (-)
40	"	18	"				

Appendix-

SSU rRNA sequences of 2 representative species in this study

>MH10

AGAGTCCGTAGTTGTGGATACAATGAAAAGGTGATGATCAAGAACACTCAG
TTTGTTAGTCGTGGCAGAGTGGACAGGGAATACAGTATAGGCTGGCGAAGA
ATGAAATCTCAAAACCCAGCTTGGACTAACGAAGGCGAAGGCAGTATTCTTG
TACAGATCTGAGGATCAAGGACGAAGGCTAGAGGATCGAAGGTGATTAGAG
ACCACTGTAGTTCTAGCAGTAAACTATGCCAACATTCTCTATCATTGAGATG
GGGAAGGGAGAAATCTTAGTTTTTGGGCCACGGGGATAGTACGTTCGCAAG
GAAGAAACTTAAACTGAGAT

>MH11

AGAGTCCGTAGTTGTGGATACAATGAAAAGGTGATGATCAAGAACACTCAG
TTTGTTAGTCGTGGCAGAGTGGACAGGGAATACAGTATAGGCTGGCGAAGA
ATGAAATCTCAAAACCCAGCTTGGACTAACGAAGGCGAAGGCAGTATTCTTG
TACAGATCTGAGGATCAAGGACGAAGGCTAGAGGATCGAAGGTGATTAGAG
ACCACTGTAGTTCTAGCAGTAAACTATGCCAACATTCTCTATCAGTGAGATG
GGGAAGGGAGAAATCTTAGTTTTTGGGCCACGGGGATAGTACGTTCGCAAG
GAAGAAACTTAAACTGAGAT