

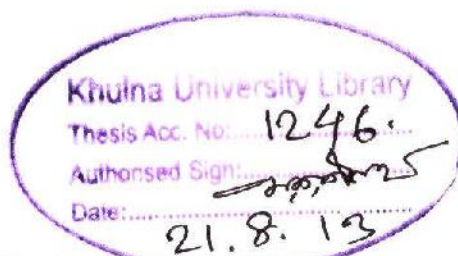
BIOCHEMICAL AND PCR ASSAY FOR DETECTION OF PATHOGENIC BACTERIA IN SHRIMP AND SHRIMP FARMS

A Thesis By

Harekrishna Adhikari

Examination Roll No: MS 120609

Session: 2011-2012



The Thesis submitted to the Fisheries and Marine Resource Technology Discipline in
partial fulfillment of the requirements for the degree of

Masters of Science in Fish Genetics and Biotechnology

Khulna University

Khulna, Bangladesh

December, 2012

BIOCHEMICAL AND PCR ASSAY FOR DETECTION OF PATHOGENIC BACTERIA IN SHRIMP AND SHRIMP FARMS

Approved as to style and content

By



Professor Dr. Md. Nazmul Ahsan

Head

Fisheries and Marine Resource Technology Discipline

Life Science School, Khulna University

Khulna-9208, Bangladesh.

DECLARATION

Biochemical and PCR assay for detection of pathogenic bacteria in shrimp and shrimp farms

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

Candidate

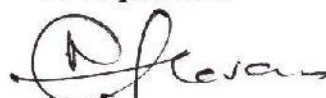


Harekrishna Adhikari

Examination Roll No. MS 120609

Session: 2011-2012

Co-supervisor



Dr. Md. Nazmul Ahsan

Professor

Fisheries and Marine Resource Technology Discipline
Khulna University, Khulna-9208, Bangladesh



Supervisor



Dr. Md. Golam Sarower

Professor

Fisheries and Marine Resource Technology Discipline
Khulna University, Khulna-9208, Bangladesh

December, 2012

Dedicated to
My Beloved parents



Acknowledgement

At first, all my admiration goes to the supreme creator and Ruler of the universe, the Almighty God, for His great mercy to keep me alive and enables me to pursue my education in Fisheries and Marine Resource Technology Discipline as well as to complete my project thesis for the degree of Master of Science in Fisheries.

I would like to express my sincere gratitude and profound appreciation to my supervisor, **Dr. Md. Golam Sarower, Professor,** Fisheries and Marine Resource Technology Discipline for his continuous guidance, valuable suggestions and sustained support throughout the experiment. I am really grateful to him for introducing me to the field of PCR technology; his skill and compassion as a teacher are unsurpassed.

I would like to express my gratitude to Vhabanandho Biswas for his valuable suggestions and providence of all necessary facilities.

I am grateful and deeply thankful to Debashis Roy for his cordial co-operation and generous help at all phases of the experiment.

Finally, I would like to express my earnest appreciation to my parents, for their immeasurable blessing, continuous inspiration and financial support throughout the study.

Harekrishna Adhikari

December, 2012

Abstract

The study was conducted to detect faecal coliforms and pathogenic bacteria in shrimp and shrimp farms. A multiplex PCR system was used for the rapid detection of pathogenic *Aeromonas sp.*, *Clostridium sp.*, *Listeria sp.*, *Salmonella sp.*, *Shigella sp.*, *Vibrio sp.* and *Staphylococcus sp.* in the water, sediment and shrimp. The target genes chosen for this investigation were Aer gene for *Aeromonas sp.*, hilA gene for *Salmonella sp.*, virA gene for *Vibrio sp.*, Sec gene for *Staphylococcus aureus*, neurotoxin gene type A,B,E,F for *Clostridium botulinum*, internalin gene for *Listeria sp.* and ipaH gene for *Shigella sp.* respectively. Seven pairs of specific primers were used to amplify internal fragments of the genes by PCR. The PCR products were then analyzed and confirmed with relative ease by gel electrophoresis. *Aeromonas* and *Vibrio sp.* were found in the water, sediment as well as in the shrimp being the dominant bacteria. On the other hand, *Salmonella* and *Clostridium* were found in the sediment of one sample but there was no evidence of *Listeria*, *Staphylococcus* and *Shigella* in the farms. All samples contained relatively large number (1100 to ≥ 2400 mpn/gm) of coliform bacteria. The result revealed that the PCR-based rapid detection system could be a powerful method for routine monitoring and risk assessment of water quality in the shrimp farms.

Table of Contents

	Page
Acknowledgement	i
Abstract	ii
Table of contents	iii- iv
List of tables	v
List of figures	vi-vii
Chapter 1 Introduction	1-8
Chapter 2 Literature Review	9-13
Chapter 3 Materials and methods	14-25
3.1 Study area	
3.2 Laboratory Requirements	
3.2.1 Materials	
3.2.2 Reagents and chemicals	
3.3 Sample collection	
3.4 Biochemical test	
3.4.1 Total Coliform	
3.4.1.1 Culture Media	
3.4.1.1.1 Lauryl Sulphate Tryptose Broth (LSTB):	
3.4.1.1.2 Procedure for Total Coliform	
3.4.2 Faecal Coliform (FC)	
3.4.2.1 Culture Media	
3.4.2.1.1 Brilliant Green Bile Broth (BGBB)	
3.4.2.1.2 Tryptone Water	
3.4.2.2 Procedure	
3.4.3 <i>Vibrio cholerae</i>	
3.4.3.1 Culture Media	
3.4.3.1.1 Alkaline Peptone Water	
3.4.3.1.2 Thio-sulphate Citrate Bile Salts Sucrose Agar	



3.4.3.2 Procedure for *Vibrio Cholera*3.4.4 *Salmonella sp.*

3.4.4.1 Culture media

3.4.4.1.1 Selenine Cystine Broth

3.4.4.1.2 Tetrathionate Brilliant Green Broth

3.4.4.1.3 Xylose Lysine Deoxycholate (XLD) Agar

3.4.4.1.4 Brilliant Green Agar

3.4.4.2 Procedure for salmonella:

3.5 PCR test

3.5.1 Preparation of media and culture of organism

3.5.2 DNA Extraction

3.5.3 Qualitative and quantitative determination of extracted DNA

3.5.4 Components for DNA amplification in PCR

3.5.4.1 Description of Target genes and primers

3.5.5 Top DNA polymerase enzyme

3.5.6 Deoxynucleotide Triphosphates (dNTPs) mixture

3.5.7 Buffers used for PCR

3.5.8 DNA amplification by a thermal cycler

3.5.9 Evaluation of PCR products

Chapter 4	Results & Discussion	26-36
Chapter 5	Conclusion	37
Chapter 6	References	38-46
Chapter 7	Appendixes	47-50

List of Tables

Table No.	Title	Page
3.1	Specific gene for pathogenic bacteria detection.	22
3.2	Primers and expected size of PCR amplified gene targets of pathogenic bacteria	23
3.3	The component of reaction mixture for the PCR.	24
4.1	The biochemical test result of the total coliform ,faecal coliforms and the presence of <i>vibrio cholerae</i> and <i>salmonella sp.</i> in 5 water sample, 5 sediment sample and 5 shrimp sample are represented below in graphically..	27
4.2	Summery of the PCR results	28

List of Figures

Table	Title	Page no.
3.1	Sampling area at Bagerhat sadar upzlla; the circle indicates the approximate location of sampling farm	14
4.1	UV visualization of PCR product of farm 3 following electrophoresis in a 2% agarose gel. Lanes 1 to 7, 8 to 14 and 15 to 21 represent water, sediment and intestine of shrimp respectively where 7 primers (Aero, Clos, List, Salm, Shig, Vibr, Stap) is used for each sample. Lane M represents 100 bp DNA ladder or marker	29
4.2	UV visualization of PCR product of farm 3 following electrophoresis in a 2% agarose gel. Lanes 1 to 7, 8 to 14 and 15 to 21 represent water, sediment and intestine of shrimp respectively where 7 primers (Aero, Clos, List, Salm, Shig, Vibr, Stap) is used for each sample. Lane M represents 100 bp DNA ladder or marker	30
4.3	UV visualization of PCR product of farm 3 following electrophoresis in a 2% agarose gel. Lanes 1 to 7, 8 to 14 and 15 to 21 represent water, sediment and intestine of shrimp respectively where 7 primers (Aero, Clos, List, Salm, Shig, Vibr, Stap) is used for each sample. Lane M represents 100 bp DNA ladder or marker.	31
4.4	UV visualization of PCR product of farm 4 following electrophoresis in a 2% agarose gel. Lanes 1 to 7, 9 to 15 and 17 to 23 represent water, sediment and intestine of shrimp respectively where 7 primers (Aero, Clos, List, Salm, Shig, Vibr, Stap) is used for each sample. Lane M represents 100 bp DNA ladder or marker.	32
4.5	UV visualization of PCR product of farm 5 following electrophoresis in a 2% agarose gel. Lanes 1 to 7 and 8 to 14 represent water, sediment respectively where 7 primers (Aero, Clos, List, Salm, Shig, Vibr, Stap) is used for each sample. For intestine of shrimp sample Lane 1 to 7 which represent <i>Aero</i> , <i>Clos</i> , <i>List</i> , <i>Salm</i> , <i>Shig</i> , <i>Vibr</i> , <i>Stap</i> serially. Lane M represents 100 bp DNA ladder or marker.	33

Chapter One

INTRODUCTION



1. Introduction

1.1. Background of the Study

Fish is the primary source of animal proteins for millions of people in Bangladesh. As a riverine country, Bangladesh poses an immense potential for fisheries which provide 60% of total animal protein supply needed in the nationwide. The fishery sectors in Bangladesh currently contribute about 4.43% of the Gross Domestic Production and more than 2.73% of the export earning. Shrimp culture is one of the most important divisions in the total fishery sector. In the 2011-2012 fiscal years, the total production of shrimp is about 239460 mt. ton in which 52.05% is from culture basis (DoF, 2012).

The main cultured species is the tiger shrimp, *Penaeus monodon* that is a marine shrimp and is cultivated in brackish water. With strong international demand and high prices culture of this shrimp in the brackish-water habitats in the coastal areas has become popular. Currently *P. monodon* culture is practiced not only in Khulna region but also in the district of Cox's Bazar.

South-western region of Bangladesh, especially Khulna, Satkhira, Bagerhat are most suitable for aquaculture and particularly for shrimp culture. Ponds, Ghers (Closed water field), and rivers are the main source of aquatic foods among the coastal communities of Bangladesh. The major shrimp producing districts are Bagerhat, Satkhira, Pirojpur, Khulna, Cox's Bazar and Chittagong, recently farmers especially in the Bagerhat and Pirojpur districts have begun shrimp farming in their paddy fields. Traditionally shrimp farming began by trapping tidal waters in nearby coastal enclosures known as 'Gher' where no feed, fertilizers or other inputs were applied, with an increasing demand from both national and international markets farmers started to switch over into improved extensive and semi-intensive systems.

Shrimp farming in the south and southeastern coastal belt of Bangladesh began in the early 1970s. From less than 20,000 ha of brackish water ponds in 1980, the area under cultivation expanded to approximately 140 000 ha by 1995. The last complete survey to estimate the total

area under shrimp cultivation was carried out in 1993–94; it has not been updated since then. Paul (2007) estimated that the total area under farming has expanded to 203 071 ha in 2003–2004.

Shrimp farming concentrated largely in tropical developing countries for export to the west, has experienced spectacular growth over recent decades. Currently, aquaculture industry in Bangladesh and other parts of the world especially southwest region has been facing serious problems due to microbial diseases. In aquatic environments, diseases in fishes and shrimps are caused by opportunistic pathogens (Lundin, *et al.*, 1996). In culture conditions various diseases are found which are acting like as natural disaster. Bacterial and viral diseases have most serious losses occurred. Like fish, shrimp lack an antigen/antibody system and so cannot be vaccinated in the way fish can be. Prevention is the only cure.

Coliforms are bacteria that are always present in the digestive tracts of animals, including humans, and are found in their wastes. They are also found in plant and soil material.

Total coliforms include bacteria that are found in the soil, in water that has been influenced by surface water, and in human or animal waste.

Fecal coliforms are the group of the total coliforms that are considered to be present specifically in the gut and feces of warm-blooded animals. Because the origins of fecal coliforms are more specific than the origins of the more general total coliform group of bacteria, fecal coliforms are considered a more accurate indication of animal or human waste than the total coliforms.

Escherichia coli is the major species in the fecal coliform group. Of the five general groups of bacteria that comprise the total coliforms, only *E. coli* is generally not found growing and reproducing in the environment. Consequently, *E. coli* is considered to be the species of coliform bacteria that is the best indicator of fecal pollution and the possible presence of pathogens.

Pathogenic bacteria are bacteria that cause bacterial infection. Although the vast majority of bacteria are harmless or beneficial, quite a few bacteria are pathogenic. Pathogenic bacteria contribute to other globally important diseases. The pathogenic bacteria belonging to the group *E. coli*, *Salmonella*, *Vibrio*, *Staphylococcus*, *Shigella*, *Aeromonas*, *Listeria*, *Clostridium botulinum*, are common and widely distributed in the aquatic environment in various parts of the world. The more psychrotrophic organisms (*Listeria*) are common in colder climates, while the more mesophilic types (*V. cholerae*, *V. Parahaemolyticus*) are representing part of the natural flora of fish from coastal and estuarine environments of temperate or warm tropical and sub-tropical zones (Ingianni, *et al.*, 2001). Faecal coliform bacteria are available in water wherever it is contaminated with faecal waste of human or animal excreta. Faecal coliforms are the indicator of the presence of the presence of bacterial pathogens such as *Salmonella sp.*, *Shigella*, *Vibrio cholera* and *E. coli*. These organisms can be transmitted via digestive tract by contaminated water and may lead to diseases such as gastroenteritis, salmonellosis, dysentery, cholera and typhoid fever. Higher concentration of faecal coliforms in water will indicate a higher risk of contracting waterborne disease. For years, total coliforms and fecal coliform were the most widely used indicators but, more recently, the abundance of *E. coli* has been shown to be more related to the sanitary risk than that of coliforms (Fewtrell and Bartram, 2001).

The classical species of coliforms are *E. coli* and *Enterobacter aerogenes* which are also known as faecal coliforms have relation to other pathogenic enteric bacteria such as *Salmonella*, *Shigella*, *Klebsiella*, *Proteus*, *Serratia* etc. that may cause dangerous fever, diarrhea and dysentery (Pelczar, *et al.*, 1998). Even members of coliforms are sometimes opportunistic to cause enteric diseases, urinary tract infections, wound infections and bacteraemia (Stanier, *et al.*, 1995). In contrast to non-faecal, faecal coliforms have the capability to grow at elevated

temperatures (44–44.5°C) and are associated with the faecal materials of warm-blooded animals (Pelczar, *et al.*, 1998).

There are various types of bacteria that present in the water body and create various types of disease that economically very harmful in our country. Disease is inevitable in uncontrollable tradition and semi-intensive culture systems. In addition, the use of antibiotics to control bacteria population and maintain healthy environment for shrimp culture becomes popular. A wide range of antibiotics is now being used to treat bacterial diseases and to control bacterial population in the hatcheries and prawn farms (Lightner, *et al.*, 1996). The potential consequences of used antibiotics for treatment may arise various antibiotic resistant, antibiotic-resistant bacteria. The phenomenon resistance was transfer to pathogenic bacteria, and led to reduce efficacy of antibiotic treatment for disease caused by the resistant pathogens (Frappalo, *et al.*, 1986). Moreover, all the fish have normal bactericidal activity in their blood serum. Current state of knowledge regarding fish health is still greatly needed. Few attempts have been taken in order to assess the bacterial population in aquatic environment and their involvement in causing disease. Representatives of upwards of 25 bacterial genera have been implicated at various times as pathogens of fresh water, marine shrimp and fish species (King, *et al.*, 1997).

Types and levels of bacterial populations associated with farmed brackish water shrimp (*P. monodon*) are the important indicators for the assessment of quality and safety of shrimp. In addition, most diseases in *P. monodon* are caused by opportunistic pathogens which are prevalent in the rearing environment (Jayasree, *et al.*, 1999).

Identification of bacteria is essential for the diagnosis of diseases in shrimp. Conventional identification of bacteria involves cultivation of bacteria followed by their biochemical identification. Biochemical identification methods are laborious, time-consuming and at times

misleading due to the presence of variants among bacterial species (Fredrickson, *et al.*, 2004). There are also many bacterial species that are not cultivable by standard methods. PCR has been proved to be a simple and rapid way to identify bacteria.

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

In the 40 years since the advent of molecular biology, developments in DNA technology have revolutionized biological research. The study of fish disease is no exception and great advances have been made in this field, particularly in the last 10 years. The time has now come to reap the benefits of work that has provided the means to improve the diagnosis and control of fish disease (Leong, *et al.*, 1995). Prior to the advent of molecular methods, diagnosis of disease relied largely on culture of the causative organism in media or cells, analysis of phenotypic or serological properties of the pathogen or histological examination of the effects on host tissue. Nucleic acid technology presents the opportunity to detect the pathogen directly, targeting the genetic material and augmenting or replacing culture, serological or histological techniques (Wagener, *et al.*, 1997; McKeever and Rege, 1999).

The presence or absence of a product following PCR may be sufficient to indicate whether a sample is infected by a certain pathogen. In other situations, the specificity of diagnosis can be improved by further manipulations of PCR products and the amplification is only carried out in order to enable these other reactions. Restriction digestion, probe hybridization or even

nucleotide sequencing using PCR products can often provide further detail on the identity of the original sample material (McBeath, *et al.*, 2000). When dealing with pathogens, such as *Renibacterium salmoninarum*, which require lengthy culture prior to identification by other methods, PCR may greatly increase the speed of diagnosis. (Patel, *et al.*, 1997).

The specificity of assays using primers is usually conferred by further manipulations such as digestion or sequencing. Despite their ready availability for prokaryotic and eukaryotic organisms, universal primers carry an increased risk of false positive results, as they are potentially able to anneal to a wide variety of bacteria or parasites, thus detecting harmless organisms in addition to pathogens. Template preparation is an important step of any diagnostic test. Traditional methods of nucleic acid preparation involve extractions that use harmful chemicals and may take a considerable time to perform. Many commercial extraction kits are now available that are more user-friendly, but can result in reduced yields and therefore may affect the sensitivity of PCR. The reaction conditions must be optimized. In many cases, development work is undertaken using pure cultures or cloned DNA. The practical application of PCR in diagnostic testing does require significant attention to development of suitable methodologies and validation to ensure greatest possible sensitivity and specificity are obtained. Despite the possible difficulties involved with this technique, PCR will play an integral part in molecular diagnosis of fish and shellfish diseases (Cunningham, 2002).

The PCR has seen numerous recent applications to pathogen detection and shrimp pathology research. In PCR, small, often undetectable, amounts of DNA can be amplified to produce detectable quantities of the target DNA. This is accomplished by using specific oligonucleotide primers designed for the target DNA sequence. The resultant PCR product may then be compared to a known standard using gel electrophoresis, by reaction with a specific DNA probe

of PCR products blotted directly onto a membrane or to the PCR products in southern transfers (Perkins and Martin, 1999).

Fish importers often complain that pathogenic bacteria are found in the sea foods imported from Bangladesh. Multifarious gastroenteritis problem is common among the coastal communities. It is believe that the main sources of these diseases are water and aquatic foods. So, it is very important to focus a special research on the identification of all possible sources of pathogenic bacteria in aquatic food chain and aquatic environment in the coastal region of Bangladesh.

One of the inherent difficulties in the detection of pathogens in foods and environment is that they are generally present in very low numbers (<100 cfu/g) in the midst of up to a million or more other bacteria and these microbes may be lost among a background of indigenous microflora in the sample. The isolation of these bacteria in the samples is frequently complicated by the presence of nontoxigenic strains that phenotypically and getically resemble.

For pathogenic bacteria severasl PCR based detection methods have been reported during the last decade (Kannan, *et al.*, 2001; Lindstro, *et al.*,2001; Ingianni, *et al.*,2001; Pathmanathan, *et al.*,2003; Thiem, *et al.*,2004; Lovseth, *et al.*,2004; Tarr, *et al.*,2007; Panicker, *et al.*,2005). Compared to conventional methods these protocols provide rapid and sensitive detection of these organisms. The Multiplex PCR method targeting species specific gene may provide a more sophisticated approach, enabling a simultaneous and specific detection of pathogenic bacteria.

1.2. Objective of the Study:

The specific objectives of the study were:

- To determine the total coliforms, faecal coliforms, *salmonella sp.* and *vibrio sp.* by biochemical method.
- to detect pathogenic bacteria (*Aeromonas sp.*, *Clostridium sp.*, *Listeria sp.*, *Salmonella sp.*, *Shigella sp.*, *Vibrio cholera* and *Staphylococcus aureus*) by PCR in shrimp and shrimp farms.



Chapter Two

LITERATURE REVIEW

2. Literature Review

Aeromonas sp. comprises mesophilic motile and psychrophilic nonmotile gram-negative ubiquitous bacteria. (Cesar, et al., 1999) evaluate a various multiplex protocol for detection of pathogenic *Aeromonas* Species (accession no.m84709).

Aeromonas hydrophila is a gram negative, facultatively anaerobic, motile, waterborne bacterium with wide distribution. They have long been known to be pathogenic to cold and warm-blooded fauna. Since 1960 the potential of these organisms to cause diseases in human has been recognized. In humans and other mammals, *A. hydrophila* has been reported as the causative agent of numerous manifestations associated with gastroenteritis, systemic infections and bacterial endocarditis. In amphibians, reptiles, and fishes, these organisms have reportedly been associated with necrotic septicaemic and ulcerative diseases (Austin and Austin, 1993; Inglis, et al., 1993; Olafse, et al., 1993).

Bin Kingombe, et al., (1999) examined many *Aeromonas* isolates, but a large scale trial of this method with a larger number of bacteria pathogenic to fish will be required to demonstrate the value of this as a diagnostic assay for use in fish health. Amplification of genes encoding virulence determinants appears to be a promising approach for the detection of pathogenic *Aeromonas* species or strains. Once virulence determinants have been identified and defined in other bacteria, this approach may be particularly valuable in the identification of pathogenic strains and their differentiation from other innocuous strains that have been identified as the same organism. The presence of a certain virulence factor gene or genotype could potentially be detected by PCR or PCR combined with RFLP or probes.

Shigella constitutes a genus of gram-negative, nonmotile, non-spore-forming rod-shaped bacteria with four species or serotypes, namely, *S. boydii*, *S. dysenteriae*, *S. flexneri*, and *S. sonnei*.

Virulent *Shigella* organisms cause the human illness known as bacillary dysentery, as do enteroinvasive *E. coli* (EIEC) strains. According to Eduardo and Antonio (1998) the use of PCR to amplify a specific *virA* gene fragment serves as a highly specific and sensitive method to detect virulent bacteria of the genus *Shigella* and enteroinvasive *E. coli*. Amplification of a 215-bp DNA band was obtained by using isolated genomic DNA of *Shigella*, individual cells of *Shigella dysenteriae*, and mayonnaise contaminated with *S. dysenteriae*. Moreover, a multiplex PCR with specific (*virA*) and bacterium-restricted (16S ribosomal DNA) primers generated an amplification product of approximately 755 bp for all bacteria tested and an additional 215-bp product for *Shigella* and enteroinvasive *E. coli*.

Shrimp and other fishes or seafood can be jeopardous for health and life by carrying pathogenic or potentially pathogenic bacteria like *Enterobacteriaceae*, *Staphylococcus* etc (Ayulo, *et al.*, 1994). *Enterobacteriaceae* is a large family comprising heterogeneous group of gram-negative rods as coliforms, *Salmonella*, *Shigella*, *Yersinia* and so on (Pelczar, *et al.*, 1998; Stanier, *et al.*, 1995, Tortora, *et al.*, 1995), and can be contaminated in shrimp or seafood from animal or human reservoir. Therefore, fresh shrimps should have a specification of 10 cfu/g (WHO specification) and <102 cfu/g (Thai specification) for coliforms, and <3 cfu/g for *E. coli* (Suwansonthichai and Rengpipat, 2003).

Coliforms which are Gram-negative, aerobic or facultative anaerobic, non-spore forming bacteria that ferment lactose with the production of acid and gas at 37°C within 24–48 h (Cappuccino and Sherman, 1999; Stanier, *et al.*, 1995, Tortora, *et al.*, 1995) are widely used as indicator organisms for bacteriological analysis of food, drinks, beverage and drinking water (Pelczar, *et al.*, 1998).

The classical species of coliforms are *E. coli* and *Enterobacter aerogenes* which are also known as faecal coliforms have relation to other pathogenic enteric bacteria such as *Salmonella*, *Shigella*, *Klebsiella*, *Proteus*, *Serratia* etc. that may cause dangerous fever, diarrhea and dysentery (Pelczar, *et al.*, 1998). Even members of coliforms are sometimes opportunistic to cause enteric diseases, urinary tract infections, wound infections and bacteraemia (Nataro and Kaper, 1998; Stanier, *et al.*, 1995). In contrast to non-faecal, faecal coliforms have the capability to grow at elevated temperatures (44-44.5°C) and are associated with the faecal materials of warm-blooded animals (Pelczar, *et al.*, 1998).

Giovanna and Paolo (1996) developed and applied a PCR for the detection of *Clostridium botulinum* type C in 18 avian and environmental samples collected during an outbreak of avian botulism, and the results were compared with those obtained by conventional methodologies based on the mouse bioassay.

Salmonellosis is responsible for large numbers of infections in both humans and animals (Keusch, 2002). *Salmonella* strains are not detectable in certain clinical samples that contain small numbers of organisms (Fricker, 1987). However, the number of salmonellae present in the faeces of an infected individual is large, i.e. approx. 10^9 g^{-1} . Several PCR assays have been developed for the detection of *Salmonella* strains in faeces (Chiu & Ou, 1996; Gentry-Weeks, *et al.*, 2002; Stone *et al.*, 1994; Widjoatmodjo, *et al.*, 1992).

Vibrio harveyi is a Gram-negative, luminous bacterium. It is widely distributed in aquatic environments and is considered to be an important causative agent of luminous disease in marine organisms. Over the past decade, strains of this species have been reported to be significant pathogenic agents and one cause of the high rates of shrimp mortality in the shrimp culture industry worldwide (Karunasagar, *et al.*, 1994; Saeed, 1995; Liu, *et al.*, 1996a.)

Vibrio sp. occurs naturally in aquatic environments and is one of the most commonly-occurring bacteria during shrimp farming (Vandenberghe, *et al.*, 2003). The number of reported *Vibrio* species has increased rapidly in the last decade. Thompson (2004) has reported 63 environmental species comprising the genus *Vibrio*. Ten of them are of human concern (Twedt, 1989) since they have been associated with skin infections and severe gastrointestinal disorders (Andrews, 2004). Some of the pathogenic *Vibrio* species have also been reported as the causal agents of shrimp infections (Goarant, *et al.*, 1999). In contrast, other species such as *Vibrio alginolyticus* have been reported as probiotics for shrimp aquaculture (Vandenberghe., 2003, Direkbusaram, *et al.*, 1998).

The genus *Listeria* is composed of six species including *Listeria monocytogenes*, *Listeria innocua*, *Listeria ivanovii*, *Listeria seeligeri*, *Listeria welshimeri* and *Listeria grayi*. Only two of these species, namely *L. monocytogenes* in humans and *L. ivanovii* in other mammals, are considered pathogenic (Gasarov, *et al.*, 2005).

Kannapiran (2009) reported that population dynamics and distribution profile of luminous bacteria and total heterotrophic bacteria in the water, sediment and animal samples were monitored. Luminous bacteria associated with exoskeleton, gills and gut were isolated and quantified. The total heterotrophic bacterial counts ranged from 1.3×10^4 to 25.3×10^4 CFU ml⁻¹ in water and 1.5×10^6 to 26.2×10^6 CFU g⁻¹ in sediment. The *V. harveyi* population density varied between 0.6×10^4 and 8.8×10^4 LCFU ml⁻¹ in water and from 1.2×10^6 to 10.4×10^6 LCFU g⁻¹ sediment respectively. The high *V. harveyi* density observed in this study at the end of the culture period correlated with the outbreak of white spot disease.

Thaithongnum, *et al.* (2006) stated that the *gyrB* gene of *V. harveyi* NICA, isolated from shrimp in Thailand, was sequenced. The Most Probable Number (MPN) technique was applied to enumerate *V. harveyi*. They have demonstrated that when PCR was applied directly to the enrichment broth of shrimp artificially inoculated with *V. harveyi*, the MPN value was no different from the MPN value obtained using the standard technique with selective agar. This technique was employed to enumerate *V. harveyi* in postlarvae and hatchery tank water. *V. harveyi* were detected in 18 out of 21 postlarval samples and in 14 out of 21 tank water samples. This technique suggested as a measure to prevent the catastrophic losses caused by *V. harveyi* disease.

Barski (1996) established a PCR method for rapid detection of pathogenic *Staphylococcus aureus*.

Bettelheim (1992) reported that the method which is widely used has several disadvantages especially in relation to its lack of specificity. First, it does not distinguish pathogenic from nonpathogenic strains of *E. coli*. Second, some strains of *Salmonella*, *Shigella* and *Yersinia* which are capable of splitting MUG will also be detected.

Chapter Three

MATERIALS AND METHODS

3. Materials and Methods

The Experiment was conducted at Genetics Laboratory of Fisheries and Marine Resource Technology (FMRT) Discipline of Khulna University from July to October 2012.

3.1 Study area

Samples (water, sediment and fresh shrimp) were collected from five different shrimp farms which are situated at Khesra and Jalalpur Union of Tala upazilla of Satkhira district.

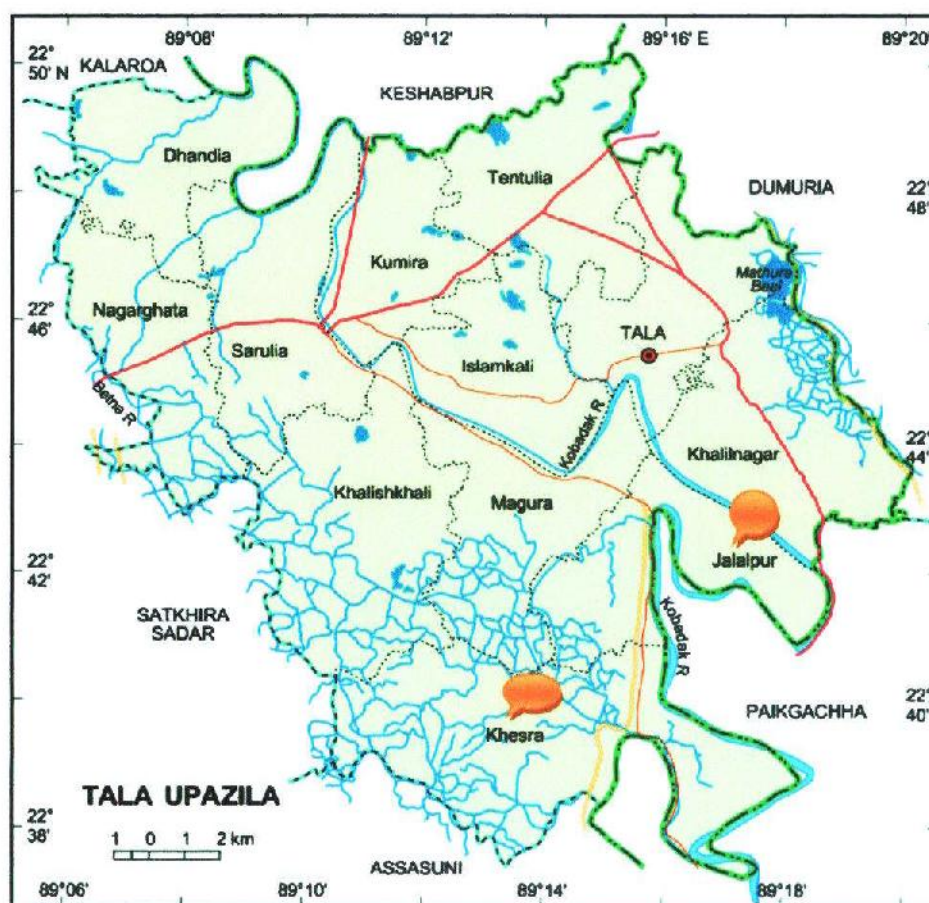


Figure 3.1: Sampling area at Tala upazilla; the circle indicates the approximate location of sampling farm

3.2 Laboratory Requirements

3.2.1. Materials

- Autoclave machine.
- Centrifuge machine, Electric balance.
- Spirit lamp and burning spirit.
- Petri dish.
- Biological dissection box.
- Solution mixer.
- Bacteria isolating loops.
- TCBS agar, XLD agar, LSTB agar, BGA, PCA, Nutrient agar,
- Refrigerator.
- Bio-safety cabinet.
- Micro-pipette, Tips and tips box.

3.2.2 Reagents and chemicals

- ❖ Peptone water.
- ❖ Absolute alcohol, Ethyl alcohol (75%)
- ❖ Distilled water, Peptone water.
- ❖ Detergent powder.
- ❖ Luria-Bertani (LB) broth (Difco Laboratories, MI, USA)
- ❖ 10x Reaction buffer, dNTP mixture
- ❖ Agarose powder, 50x TAE buffer, Ethidium Bromide and gel loading buffer
- ❖ Top DNA polymerase

3.3 Sample collection

The samples were collected from five different location of shrimp farm. Mainly water, sediment and shrimp samples were collected. During sampling, the some other information were also checked, such as, the size of the farm, status of sanitation system, culture system, water source, stocking density, disease problem, as well as production of each farm.

Both water and sediment sample were collected from five different point of each shrimp farm by a pasteurized tube and was kept in a sterile 10 ml plastic vial. Fresh shrimp, *Penaeus monodon* (average body weight 15.7 ± 2.5 g) also collected from each farm which was kept in individual polythene bag. The sample containing plastic vials were transported to Molecular Biology Laboratory, FMRT Discipline, Khulna University. Intestine of the collected shrimp was separated by dissecting the body and was kept each in 1.5 ml tube with 1 ml distill water. To avoid cross contamination, separate surgical blade, dissection tray were used for each individual. All the samples were properly labeled and stored in 4°C temperature.

3.4. Biochemical test

Biochemical test for total coliform, faecal coliform, *Vibrio cholera* and *Salmonella sp* was done in FMRT Laboratory.

3.4.1 Total Coliform

3.4.1.1 Culture Media

3.4.1.1.1 Lauryl Sulphate Tryptose Broth (LSTB):

LSTB (3.56 gm) was dissolved in 100ml of distilled water and then was distribute into test tubes with inserting Durham tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minute.

3.4.1.2 Procedure for Total Coliform:

Twenty gram pooled sample (water) was weighed and aseptically taken into a sterile warming blender jar and then blended. After blending, the sample was added into conical flask containing 180 ml. of 0.1% peptone water. Ten fold serial dilutions were made with this suspension in McCarty's bottles containing 9.0 ml of 0.1% peptone water. One milliliter of each dilution was transferred into three screw-capped test tubes autoclaved LSTB (10.0 ml) with Durham's tubes.

The tubes were inverted to ensure that Durham's tubes did not contain gas bubbles. These tubes were incubated at 37 °C for 24 hours. The formation of gas within 24 to 48 hours was considered as evidence for the presumptive positive test for Coli form organisms.

A loopful of suspension from all LSTB broth tube was transferred into 10. ml. volumes of Brilliant Green Lactose Bile (BGLB) broth. These tubes were incubated at 37 °C for 24 hours.

Gas production in BGLB tubes considered as sufficient evidence for the confirmatory test for Total Coli form organisms. Number of positive tubes out of three was recorded for each dilution.

Using the MPN charts, the MPN of Total Coliform was calculated based on the portion of conformed LSTB tubes (gas production) for three consecutive dilutions in BGLB broth at 37° C.

3.4.2 Faecal Coliform

3.4.2.1 Culture Media

3.4.2.1.1 Brilliant Green Bile Broth (BGBB)

Four grams was suspended in 100 ml distilled water. It was mixed well and later it was distributed in fermentation tubes and sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

3.4.2.1.2 Triptone Water

One gram was suspended in 100 ml distilled water. Then it was heated to boiling to dissolve the medium completely. Sterilize by autoclaving for 15 minutes at 15 lbs pressure (121°C).

3.4.2.2 Procedure

Those tubes of LSTB, which were positive for gas production in total Coliform estimation tests, were considered. Subcultures were made from all positive tubes of LSTB into a 10 ml volume of BGLB and incubated at 44.5 + 0.5 ° C up to 48 hours. Any tube showing gas production was considered positive for the presence of presumptive faecal coliforms.

All positive tubes were sub-cultured by streaking onto plates of EMB agar. Then these plates were incubated at 37° C for 18 - 24 hours. After incubation, the plates were examined for suspicious Faecal Coliform colonies i.e. black or dark centered with the greenish metallic sheen.

3.4.3 *Vibrio cholerae*

3.4.3.1 Culture Media

3.4.3.1.1 Alkaline Peptone Water

Alkaline Peptone Water (4.5 gm) was suspended in 225 ml distilled water and slightly heated to dissolved completely and sterilized by autoclaving at 15lb pressure at 20 minutes.

3.4.3.1.2 Thio-sulphate Citrate Bile Salts Sucrose Agar (TCBS)

TCBS agar (4.45 gm) was first boiled with 50 ml of distilled water to dissolve and then boiled for 1 minute. Then the media was dried to 60°C for 15 minutes and was poured into Petri dishes and allowed to solidify.

3.4.3.2 Procedure for *Vibrio Cholera*:

Twenty five gram's of the sample was added with approximately 225 ml of alkaline peptone water in a warring blender flask. Then the sample containing alkaline peptone water was blended for 1 minute. The sample was incubated at 37° C for 6-8 hrs.

At the end of the incubation period, a loopful of the alkaline peptone water was streaked onto TCBS agar plates. The plates were then incubated at 37° C for 24 hrs. At the end of incubation, plates were checked for the characteristic colony of *Vibrio Cholerae*. On TCBS agar colonies appeared as large as (2-3mm), smooth and yellow, slightly flattened, with opaque center and translucent peripheries.



3.4.4 *Salmonella* sp.

3.4.4.1 Culture media

3.4.4.1.1 Selenine Cystine Broth

Part A	Part B
Tryptone - Sgm/litre	Sodium Acid selenine - 4 gm litre
Lactose - 4 gm/litre	
Sodium - 1 Ogm/litre	

Two gram of part A and 1.33 gm's of part B of selenite broth was suspended in 100 ml distilled water. It was warmed to mix well. It was dispensed and sterilized in a boiling water bath or in free flowing steam for 10 minutes. It was not done in autoclave because excessive heating is detrimental.

3.4.4.1.2 Tetrathionate Brilliant Green Broth

Nine grams of broth was suspended in 100 ml distilled water. It was heated to dissolve and keep in free flowing steam for 30 minutes. It was kept at 45°C and added 4 ml iodine solution containing 0.5 grams iodine and 0.8 grams potassium iodine. It was mixed well and dispensed as desired.

3.4.4.1.3 Xylose Lysine Deoxycholate (XLD) Agar

XLD agar (2.5 g) was suspended in 50 ml distilled water and was heated with frequent agitation until the medium boiled and then transferred to a water bath at 60°C for 15 minutes. After cooling, the medium was roused into sterile Petri dishes.

3.4.4.1.4 Brilliant Green Agar

BGA (5.8 g) was suspended in 50 ml of distilled water and boiled to dissolve completely and then sterilized by autoclaving at 15lbs pressure for 15 minutes.

3.4.4.2 Procedure for salmonella:

Twenty five gram of sample was weighed and taken into a warring blender flask. 225 ml. of buffered peptone water was added into the sample and then homogenized for 1 minute. Sample was incubated at 37+1 C for 24 hours.

The resuscitated culture was gently mixed. 1.0 ml of the mixture was added each to 10.0 ml. of Selenite broth and 10.0 ml of Tetrathionate broth .The selective enrichment broth was then incubated at 37⁰C for 24 hours. After incubation loopful of each of the two selective enrichment broths were transferred to the surface of the selective agar media, i.e. XLD agar. The plates were then incubated at 37⁰ C for 24 hours and observed for characteristics colonies for *Salmonella sp.* After 24-48 hours; *Salmonella sp.* shows pink colonies with black centre of H₂S on XLD.

3.5 PCR test

3.5.1 Preparation of media and culture of organism

Luria-Bertani (LB) broth (Difco Laboratories, MI, USA) was prepared by mixing 2g LB powder in 100 ml water and then it was autoclaved (121⁰ C, 20 min), then 1 ml LB broth was taken into 1.5 ml centrifuge tube (Eppendorf, Hamburg, Germany) where 10 µl samples (water, sediment and shrimp intestine) were added and culture was carried out in a shaking incubator at 37⁰C overnight. A pair of 1.5 ml tube was taken into culture for each sample (water, sediment and shrimp intestine). Following overnight culture, the bacteria sample was used for DNA extraction (Ausubel *et al.*, 1990).

3.5.2 DNA Extraction

According to the Invitrogen manual for DNAzol Reagent, bacterial DNA was extracted from the cultured bacteria. DNAzol Reagent is a complete and ready-to-use reagent for the isolation of genomic DNA isolation solid and liquid samples. The DNAzol Reagent procedure is based on

the use of a novel guanidine-detergent lysing solution which permits selective precipitation of DNA from a cell lysate. DNAzoL Reagent is an advanced DNA isolation reagent that combines both reliability and efficiency with simplicity of the isolation protocol. The DNAzoL Reagent protocol is fast and permits isolation of genomic DNA from a large number of samples of small or large volumes.

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

3.5.3 Qualitative and quantitative determination of extracted DNA

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

3.5.4 Components for DNA amplification in PCR

3.5.4.1 Description of Target genes and primers

Target genes: The target genes were chosen for this investigation included: Aer gene for *Aeromonas*, hilA gene for *Salmonella sp.*, virA gene for *Vibrio*, Sec gene for *Staphylococcus aureus*, neurotoxin gene type A,B,E,F for *Clostridium botulinum*, internalin gene for *Listeria* ipaH gene for *Shigella*. Seven pairs of specific primers were designed from above mentioned gene sequence (Table 3.1).

Table 3.1: Specific gene for pathogenic bacteria detection.

Specie	Specific Gene	Accession No.	References
<i>Aeromonas sp.</i>	aerolysin toxin (Aer) gene	M 84709	Kannan <i>et al.</i> ,2001
<i>Clostridium Botulinam</i>	botulinum neurotoxin gene type A, B, E, or F		Lindstrom <i>et al.</i> , 2001
<i>Listeria monocytogenes</i>	internalin gene	M67471	Ingiani <i>et al.</i> ,2001
<i>Salmonella sp.</i>	hilA gene		Pathmanathan <i>et al.</i> ,2003
<i>Shigella sp.</i>	invasion plasmid antigen H gene (ipaH)	M32063	Thiem <i>et al.</i> ,2004
<i>Vibrio sp.</i>	rpoB, toxR gene, vvhA gene, virA gene	D26468	Tarr <i>et al.</i> ,2007
<i>Staphylococcus aureus</i>	Sec gene		Mehrotra <i>et al.</i> ,1999

Oligonucleotide primers: The DNA fragment to be amplified is determined by selecting primers. Primers are short, artificial DNA strands that are complementary to the beginning and the end of the DNA fragment to be amplified. They anneal to the DNA template at these starting

and ending points, where the DNA-polymerase binds and begins the synthesis of the new DNA strand. In this study, seven pairs of primers were used. Sequences of the seven PCR primer pairs and size of expected amplification products are shown in table 3.2.

Table 3.2: Primers and expected size of PCR amplified gene targets of pathogenic bacteria.

Specie	Primers	Sequence (5' → 3')	Product size (bp)	Temp
<i>Aeromonas</i>	Aero-F	ATGACCCAGTCCTGGCACGG	480	61
	Aero-R	GCCGCTCAGGGCGAAGCCGC		
<i>Clostridium Botulinam</i>	Clos-F	AGCTACGGAGGAGCTATGTT	782	52.5
	Clos-R	CGTATTTGGAAAGCTGAAAAGG		
<i>Listeria monocytogenes</i>	List-F	ACTATCTAGTAACACGATTAGTGA	250	51
	List-R	CAAATTTGTTAAAATCCCAAGTGG		
<i>Salmonella sp.</i>	Salm-F	CGGAACGTTATTTGCGCCATGCTGAGGTAG	784	65.5
	Salm-R	GCATGGATCCCCGCCGCGAGATTGTG		
<i>Shigella sp.</i>	Shig-F	TGCTGATGCCACTGAGAGCT	939	51.5
	Shig-R	CGTCGATTGTTATATCAGTCTA		
<i>Vibrio sp.</i>	Vibr-F	CGGTGAAATGCGTAGAGAT	663	49.5
	Vibr-R	TTACTAGCGATTCCGAGTTC		
<i>Staphylococcus aureus</i>	Stap-F	AGATGAAGTAGTTGATGTGTATGG	451	50
	Stap-R	CACACTTTTAGAATCAACCG		



The final mixture for the PCR test was 25 μ l. In the mixture, to prepare 1 unit *Taq* DNA polymerase, it was 3 times diluted with de-ionized water. The components of the mixture are shown in table 3.3.

Table 3.3: The component of reaction mixture for the PCR.

Component	Final volume
1. Di- ionized water	13 μ l
2. 10X Reaction Buffer (Tris; pH 9.0, 15 mM MgCl ₂)	5 μ l
3. 10 mM dNTP mixture (each dNTP 2.5 mM)	2 μ l
4. Template	2 μ l
5. Primers	2 μ l (Forward 1 μ l + Reverse 1 μ l)
6. A <i>Taq</i> Polymerase (5 units/ μ l; Top DNA Polymerase, Bioneer, Korea)	1 μ l

3.5.5 Top DNA polymerase enzyme

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

3.5.6 Deoxynucleotide Triphosphates (dNTPs) mixture

In the present experiment, 10 mM dNTP mixture (each dNTP 2.5 mM) was used (Bioneer, Korea).

3.5.7 Buffers used for PCR

10x Reaction buffer (Tris; pH 9.0, 15 mM MgCl₂) was used for PCR test.

3.5.8 DNA amplification by a thermal cycler

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

Before PCR, made PCR sample by the following way, in Brief, 2 µl DNA extract were directly used as template in each PCR-tube for the reaction. The reaction mixture consisted of 1 µl primer (Forward 1 µl + Reverse 1 µl), 5 µl 10x Reaction buffer, 2 µl dNTPs, 2 µl 1 unit Top DNA Polymerase enzyme as well. These all components were mixed in 13 µl distill water and thus a total of 20 µl PCR sample was prepared in a 0.2 ml PCR-tube (Eppendorf, Hamburg, Germany) to operate in a thermal cycler.

3.5.9 Evaluation of PCR products

After completion of the thermal cycling, a sample of 8 µl from each PCR products was analyzed electrophoretically by running on a 2% agarose gel and the amplified product size was determined by comparison with a 100 bp DNA size marker.

Chapter Four

RESULTS & DISCUSSION

4. Results and Discussion

To analyze the bacteriological quality of fresh shrimps, five habitats (as described in materials and methods) were chosen as representative habitat in Tala Upazilla of Satkhira district of Bangladesh. The water quality of selected five farms was in unhygienic condition. Among the five farms, farm-4 was comparatively in better quality. Its water quality was good than others. Semi-intensive culture was practiced in farm four and traditional culture was practiced in other four farms.

The MPN of total and faecal coliforms in pooled samples was enumerated using LSTB and BGLB by three tubes of three different 10-fold dilutions. Table-4.1 showed that the MPN of total coliforms is at least 1.30×10^4 per 100 ml of water sample in farm-3 and highest in farm-2 that represents the MPN of total coliforms 6.50×10^4 per 100 ml of water sample. For sediment samples, the MPN of total coliforms is highest in both farm-1 and farm-2, that is 4.47×10^4 per gm of sediment and, the MPN of total coliforms is lowest in farm-3, which is 1.50×10^4 per gm of sediment. The MPN of faecal coliforms was comparatively low than total coliforms in shrimps of that farms.

However, fresh shrimps of five farms had comparatively less number of total and faecal coliforms. But for sediment and water sample, coliforms were comparatively high when we compare to fresh shrimp sample.

Besides the counting of total coliforms and faecal coliforms, we have detected and isolated the *Vibrio cholera* and *Salmonella sp.* by biochemical tests. Among the 15 samples of 5 farms, only one sample represent *Salmonella sp.* and 4 samples represent *Vibrio cholera*.

Table-4.1: The biochemical test result of the total coliform ,faecal coliforms and the presence of *vibrio cholerae* and *salmonella sp.* in 5 water sample, 5 sediment sample and 5 shrimp sample are represented below in graphically.

Farm	Sample	Total Coliforms MPN/gm	Faecal coliforms MPN/gm	Presence of	
				<i>Salmonella</i>	<i>Vibrio</i>
F-1	Water	4.39×10^4	3.10×10^4	-	+
	Sediment	4.47×10^4	2.40×10^3	-	-
	Shrimp	2.40×10^2	2.4×10^2	-	-
F-2	Water	6.50×10^4	4.50×10^3	-	+
	Sediment	4.47×10^4	2.40×10^3	+	-
	Shrimp	2.4×10^2	1.1×10^2	-	-
F-3	Water	1.30×10^4	1.70×10^3	-	+
	Sediment	1.50×10^4	1.40×10^2	-	-
	Shrimp	1.10×10^2	15	-	+
F-4	Water	2.40×10^4	1.30×10^4	-	-
	Sediment	2.20×10^4	1.40×10^2	-	-
	Shrimp	2.40×10^2	0	-	-
F-5	Water	4.40×10^4	2.50×10^4	-	+
	Sediment	2.47×10^4	5.40×10^3	-	+
	Shrimp	1.10×10^2	23	-	-

We have done PCR test to sure the presence of pathogenic bacteria in collected sample. Seven pairs of oligonucleotide primers were designed in this study to simultaneously amplify various virulence-associated genes of pathogenic bacteria (*Aeromonas*, *Clostridium botulinum*, *Listeria monocytogenes*, *Salmonella sp*, *Shigella sp*, *Vibrio cholera*, *Staphylococcus aureus*) in a single tube. In the PCR analysis, the PCR product was amplified with designed primers and bacterial extracted DNA of five shrimp farms. The summery of the results were shown in table 4.2.

Table 4.2: Summery of the PCR results

Farm		<i>Aeromonas</i>	<i>Clostridium</i>	<i>Listeria</i>	<i>Salmonella</i>	<i>Shigella</i>	<i>Vibrio</i>	<i>Staphyloccus</i>
F-1	Water	+	-	-	-	-	+	-
	Sediment	+	-	-	-	-	-	-
	Shrimp	-	-	-	-	-	-	-
F-2	Water	+	-	-	-	-	+	-
	Sediment	-	-	-	+	-	-	-
	Shrimp	-	-	-	-	-	-	-
F-3	Water	+	-	-	-	-	+	-
	Sediment	-	+	-	-	-	-	-
	Shrimp	-	-	-	-	-	+	-
F-4	Water	-	-	-	-	-	-	-
	Sediment	-	-	-	-	-	-	-
	Shrimp	-	-	-	-	-	-	-
F-5	Water	+	-	-	-	-	+	-
	Sediment	-	-	-	-	-	+	-
	Shrimp	-	-	-	-	-	-	-

Farm-01

In the case of farm 1, in water, two bands of target genes were found on electrophoresis gel at 480 bp for *Aeromonas sp* and 663 bp position for *Vibrio cholerae* which indicated the presence of pathogenic *Aeromonas sp* and *Vibrio cholerae* in water sample of farm-01. For sediment sample only *Aeromonas sp* is observed but for shrimp sample no pathogenic bacteria was observed. The water quality of farm-1 was polluted. Traditional culture was practiced and there was no monitoring of water quality.

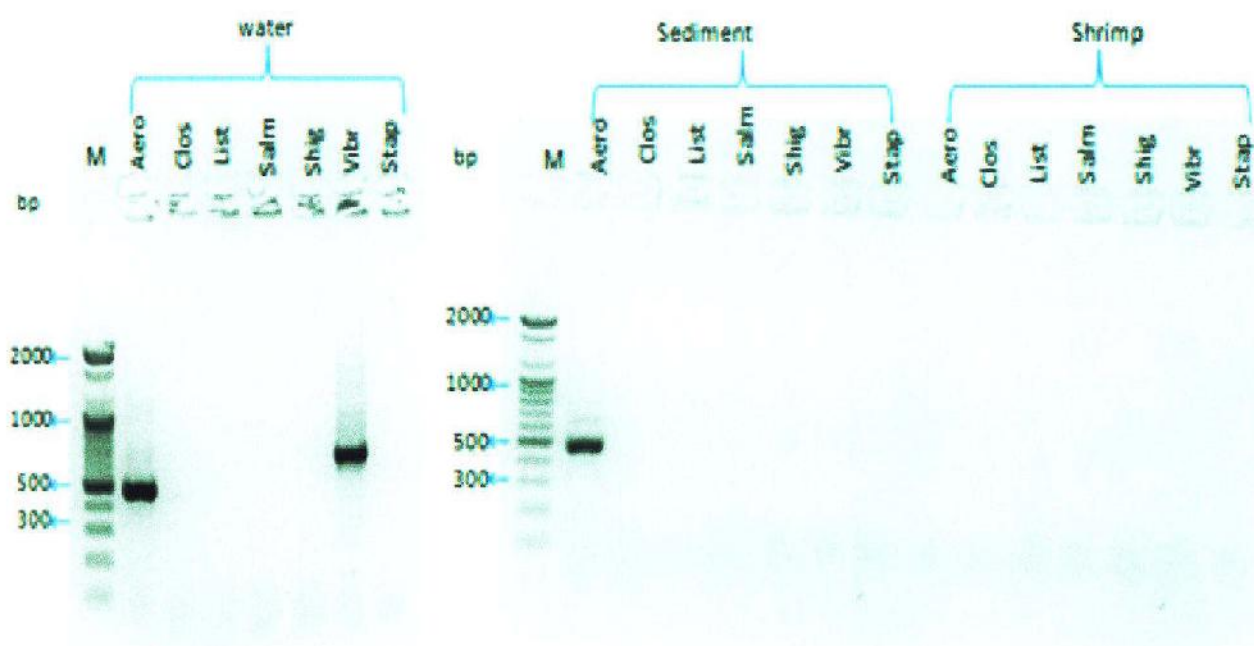


Figure 4.1: UV visualization of PCR product of farm 3 following electrophoresis in a 2% agarose gel. Lanes 1 to 7, 8 to 14 and 15 to 21 represent water, sediment and intestine of shrimp respectively where 7 primers (Aero, Clos, List, Salm, Shig, Vibr, Stap) is used for each sample. Lane M represents 100 bp DNA ladder or marker.

Farm- 02

In the case of farm 2, in water, two bands of target genes were found on electrophoresis gel at 480 bp for *Aeromonas sp* and 663 bp position for *Vibrio cholerae* which indicated the presence of pathogenic *Aeromonas sp* and *Vibrio cholerae* in water sample of farm-2. For sediment sample pathogenic *Salmonella sp* is observed at 784 bp position but for shrimp sample no pathogenic bacteria was found.

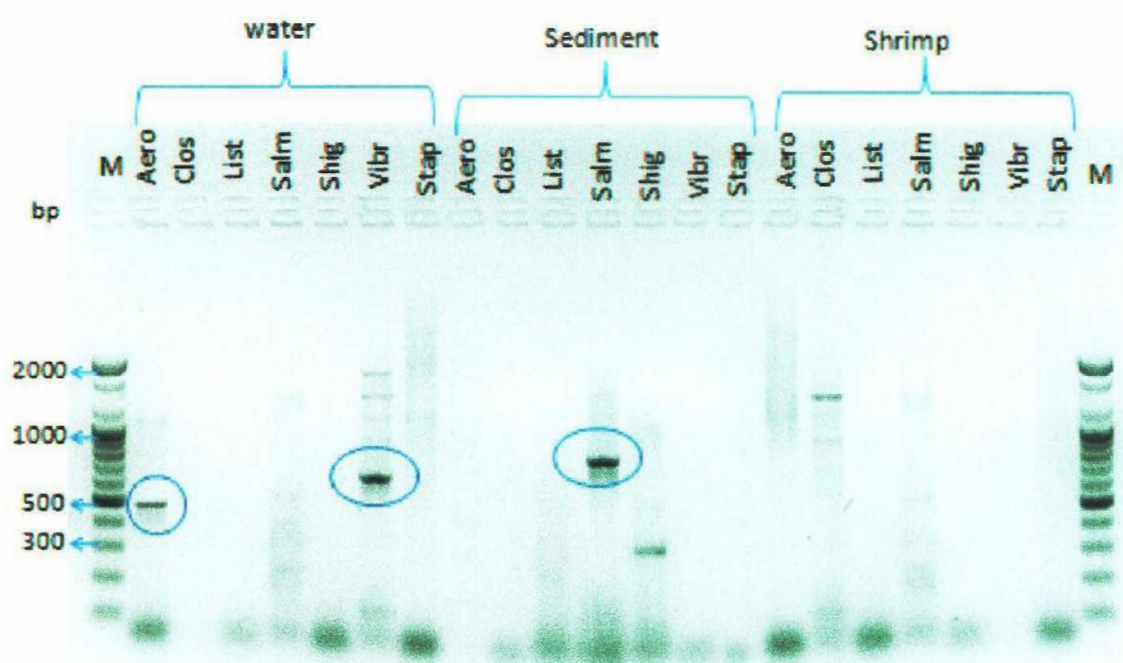


Figure 4.2: UV visualization of PCR product of farm 3 following electrophoresis in a 2% agarose gel. Lanes 1 to 7, 8 to 14 and 15 to 21 represent water, sediment and intestine of shrimp respectively where 7 primers (Aero, Clos, List, Salm, Shig, Vibr, Stap) is used for each sample. Lane M represents 100 bp DNA ladder or marker.

Farm -03

In the water sample of farm 3, two bands of target genes were found on electrophoresis gel at 480 bp for *Aeromonas sp.* and 663 bp position for *Vibrio cholerae* which indicated the presence of pathogenic *Aeromonas sp.* and *Vibrio cholerae* in water sample of farm-3. For sediment sample pathogenic *Clostridium botulinum* is observed at 782 bp position but for shrimp sample pathogenic *Vibrio cholera* was present at 663 bp position ,

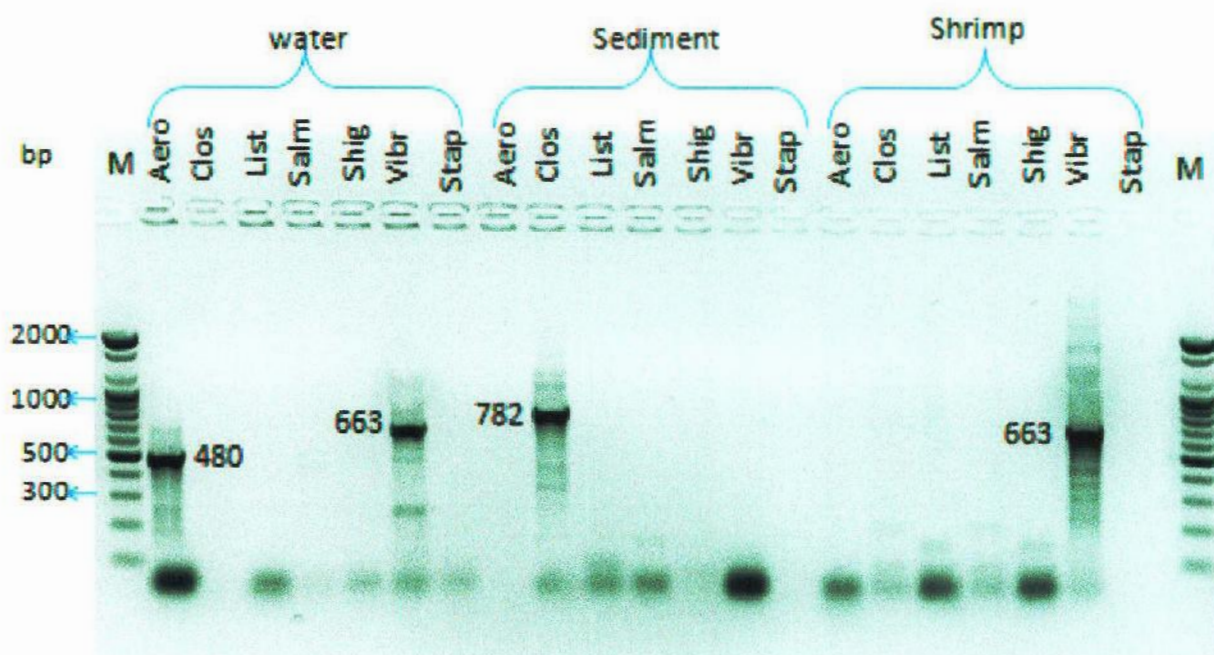


Figure 4.3: UV visualization of PCR product of farm 3 following electrophoresis in a 2% agarose gel. Lanes 1 to 7, 8 to 14 and 15 to 21 represent water, sediment and intestine of shrimp respectively where 7 primers (Aero, Clos, List, Salm, Shig, Vibr, Stap) is used for each sample. Lane M represents 100 bp DNA ladder or marker.

Farm -04

There was no evidence of pathogenic bacteria (*Aeromonas*, *Clostridium botulinum*, *Listeria monocytogenes*, *Salmonella sp*, *Shigella sp*, *Vibrio cholera*, *Staphylococcus aureus*) in the water, sediment and shrimp sample of farm 4.

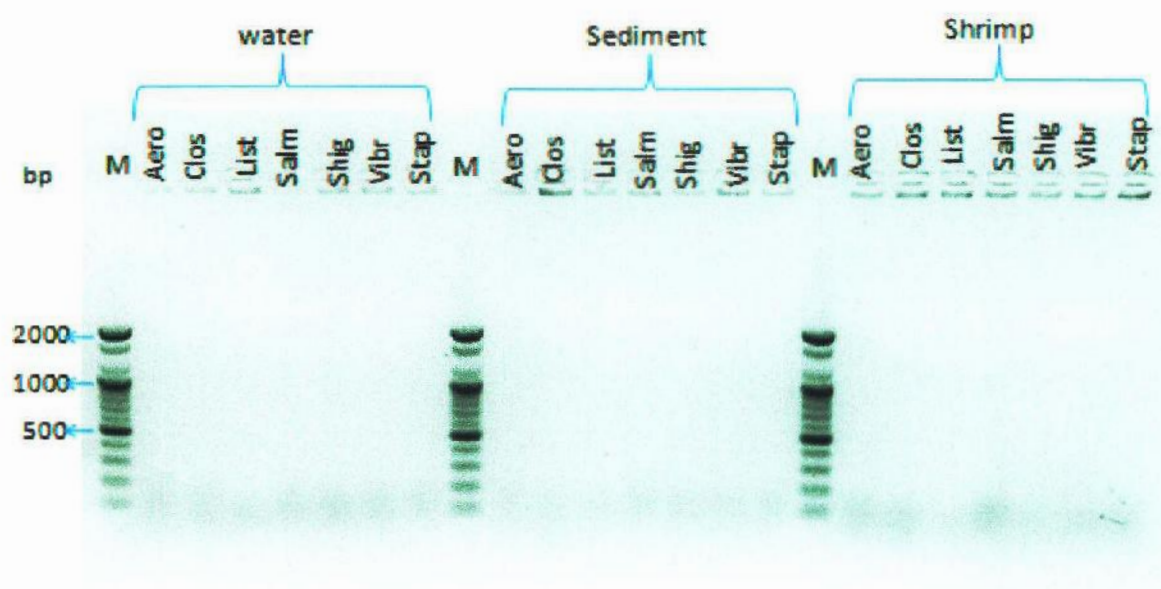


Figure 4.4: UV visualization of PCR product of farm 4 following electrophoresis in a 2% agarose gel. Lanes 1 to 7, 9 to 15 and 17 to 23 represent water, sediment and intestine of shrimp respectively where 7 primers (Aero, Clos, List, Salm, Shig, Vibr, Stap) is used for each sample. Lane M represents 100 bp DNA ladder or marker.

Farm -05

Aeromonas, *Vibrio cholera* in water sample and *Vibrio cholera* in sediment sample for farm-05 is detected by PCR where *Aeromonas* was observed on 480 bp position and *Vibrio cholera* was observed on 663 bp position. For shrimp sample, pathogenic bacteria was not found.

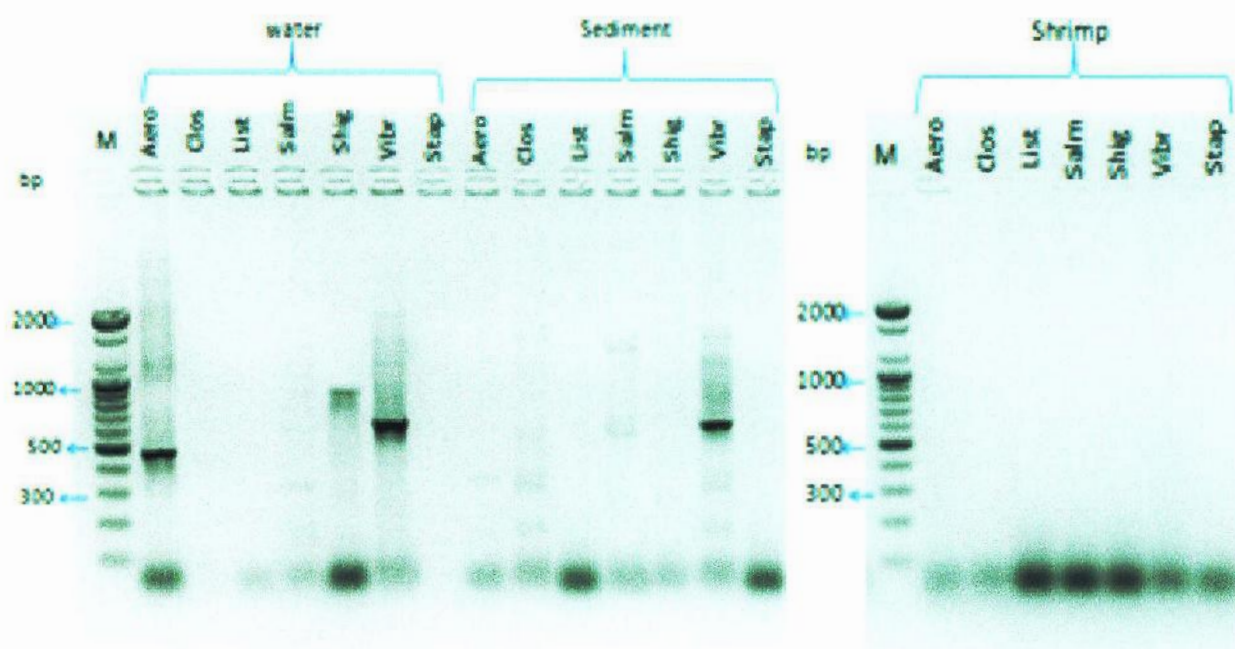


Figure 4.5: UV visualization of PCR product of farm 5 following electrophoresis in a 2% agarose gel. Lanes 1 to 7 and 8 to 14 represent water, sediment respectively where 7 primers (Aero, Clos, List, Salm, Shig, Vibr, Stap) is used for each sample. For intestine of shrimp sample Lane 1 to 7 which represent *Aero*, *Clos*, *List*, *Salm*, *Shig*, *Vibr*, *Stap* serially. Lane M represents 100 bp DNA ladder or marker.

In this study, all shrimp farms sample represent a moderate quantity of total coliform and faecal coliform. In the farm 3, 4 and 5 there were small portion of faecal coliform in shrimp intestine but high in water and sediment. Because most of the selected farms were located in unhygienic condition. There was hanging latrine and the water monitoring system is very bad. In most of the water and sediment sample pathogenic *Vibrio cholera* and *Aeromonas sp* was found.

The entire bottom water sample contains higher bacterial load then surface water samples. It may happen due to that the most of the organic materials; debris and living organism including shrimp exist in the bottom of the pond that acts as substratum for the bacteria.

In any aquatic system, environmental parameters such as temperature, salinity, pH and dissolved oxygen play a foremost part in the temperature 27-31°C; salinity 14 -25 ppt, pH 7.8-8.6 and distribution of bacteria (Palaniappan, 1982). But, pond being a confined environment the optimum environmental parameters (temperature 27-31°C; salinity 14 -25 ppt, pH 7.8-8.6 and DO 5.1 -5.9 ppm) could be maintained throughout the culture period by proper pond management involving water exchange and lime application. The bacterial loads might have changed due to the organic matter deposited at the pond bottom irrespective of environmental factors and water exchange, which was supported by Palaniappan (1982). They suggested that environmental parameters did not influence the distribution of bacterial load in the pond ecosystem because there was no dramatic change in the environmental parameters.

Due to the influence of turbidity, heavy organic matter plays a major role in the distribution of bacterial population in pond water (Sindermann C.J., 1979). High organic stuff is possible in shrimp culture ecosystem due to organic manure, fertilizers, high stocking density, feed waste, fecal matter, algal bloom and human interference (Moriarty, 1997; Lloberra *et al.*, 1991).

Shrimp ponds are stressful environments compared to estuaries or other enclosed water bodies (Direkbusararam *et al.*, 1998). This is mainly due to high organic matter and dissolved oxygen fluctuations which affect the composition of natural bacterial communities. Under normal conditions, temperature increments will also bring about a greater diversity of *Vibrio* species (Barbieri, *et al.*, 1999; Pfeffer, *et al.*, 2003). When high temperatures and high salinity conditions prevail, some species such as *Vibrio parahaemolyticus* may predominate (Williams and LaRock, 1985).

In shrimp culture ecosystem, most of the bacteria play a negative role as they compete with shrimps for food and oxygen, causing stress and disease (Moriarty, 1997). Generally gram-negative bacteria were found to be the dominant forms in the shrimp culture ponds (Sung *et al.*, 2003) as noticed in the present investigation.

The Total Coliform, *Salmonella* and *Vibrio* bacteria play a vital role in shrimp culture ecosystem (Ruangpan and Kitao, 1991) as they damage water quality causing diseases and mortality to the shrimp as primary and secondary pathogens. *Vibrio* sp. is the autochthonous flora of the coastal pond ecosystem (Ruangpan and Kitao, 1991). A sudden increase of bacterial load could develop bacterial infection directly and making shrimps susceptible to infection indirectly as found by Ping and Meimei (1994).

Efficient sample treatment methods are needed to fully exploit the potential of the PCR technique to detect pathogens due to the presence of PCR inhibitors (Rossen *et al.*, 2002). Consequently, several treatments to concentrate DNA for the PCR detection of pathogenic bacteria strains (*Salmonella*, *Shigella*, *Vibrio*, *Listeria* sp.) in shrimp farms and to remove PCR inhibitors have been suggested e.g., lysozyme, proteinase K, detergents by centrifugation and

filtration (Kroll, 1993), use of proteinase K and organic solvents such as phenol, chloroform and iso-amyl alcohol for deproteinizing cell digests (Lo *et al.*, 1997).

During the baseline survey, hanging toilet, cattle field were observed near the farms or at the canals or rivers which is the main source of water in shrimp farms. So, these could be the possible source of *E. coli* in the farms (Karunasagar *et al.*, 1999).

The present study was carried out to investigate the availability of pathogenic seven types of bacteria strains in the shrimp farms. The presence of various strains was observed among the four studied farms except farm 4.

However considering higher count and prevalence of total and faecal coliforms in almost all of the samples under investigation, it can be inferred that the shrimp cultures in Satkhira district of Bangladesh are exposed to faecal contamination, indicating the contamination by other pathogens also, and further suggested that these shrimps may contain bacterial toxin even after processing and may cause risk for health. The PCR test of pathogenic bacteria confirmed some pathogenic strain in the samples that show the unhygienic condition of the farms.

Depending on the results of this study, the following suggestions may be made to prevent bacterial contamination of shrimps- (i) hygienic conditions for shrimp cultivation should be maintained, (ii) routine microbial analysis of feed is necessary before use, (iii) the feed manufacturer should maintain the microbial quality, (iv) aseptic shrimp seed multiplication farm should be established, (v) water quality should be routinely tested and (vi) awareness should be developed among the personnel associated with this industry. This study will contribute to detect pathogenic bacteria in short time, so that one can understand the fact of bacteriological quality of fresh shrimps and shrimp farms, and to adopt strategies to improve the shrimp quality.



Chapter Five

CONCLUSION

5. Conclusion

Bangladesh is a country with high incidence of bacterial diseases in shrimp farms due to the most of our water body is not hygienic. Every year vast amount of shrimps is affected in bacterial diseases. It is threatened of human health and disease our total shrimp production. From our present study it may be concluded that shrimp culturing ponds in southern part of Bangladesh are getting contaminated with pathogenic bacteria. Therefore, it is very important to pay attention to good rearing practice, hygiene, use of antibiotic supplemented feed and pollution in the shrimp rearing ponds. Furthermore, investigation and research are required to find out the root cause of different pathogenic bacteria such as *Salmonella sp*, *Vibrio cholera*, *Shigella sp*, *Aeromonas sp*, *E.coli* and *Staphylococcus sp*. in shrimp rearing ponds in Satkhira, Bangladesh. The application of PCR based techniques is beginning a new era of excellence in the field of fish disease analysis. The findings reported in this study describe a versatile, reliable and highly sensitive PCR system for the rapid detection of pathogenic bacterial species in shrimp farm samples. At present, the main goal of the shrimp industry is to meet the growing demand in a sustainable manner without damaging the environment. Further research is required to enable high-quality validation and trial of these methods as we move into the next century of shrimp health research and development.

Chapter Six

REFERENCES

6. Reference

- Andrews, L.S. 2004. Strategies to control *Vibrio* in Molluscan Shellfish. *Food Protection Trends* 24:70-76
- Austin, B. and Austin, D. 1993. *Bacterial Fish Pathogens: Disease in farmed and wild fish* (Ed. Laird, M.). Ellis Horwood, Chichester U.K. pp. 264-269
- Ayulo, A. M., Machado, R. A. and Scussel, V. M. 1994. Enterotoxigenic *Escherichia coli* and *Staphylococcus aureus* in fish and seafood from the southern region of Brazil. *International Journal of Food Microbiology* 24:1-2
- Barbieri, E., Falzano, L., Fiorentini, C., Pianetti, A., Matarrese, P., Casiere, A., Katouli, M., Kuhn, I., Mollby, R., Bruscolini, F. and Donelli, G. 1999. Occurrence, Diversity, and Pathogenicity of Halophilic *Vibrio spp.* and Non-O1 *Vibrio cholerae* from Estuarine Waters along the Italian Adriatic Coast. *Applied and Environmental Microbiology* 65:2748-2753
- Barski, P., Piechowicz, L., Galinski, J. and Kur, J. 1996. Rapid assay for detection of methicillin-resistant *Staphylococcus aureus* using multiplex PCR. *Molecular Cellular Probes* 10:471-475
- Bettelheim, K. A. 1992. *The Genus Escherichia*. In *The Prokaryotes* (Vol. III), eds. Balows, A., Truper, H. G., Dworkin, M., Harder, W. and Schleifer, K.H, Springer, New York, pp.2696-2736
- Kingombe, C. I., Huys, G., Tonolla, M., Albert, M. J., Swings, J., Peduzzi, R. and Jemmi, T. 1999. PCR detection, characterization and distribution of virulence genes in *Aeromonas spp.* *Applied Environmental Microbiology* 65:5293-5302

- Cappuccino, J. G., Sherman, N. 2007. Microbiology: A Laboratory Manual. The Benjamin Cummings Publishing, Redwood City, California.
- Cesar, K. B., Geert, B. and Thomas, J., 1999, PCR Detection, Characterization, and Distribution of Virulence Genes in *Aeromonas* spp. *Applied and Environmental Microbiology*, 65:5293–5302.
- Chiu, C. H., and Ou, J. T., 1996. Rapid identification of *Salmonella* serovars in feces by specific detection of virulence genes, *invA* and *spvC*, by an enrichment broth culture-multiplex PCR combination assay. *Journal of Clinical Microbiology*, 34: 2619–2622.
- Cunningham, C. O., 1997. Species variation within the internal transcribed spacer (ITS) region of *Gyrodactylus* (Monogenea; Gyrodactylidae) ribosomal RNA genes. *Journal of Parasitology*, 83:215–219.
- Cunningham, C. O., 2002. Molecular diagnosis of fish and shellfish diseases: present status and potential use in disease control. *Aquaculture Journal*, 206:19–55.
- Direkbusaram, S., Yoshimizu, M., Ezura, Y., Ruangpan, L. and Danayadol, Y., 1998. *Vibrio* sp, the dominant flora in shrimp hatchery against some fish pathogenic viruses. Short Communication.
- DoF (Department of Fisheries), 2012. *Jatio Matsha Soptah* 2012. Ministry of Fisheries and Livestock. Government of the Peoples Republic of Bangladesh, pp. 102-107. (In Bangla)
- Eduardo, V. and Antonio, T. 1998. PCR for Detection of *Shigella* spp. in Mayonnaise. *Applied and Environmental Microbiology*, p. 1242–1245
- Fewtrell, L. and Bartram, J., 2001. *Water Quality: Guidelines, Standards and Health*. World Health Organization Water Series IWA Publishing, London, UK

- Frappalo, P. J. and Guest, G. B. 1986. Regulatory status of tetracyclines, penicillin and other antimicrobial microbials drugs in animal feeds. *Journal of Animal Sciences* 62: 86-92
- Fredrickson, J., Zachara, J. and Balkwill, D. 2004. Bacteria. *Applied Environment Microbiology* 70:4230-4241
- Fricker, C. R. 1987. The isolation of *salmonellas* and *campylobacters*. *Journal of Applied Bacteriology* 63: 99-116
- Gasanov, U., Hughes, D. and Hansbro, P. M. 2005. Methods for isolation and identification of *Listeria spp.* and *Listeria monocytogenes*: a review. *FEMS Microbial Reviews* 29: 851-875
- Gentry-Weeks, C., Hutcheson, H. J., Kim, L. M., Bolte, D., Traub- Dargatz, J., Morley, P., Powers, B. and Jessen, M. 2002. Identification of two phylogenetically related organisms from feces by PCR for detection of *Salmonella spp.* *Journal of Clinical Microbiology* 40: 1487-1492
- Giovanna, F. and Paolo, A. 1996. PCR for Detection of *Clostridium botulinum* Type C in Avian and Environmental Samples. *Journal of Clinical Microbiology* 34: 882-885
- Goarant, C., Merien, F., Berthe, F., Mermoud, I. and Perolat, P. 1999. Arbitrarily Primed PCR To Type *Vibrio spp.* Pathogenic for Shrimp. *Applied and Environmental Microbiology* 65:1145-1151
- Ingianni, A., Floris, M., Palomba, P., Madeddu, M.A., Quartuccio, M. and Pompei, R. 2001. Rapid detection of *Listeria monocytogenes* in foods, by a combination of PCR and DNA probe. *Molecular and Cellular Probes* 15: 275-280
- Inglis, V., Roberts, R.J. and Bromage, N.R. 1993. Bacterial diseases of fish. *Blackwell Scientific Publications Oxford*



- Kannan, S., Kanna, S. P., Karkuzhali, K., Chattopadhyay, U. K. and Pal, D. 2001. Direct detection of diarrheagenic *Aeromonas* from faeces by polymerase chain reaction (PCR) targeting aerolysin toxin gene. *European Review for Medical and Pharmacological Sciences* 5: 91-94
- Kannapiran, E., Ravindran, J., Chandrasekar, R. and Kalaiarasi, A. 2009. Studies on luminous, *Vibrio harveyi* associated with shrimp culture system rearing *Penaeus monodon*. *Journal of Environmental Biology* 30(5):791-795
- Karunasagar, I., Karunasagar, I. and Reily, A. 1999. *Aquaculture and Biotechnology*. Oxford and IBH publishing Co. Pvt. Ltd. New Delhi, India, Pp. 71-86
- Karunasagar, I., Otta, S.K. and Karunasagar, I. 1996. Biofilm formation by *Vibrio harveyi* on surfaces. *Aquaculture* 140: 241-245
- Karunasagar, I., Pai, R., Malathi, G.R. and Karunasagar, I. 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. *Aquaculture* 128: 203-209
- Keusch, G. T. 2002. Systemic gastro-intestinal infections: a clinical overview. In *Molecular Medical Microbiology* 2:1357-1363
- King, M. 1997. *Fisheries Biology, Assessment and Management*. 3rd edition. Fishing News Books, Blackwell Science Ltd., London, pp111
- Korl, R. Y. C., Dung, W. F., Vrijmoed, L. L. P. and Wu, R. S. S. 1995. Co-detection of three species of waterborne bacteria by multiplex PCR. *Marine Pollution Bulletin* 31: 317-324
- Leong, J.C. 1995. Molecular biological tools to detect fish pathogens. *Journal of Fish Biology* 47: 61-75

- Lightner, D. V. 1998. *Diseases of cultured Penaeid shrimps and prawns*. In Disease diagnosis and control in North American marine aquaculture, second edition
- Lightner, D.V. Ed. 1996. *A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases of Cultured Penaeid Shrimp*. World Aquaculture Society, Baton Rouge, LA, USA, pp. 305
- Lindstro, M., Keto, R., Markkula, A., Nevas, M., Hielm, S. and Korkeala, A. H. 2001. Multiplex PCR Assay for Detection and Identification of *Clostridium botulinum* Types A, B, E, and F in Food and Fecal Material. *Applied and Environmental Microbiology* 67: 5694–5699
- Liu, P.C., Lee, K.K. and Chen, S.N. 1996. Pathogenicity of different isolates of *Vibrio harveyi* in tiger prawn, *Penaeus monodon*. *Applied Microbiology* 22: 413–416
- Liu, P.C., Lee, K.K., Yii, K.C., Kou, G.H. and Chen, S.N. 1996. Isolation of *Vibrio harveyi* from diseased Kuruma prawns *Penaeus japonicus*. *Curr. Microbiology* 33: 129–132
- Lo, C. F., Ho, C. H., Chen, C. H., Liu, K. F., Chiu, Y. L., Yeh, P. Y., Peng, S. E., Hsu, H. E., Liu, H. C., Chang, C. F., Su, M. S., Wang, C. H. and Kou, G. H. 1997. Detection and tissue tropism of white spot syndrome baculovirus (WSBV) in captured brooders of *Penaeus monodon* with a special emphasis on reproductive organs. *Diseases of Aquatic Organism* 30: 53-72
- Lovseth, A., Loncarevic, S. and Berdal, K.G. 2004. Modified Multiplex PCR Method for Detection of Pyrogenic Exotoxin Genes in Staphylococcal Isolates. *Journal of Clinical Microbiology* 42: 3869–3872
- Lundin, G. G. 1996. *Global attempts to address shrimp disease*. Marine Environmental, Land, water and Natural Habitats Division, Environment Department . The world Bank Report. p.45

- McBeath, A. J. A., Burr, K. L. A. and Cunningham, C. O. 2000. Development and use of a DNA probe for confirmation of cDNA from infectious salmon anaemia virus (ISAV) in PCR products. *Bull. European Association, Fish Pathology* 20: 130–134
- McKeever, D.J. and Rege, J. E. O. 1999. Vaccines and diagnostic tools for animal health: the influence of biotechnology. *Livestock Production Science* 59: 257–264
- Moriarty, D. J. W. 1997. The role of microorganisms in aquaculture ponds. *Aquaculture* 151: 333–349
- Nataro, J. P. and Kaper, J. B. 1998. Diarrheagenic *Escherichia coli*. *Clinical Microbiology* 11: 142–201
- Olafse, J.A., Mikkelsen, H.V., Giaver, H.M. and Hansen, G.H. 1993. Indigenous bacteria in haemolymph and tissues of marine bivalves at low temperatures. *Applied and Environmental Microbiology* 59:1848–1854
- Palaniappan, R. 1982. *Studies on the microflora of the shrimp Penaeus indicus, Milne Edwards (crustacea, decapodes, penaeidae) with reference to its digestive system*. Ph.D Thesis, Annamalai University, India
- Panicker, G. and Bej, A.K. 2005. Real-Time PCR Detection of *Vibrio vulnificus* in Oysters: Comparison of Oligonucleotide Primers and Probes Targeting *vvhA*. *Applied and Environmental Microbiology* 71:5702–5709
- Patel, S., Yates, M. and Saunders, N. A. 1997. PCR-enzyme-linked immunosorbent assay and partial rRNA gene sequencing: a rational approach to identifying mycobacteria. *Journal of Clinical Microbiology* 35: 2375–2380

- Pathmanathan, S.G., Cardona-Castro, N., Sanchez-Jime, M.M., Puthucheary, S.D. and Thong, K.L. 2003. Simple and rapid detection of Salmonella strains by direct PCR amplification of the hilA gene. *Journal of Medical Microbiology* 52: 773–776
- Paul, S. K. 1997. *Shrimp Disease Management*. Notun Paltanlane, Dhaka, pp. 1-17
- Paul, S. K., 2007. *Shrimp: Hatchery, Modern Culture Technology and Disease Management*. 3rd edition. Mamin Ofset Press, Nilkhet, Dhaka. pp. 210-236
- Pelczar, M. J., Chan, E. C. S. and Krieg, N. R. 1998. *Microbiology*. 5th Edn., Mcgraw-Hill College, 5261 Highland Road, PMB 339 Baton Rouge, La 70808, pp: 596-599
- Perkins, S. L. and Martin, J. M. 1999. Conserved polymerase chain reaction primers fail in diagnosis of parasitic infections. *Journal of Parasitology* 85: 982–984
- Pfeffer, C. S., Hite, F. M. and Oliver, J. D. 2003. Ecology of *Vibrio vulnificus* in Estuarine Waters of Eastern North Carolina. *Applied and Environmental Microbiology* 69:3526–3531
- Ping, G. and Miemei. 1994. The bacterial variations in the water environment of cultured shrimp pond. *Oceanol.Limnol.Sin.Haiyang. Yu.Huzhao* 25: 625-630
- Rossen, L., Norskov, P., Holmstrom, K. and Rasmussen, O. F. 2002. Inhibition of PCR by components of food sample, Microbial diagnostic assays and DNA-extraction solutions. *International Journal of Food Microbiology* 17: 37-45
- Ruangpan, L., and Kitao, T. 1991. *Vibrio* bacteria isolated from black tiger shrimp *Penaeus monodon* (Fabricius). *Journal of Fish Disease* 14: 383-388
- Sindermann, C. J. 1979. *Pollution-associated disease and abnormalities of fish and shell fish-A review*. Fisheries Bulletin, p:716

- Stanier, R. Y., Ingraham, J. L., Wheelis, M. L. and Painter, P. R. 1995. *General Microbiology*. 5th Edn. Macmillan Press, Ltd., London, 451: 439-441
- Stone, G. G., Oberst, R. D., Hays, M. P., McVey, S. and Chengappa, M. M. 1994. Detection of *Salmonella* serovars from clinical samples by enrichment broth cultivation-PCR procedure. *Journal of Clinical Microbiology* 32: 1742-1749
- Suwansonthichai, S. and Rengpipat, S. 2003. Enumeration of coliforms and *Escherichia coli* in frozen black tiger shrimp *Penaeus monodon* by conventional and rapid methods. *International Journal of Food Microbiology* 81: 21-113
- Tarr, C.L., Patel, J.S., Puhr, N.D., Sowers, E.G., Bopp, C.A. and Strockbine, N.A. 2007. Identification of *Vibrio* Isolates by a Multiplex PCR Assay and *rpoB* Sequence Determination. *Journal of Clinical Microbiology* 45: 134-140
- Teophilo, G. N., dos Fernandes Vieira, R. H., dos Prazeres Rodrigues, D. and Menezes, F. G. 2002. *Escherichia coli* isolated from seafood: toxicity and plasmid profiles. *International Journal of Food Microbiology* 5: 4-11
- Thaithongnum, S., Ratanama, P., Weeradechapol, K., Sukhoom, A. and Vuddhakul, V. 2006. Detection of *V. harveyi* in shrimp postlarvae and hatchery tank water by the Most Probable Number technique with PCR. *Aquaculture Journal* 261: 1-9
- Thiem, V.D., Sethabutr, O., Seidlein, L. and Trach, D.D. 2004. Detection of *Shigella* by a PCR Assay Targeting the *ipaH* Gene Suggests Increased Prevalence of Shigellosis in Nha Trang, Vietnam. *Journal of Clinical Microbiology* 42:2031-2035
- Tortora, G. J., Funke, B. R. and Case, C. L. 1995. *Microbiology-An Introduction*. 5th Edn., The Benjamin/Cummings Publishing Company, Redwood City, California, p. 753.

- Twedt, R. M. 1989. Chapter 13. *Vibrio parahaemolyticus*. Pp. 543-568. In Doyle, M.P (Eds.) Foodborne Bacterial Pathogens. Marcel Decker, Inc. USA
- Vandenberghe, J., Verdonck, J. L., Xu, H. S. and Swings, J. 1998. *Vibrios* associated with *Penaeus chinensis* (Crustacea: Decapoda) larvae and post-larvae in Chinese shrimp hatcheries. *Aquaculture Journals* 169:121-132
- Wagener, C., 1997. Molecular diagnostics. *Journal of Molecular Medicine* 75:728– 744
- Widjojoatmodjo, M. N., Fluit, A. C., Torensma, R., Verdonk, G. P. H. T. & Verhoef, J. 1992. The magnetic immuno polymerase chain reaction assay for direct detection of *Salmonellae* in fecal samples. *Journal of Clinical Microbiology* 30: 3195–3199
- Williams, L. and LaRock, P. 1985. Temporal Occurrence of *Vibrio Species* and *Aeromonas hydrophila* in Estuarine Sediments. *Applied and Environmental Microbiology* 50:1490–1495

APPENDIXES

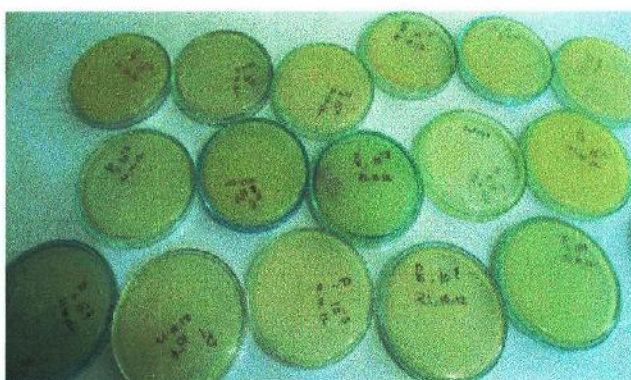
Appendix – 1 Photo Albums



Counting of total coliform & faecal coliform



Spreading of solution on media



Observation of culture media



Incubation of culture media



Fig: Bacterial colony counting

Appendix -2**Ingredients of Nutrient Agar media (NA)**

Ingredients	Amounts (gram/litre)
'Lab-Lemco' Power	1.0
Yeast extract	2.0
Peptone	5.0
Sodium Chloride	5.0
Agar	15.0
pH	7.4 ± 0.2 (at 25°C)

Appendix 3**Ingredient of Mac Conkey Agar media**

Ingredients	Amounts (gram/litre)
De gelatine	17.0g
Peptonous	3.0g
Lactosa	10g
Sales biliares	1.5g
Clorurosodico	5.0g
Agar	13.5
Rojo neutron	.03g
Cristal violeta	.001g

Appendix - 4**Ethanol wash (10%)**

Ingredient	Amounts
Ethanol	10
Distilled water	90

Appendix -5**Agarose gel electrophoresis determination of DNA integrity**

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

Materials

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

Procedure

1. **TAE buffer preparation:** To prepare 1000 ml 1x TAE buffer solution from 50x TAE buffer 20ml TAE buffer and 980 ml distill water was mixed up to make 1000 ml 1x solution.
2. **Agarose gel preparation**
 - a. Gel casting tray and samples comb were installed.
 - b. An amount of 1.5 g Agarose powder was weighted out using the electric balance and taken in a conical flask, shake for a few times and then heated in a microwave oven for 2

- minutes. To melt the powder properly, heated in the oven and shaking at a regular time (30s) interval.
- c. The mixture was cooled to about 60°C by falling cool tap water on the wall of the conical flask with continuous jerking and by keeping at room temperature
 - d. 1.5 µl Ethidium bromide was added to the solution and mixed well;
 - e. The solution was poured into the gel casting tray containing sample comb and were allowed to solidified at room temperature; (Note: with decreasing temperature gel will be solidify rapidly thus step 1.4 to 1.6 was done quickly without consuming high time.
 - f. After gel has solidified, the comb was removed, using care not to rip the bottom of the wells.
 - g. The gel could be stored at -4°C for further use.
3. The gel, still in its plastic tray, was inserted horizontally into the electrophoresis chamber and was covered by adding approximately 450 ml TAE buffer;
 4. 4 µl of the extracted DNA sample was mixed with 2 µl of loading buffer on a parafilm and were poured into a sample wells. By maintaining sample's serial number, this was done for every sample separately. To avoid cross-contamination, separate micro-tips were used for every sample.
 5. Approximately 2 µl of colored DNA size marker was poured into first sample wells on gel ;
 6. The electrophoresis chamber was covered with the lid;
 7. The power supply was switched on, only volt was fixed at a constant value of 50, the machine was run until DNA migrated to the two-third of the gel towards the positive electrode (DNA was usually blue colored based on loading buffer);
 8. After 25 to 30 minutes The power supply was switched off and the lid of the electrophoresis chamber was removed;
 9. The gel, still in its plastic tray, was removed from the electrophoresis chamber and placed on an ultraviolet (UV) transilluminator.
 10. The UV transilluminator was switched on, DNA banding pattern was visible;
 11. The resulting banding pattern was captured by using the digital camera and software.