HABITAT INFECTION ANALYSIS OF PENAEID SHRIMP WITH ENTEROVIRULENT STRAINS OF *Escherichia coli* BY USING PCR

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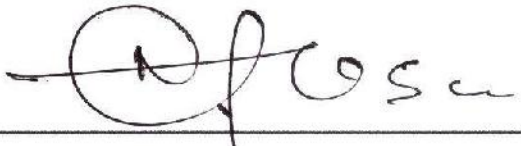
Approved as to style and content
By



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DECLARATION

HABITAT INFECTION ANALYSIS OF PENAEID SHRIMP WITH ENTEROVIRULENT STRAINS OF *Escherichia coli* BY USING PCR

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

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November, 2012

Dedicated to

My Beloved Parents

Acknowledgement

All praises are to the Supreme Being, the creator and Ruler of the Universe, the God, whose mercy 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.

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Abstract

Escherichia coli is a harmless, gram negative bacterium that is commonly found in the lower intestine of warm-blooded animals. A number of *E. coli* strains are available in nature, only a few of them is pathogenic to human. In aquatic environment, the most commonly encountered are those belonging to the enterotoxigenic (ETEC), enterohaemorrhagic (EHEC) and enteropathogenic (EPEC) subtypes. The prospective shrimp sector is facing a threat of this disease. However, the study on the virulence of *E. coli* in shrimp habitat is still scarce. In order to better determine the health risks that are associated with the exposure to some of these specific subtypes, a PCR system was used for the quick and authentic detection and typing of ETEC, EHEC and EPEC groups of *E. coli* in the water, sediment and shrimp samples of fifteen different farms of Cox's Bazar region. Six pairs of oligonucleotide primers were used for the amplification and detection of target genes *viz.*, the alkaline phophatase (Pho-A), heat-stable toxin1 (ST1), the heat-labile toxin1 (LT1) and heat-labile toxin2 (LT2) genes of ETEC, verotoxin (VT) genes of EHEC and attachment and effacement (EAE) genes of EPEC. The results revealed the presence of ETEC in sediment-farm-2 and water-farm-4 of cox's bazaar Sadar; sediment-farm-2, water-farm-4 and sediment-farm-5 of chakaria and sediment-farm-3 of moheshkhali and EPEC group in sediment-farm-3, water-farm-4 and sediment-farm-4 of cox's bazaar sadar; and water-farm-4 of chakaria region whereas no virulent strains was observed in shrimp samples. The water samples of one farm of both Cox's bazar sadar and Chakaria upazilla were found to be highly contaminated by ETEC and EPEC groups whereas, only ETEC group observed in the sediment sample farm-3 at Moheskhali upazilla. These findings of the study suggest that lack of enough sanitation system, polluted water source, unhygienic condition as well as poor management practices might be responsible for exposure of enterovirulent *E. coli* strains in this area.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Elaboration
µl	Micro liter
bp	Base pair
CFU	Colony Forming Unit
EAE	Attachment and Effacement
EEC	Enterovirulent *Escherichia coli*
EHEC	Enterohaemorrhagic *Escherichia coli*
EIEC	Enteroinvasive *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
kbp	Kilo base pair
LT1	Heat-labile toxin 1
LT2	Heat-labile toxin 2
mM	Mili Mole
ng	Nano gram
nm	Nano meter
PCR	Polymerase Chain Reaction
PhoA	Alkaline phosphatase
pmol	Pico Mole
rcf	Rotation Centrifugal Force
rpm	Rotation Per Minut
ST1	Heat-stable toxin 1
UV	Ultraviolet Transilluminator
VT	Verotoxin

Chapter One

INTRODUCTION

Chapter One

Introduction

Bangladesh is situated in such a geographic location that is blessed with numerous productive water bodies including rivers-canals, depressions and oxbow lakes, ponds and floodplains, covering a huge area of water resources of 4.575 million hectares. As an agro-based country, the contribution of fisheries sector to the national economy has always been acting as the main source of animal protein, employment opportunities, food and nutritional security, foreign earnings, aquatic biodiversity conservation and socio-economic development. Fisheries sector contributes 4.43% to GDP and 22.21% to agricultural GDP. Fish supplements to about 60% of our daily animal protein intake. About 10% of the population depends directly and indirectly on the fisheries for their livelihood (DoF, 2011).

The country has huge opportunities for the development of brackish water aquaculture boosting shrimp production and earning substantial amount of foreign currencies. Production of shrimp from culture and capture fisheries increased to a great extent in the beginning of 1980's. Since then, brackish water shrimp farming has been expanded to over 2.14 lakh ha of inland by 2011 from 1.4 lakh ha in 1980. It is expected that with the introduction of improved scientific method of shrimp culture, the present production of shrimp will be increased substantially (DoF, 2011).

The main cultured species of shrimp is the tiger shrimp, *Penaeus monodon* (locally known as bagda shrimp) contributes about 77% of export earning of whole shrimp sector. It is a marine shrimp and is cultivated in brackish water throughout the coastal areas of Bangladesh. There is ample demand in the international market for shrimp and Bangladesh is blessed with an environment congenial for shrimp culture and production. However, the industry is now at a severe stake due to several obstacles (USAID, 2005).

Almost all farmed produced shrimps are exported as processed frozen sea food and is the second largest export item in Bangladesh. The contribution of fisheries sub-sector to the total export earnings during 2010-11 was 2.73%. Presently 82 processing plants are in operation, all are HACCP certified and licensed by DoF for export of fish and fish products to European Union (EU), USA, Japan, Russia, Korea, China and India. (DoF, 2011).

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 started 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 are applied. However, with an increasing demand from both national and international markets, farmers started to switch over into improved extensive and semi-intensive systems.

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, 1996). In culture conditions various diseases are found which are acting like as natural disaster. Bacterial and Viral diseases are the most serious hindrances that might cause severe loss for the farmers. Unlike fish, shrimp lack an antigen/antibody system and so cannot be vaccinated in the way fish can be and thus prevention is the only cure to ensure better production (Lundin, 1996).

Bacteria, viruses, parasites and naturally occurring toxins in fresh and processed shellfish can cause food borne illnesses. Shellfish also can be contaminated by substances or

E. coli which are widely distributed in the aquatic environment have been universally accepted as an indicator of faecal pollution because of its presence in higher numbers in the gut of mammals. *E. coli* are a gram negative bacterium that is commonly found in the lower intestine of warm-blooded animals. Most of *E. coli* strains are harmless, but only a small percentage is pathogenic to human. The widespread occurrence of waterborne infections of *E. coli* origin in humans has become one of the major health problems worldwide. To date, several types of enterovirulent *E. coli* have been recognized as the aetiologic agents of various gastrointestinal infections in human. There are four classes of enterovirulent *E. coli* collectively known as the EEC group. This group is comprised of enterohemorrhagic *E. coli* (EHEC), which produces Shiga-like toxin and attaching-effacing lesions; enterotoxigenic *E. coli* (ETEC), producing heat-stable or heat-labile enterotoxins; enteroinvasive *E. coli* (EIEC), that causes bacillary dysentery via invasion of intestinal epithelial cells or by cytotoxin and enterotoxin; and enteropathogenic *E. coli* (EPEC), whose mechanism of virulence is thought to be unrelated to the excretion of typical *E. coli* enterotoxins (Kong *et al.*, 1999).

Given the importance of *E. coli* in water and product quality monitoring, a number of culture-based and immunological methods have been developed for its rapid detection. A fluorogenic method based on the enzymic cleavage of 4-methylumbelliferyl-β-D-glucuronide (MUG) has been reported (Edberg *et al.*, 1990). However, 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 (Bettelheim, 1992); and third, phenotypically MUG-negative *E. coli* (Chang *et al.*, 1989) for example, EHEC strains (Doyle and Schoeni, 1984) is not detected by the method. For the specific detection of pathogenic ETEC and EHEC strains which

produce different types of enterotoxins, several ELISA-based methods have been developed. They include ELISAs for the detection of heat-labile LT (Ristaino *et al.*, 1983), heat-stable STI (Carroll *et al.*, 1990) and heat-stable STII (Urban *et al.*, 1990) enterotoxins of ETEC; and verotoxins VT1 and VT2 (Downes *et al.*, 1989) of EHEC strains. However, some of the tests are known to possess variable sensitivities, the reliability of which depends upon the relative levels of gene expression of the target gene products under selective culture conditions, which has the added disadvantage of rendering non-culturable cells non-detectable (Roszak and Colwell, 1987).

Many methods have been developed to detect *E. coli* in food and water matrices, including traditional culturing with selective media, serotyping with specific antibodies to O_{157} and H_7 antigens, amplification of specific genes by Polymerase Chain Reaction (PCR) or hybridization of virulence genes by DNA microarrays.

PCR is a simple but effective method for detecting presence of marine bacteria from natural samples. However, the method requires species-specific primers and thus DNA-sequences that are unique for the respective species must be known in order for these primers to be designed and manufactured. For many pathogenic and well-studied bacteria there are several designed and well known primers to be found in the scientific literature, and for many of them there are not only species-specific primers but also primers that can detect strains that contain certain toxic genes only (Saravanan et al., 2007). Since many bacteria, when taken out of their natural environment, enter a viable but not culturable (VBNC) state, PCR may be a more accurate way of detecting marine bacteria, compared to specific plate growth (Campbell & Wright, 2003).

Current microbiological methods of culture and biochemical characterization are time consuming and labour intensive. Diagnostic methods using immunological techniques such as agglutination or the ELISA are comparatively quicker. PCR is not only more

sensitive, but also faster, cost and labour effective when compared with biochemical tests and immunoassays.

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).

In the recent years, use of the PCR and gene probe technology has provided rapid and highly sensitive methods for the specific detection of pathogenic ETEC (Pickett *et al.*, 1989; Victor *et al.*, 1991) and EHEC strains (Woodward *et al.*, 1992). More recently, various multiplex PCR protocols to simultaneously detect segments of different toxin genes of ETEC and/or EHEC strains have also been developed. For example, multiplex detection of LT1/LT2 (Kong *et al.*, 1995), LT1/ST1 (Stacy-Phipps *et al.*, 1995) and LT1/ST2 (Tsen *et al.*, 1998) genes of ETEC strains; LTI/VTI/VTII genes of ETEC and EHEC strains (Lang *et al.*, 1994); and VT1/VT2/HlyA/EAE-A/Rfb0111/Rfb0157 genes of EHEC strains (Paton and Paton, 1998) have been reported. There are different types of methods developed that are used to detect *E. coli* However, some of the tests are known to possess variable sensitivities, the reliability of which depends upon the relative levels of gene expression of the target gene products under selective culture conditions, which has the added disadvantage of rendering unculturable cells non-detectable (Roszak and Colwell, 1987).

The southern coastal belts of Cox's bazaar (20% of the area) shrimp culture area in Bangladesh has witnessed a threefold increase in production for the last decade. Now a

days shrimp are being cultivated in an area of 32,018 hectors in Cox's Bazar area. (DoF, 2011). A large number of people are involved directly or indirectly with shrimp sector of this district. During the baseline survey, mostly traditional culture system was observed in most of the farms in this region. A very little amount of people practice improved traditional culture system and the size of culture pond is extremely large. As a result poor management system, unhygienic environment might be making vulnerable to various virulent pathogens. Traditionally cultured shrimp farms in Bangladesh especially southeast region are highly susceptible to be contaminated with such pathogenic *E. coli* strains because of lack of proper sanitation near these farms. In addition, *E. coli* can be contaminated from nearby cow-shed or uncontrolled use of cow dung as fertilizer (Karunasagar *et al.*, 1999). Shrimp contaminated with one of these *E. coli* strains will not be accepted in international market that would lead adverse effect on shrimp industries in Bangladesh. So it is mostly important to detect whether any of enterovirulent strains of *E. coli* are present in the water, sediment or intestine of shrimp sample in this area.

Objectives of the study

The objective of the study was to analyze the habitat infection with *E. coli* through identify the enterovirulent *E. coli* strains (ETEC, EHEC and EPEC) by a rapid detection method using PCR in the shrimp farms of Cox's Bazar Region.

Chapter Two

LITERATURE REVIEW

Chapter Two

Literature Review

Ahmed *et al.* (2002) stated that the southern coastal belts of Cox's bazaar (20% of the area), Bagerhat, Khulna and Satkhira (80% of the area) is under shrimp culture in Bangladesh has witnessed a three fold increase in the last decade. It now covers about 145 thousand hectares sprawled over 9000 farms, 18% of the total farms in the world.

Hossain (1995) stated that about 5% of the coastal brackish water region is under coastal aquaculture mainly for shrimp farming (Hossain, 1995). Gradually shrimp production is increasing through horizontal expansion of the farming area.

Roszak and Colwell (1987) reported some of the tests are known to possess variable sensitivities, the reliability of which depends upon the relative levels of gene expression of the target gene products under selective culture conditions, which has the added disadvantage of rendering unculturable cells non-detectable.

Pickett *et al.* (1989) observed the polymerase chain reaction (PCR) and gene probe technology has provided rapid and highly sensitive methods for the specific detection of pathogenic ETEC.

Edberg *et al.* (1990) given the importance of *E. coli* in water quality monitoring, a number of cultures based and immunological methods have been developed for its rapid detection. They have been reported a fluorogenic method based on the enzymic cleavage of 4 methylumbelliferyl-β-D-glucuronide(MUG).

Victor *et al.* (1991) evaluated the use of the polymerase chain reaction (PCR) and gene probe technology has provided rapid and highly sensitive methods for the specific detection of pathogenic ETEC.

Etchegaray *et al.* (1991) reported characterisation of species pathogenic to fish has not centred on examination of the rRNA genes. Previously unidentified regions of bacterial genomes have been isolated from genomic DNA libraries and successfully used as targets for probes and PCR in detection of *R. salmoninarum* and *A. salmonicida.*

Lang *et al.* (1994) and Paton and Paton (1998) evaluated a various multiplex PCR protocols to simultaneously detect segments of different toxin genes of ETEC and/or EHEC strains have also been developed. For example, multiplex detection of genes ETEC strains; LTI/VTI/VTII genes of ETEC and EHEC strains and VT1/ VT2/HlyA/EAE-A/Rfb0111/Rfb0157 genes of EHEC respectively.

Kong *et al.* (1995) conducted a various multiplex PCR protocols to simultaneously detect segments of different toxin genes of ETEC and/or EHEC strains have also been developed. For example, multiplex detection of LT1/LT2. Same procedure was followed by Stacy-Phipps *et al.* (1995) to detect LT1/ST1 and Tsen *et al.* (1998) to detect LT1/ST2.

Austin (1997) investigated a brief overview of developments in the technology used in bacterial fish disease diagnosis and control. A large body of work now exists, describing the development of molecular methods for the study of bacteria. The molecular biology of *A. salmonicida* has received particular attention.

Hanninen and Hirvela-Koski (1997b) investigated Genomic DNA of *Aeromonas* species and strains can also be characterized by pulsed field gel electrophoresis .This method, and also detailed analysis of several genes or both molecular and phenotypic characters may not always be suitable for routine diagnostic purposes, but the information revealed in these studies is a valuable contribution in processes of validation and epidemiological

investigations. High molecular weight DNA is also valuable in characterization and grouping of different isolates of other bacterial species.

Rhodes *et al.* (1998) reported that the rRNA genes have been used in PCR assays for *R. salmoninarum*. The potential utility of conserved regions to identify or amplify the rRNA genes, followed by exploitation of more variable regions of the genes or spacers to detect or identify bacteria that may be difficult or even impossible to culture has long been recognized.

Oakey *et al.* (1999) reported RAPD has been applied as the first step in identifying regions of the genome that are useful as probes or targets for PCR for detection *of A. salmonicida* and *Y. ruckeri.*

Kong *et al.* (1999) evaluated a very sensitive multiplex PCR system for the rapid detection and typing of ETEC, EHEC and possibly EPEC strains of *E. coli* in the aquatic environment. The target genes chosen for this investigation included: the PHO-A housekeeping gene (present in all *E. coli*); the LT1, LT2 and ST1 genes of ETEC; the VT1 and VT2 verotoxin, and EAE virulence genes of EHEC and EPEC, respectively. The results showed that the six sets of PCR primers were highly specific for their target genes and produced specific amplimers of the expected size from several control strains of *E. coli* - ATCC 35401 (LT1+/ST1+); SA53 (LT2+/VT2+); and O157 (VT1+/VT2+/EAE+). Multiplex PCR analysis of seawater samples indicated the presence in all four samples of *E. coli* bacteria that were positive for LT1, ST1, VT1 and EAE virulence genes.

Prere and Fayet (2005) evaluated the Genotype EHEC test a new rapid system based on DNA multiplex amplification and further hybridization for the detection of shigatoxin *stx1*, *stx2* genes, intimin *eae* gene and invasin *ipaH* gene harbored by *Shigella* and enteroinvasive *E. coli* (EIEC). *E. coli* strains of various serogroups isolated from children

with acute gastroenteritis, hemorrhagic colitis or hemolytic–uremic syndrome were tested. The strains collection included 11 STEC/EHEC and nine EPEC. The same strains were tested with Genotype EHEC. For all the strains, the hybridization banding pattern obtained by Genotype EHEC correlated with their expected genotypic characteristics. No specific equipment is required, except a thermocycler. Absence of electrophoresis system, of ethidium bromide staining and imaging system is a clear-cut advantage of Genotype EHEC.

Dow *et al.* (2006) stated that *Escherichia coli* strains isolated in a Libyan hospital (20 from children with diarrhoea and 30 from healthy children) were investigated for their pathotypes and virulence traits. Altogether nine *eae*-positive (enteropathogenic *E. coli*, EPEC) and nine *agg*R-positive (enteroaggregative *E. coli*, EAEC) strains were identified. while six EAEC strains were identified from diarrhoeal and three from healthy children. Typical (*eae*+, EAF+, *bfp*+) EPEC strains (n=6) belonged to classical EPEC serogroups O55, O114, O127 and showed localized adherence on Hela cells. EAEC strains revealed genetic heterogeneity but uniformly adhered to HeLa cultures in an enteroaggregative adherence pattern. Antibiotic resistance frequently, characterized the strains. Sixty-eight percentages of the strains were resistant against at least one antibiotic and 30% harbored a class 1 integron independently of their clinical background.

Sorsa *et al.* (2007) stated that extra intestinal pathogenic *Escherichia coli* (ExPEC) are a major cause of urinary tract infections, sepsis, and neonatal meningitis; they recently detected short DNA fragments, which were significantly associated with the extraintestinal virulent phenotype. In this study, they identified four novel genomic DNA regions of the highly virulent uropathogenic *E. coli* strain JS299 carrying these previously identified DNA fragments. The prevalence of 15 previously uncharacterized genes was

determined in a collection of clinical ExPECs and commensal *E. coli* strains by means of DNA microarray analyses. They identified DNA sequences shared by both ExPEC and enteropathogenic *E. coli* (EPEC). The results support the idea of a considerable genetic variability among ExPEC strains and suggest that the novel genomic determinants contribute to the ExPEC virulence.

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 x 104 to 25.3 x 104 CFU ml^{-1} in water and 1.5 x 106 to 26.2 x 106 CFU g^{-1} in sediment. The *V. harveyi* population density varied between 0.6 x 104 and 8.8 x 104 LCFU ml^{-1} in water and from 1.2 x 106 to10.4 x 106 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.

Chapter Three

MATERIALS AND METHODS

Chapter Three

Materials and Methods

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

3.1 Study area

Cox's Bazar Sadar, Chakaria and Maheshkhali Upazila of Cox's Bazar District were selected for the experiment. Samples (water, sediment and fresh shrimp) were collected from three different shrimp farms of each area. Briefly, the farms were situated in Ghomatoli of Cox's Bazar Sadar upazila, Badarkhali of Chakaria upazila, and southern region of Maheshkhali upazila. During sampling, 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, chemical and medicine usage as well as production of each farm.

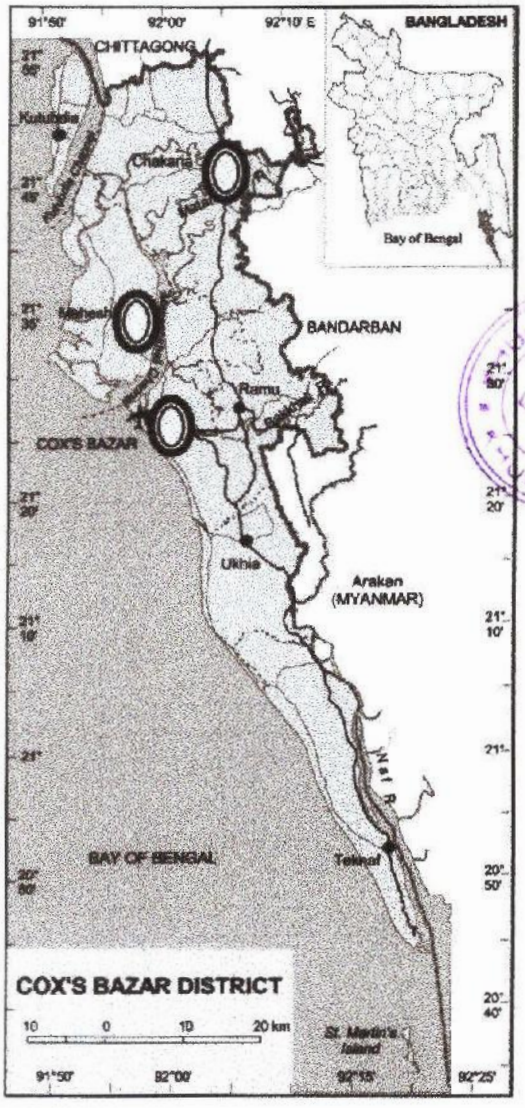


Figure 3.1: Sampling area at Cox's Bazar district; the circle indicates the approximate location of sampling farm

3.2 Experimental design

The presence of enterovirulent *E. coli* was identified by some strain specific genes, which were amplified by Polymerase Chain Reaction (PCR). For the detection of the bacteria, the target genes were chosen as: The Alkaline phosphate (Pho-A) gene (present in all *E. coli*); the LT1, LT2 and ST1 genes of ETEC; the VT verotoxin of EHEC and EAE virulence genes of EPEC. In this study, six pairs (forward and reverse) of specific oligonucleotide primers were designed from aforementioned six genes namely PhoA-F, PhoA-R, ST1-F, ST1-R, LT1-F, LT1-R, LT2-F, LT2-R, VT-F, VT-R, EAE-F and EAE-R. All the primer was used for each sample. Therefore, a farm having water, sediment and intestine of shrimp sample required 18 PCR units (6 primer X 3 samples) (Fig).

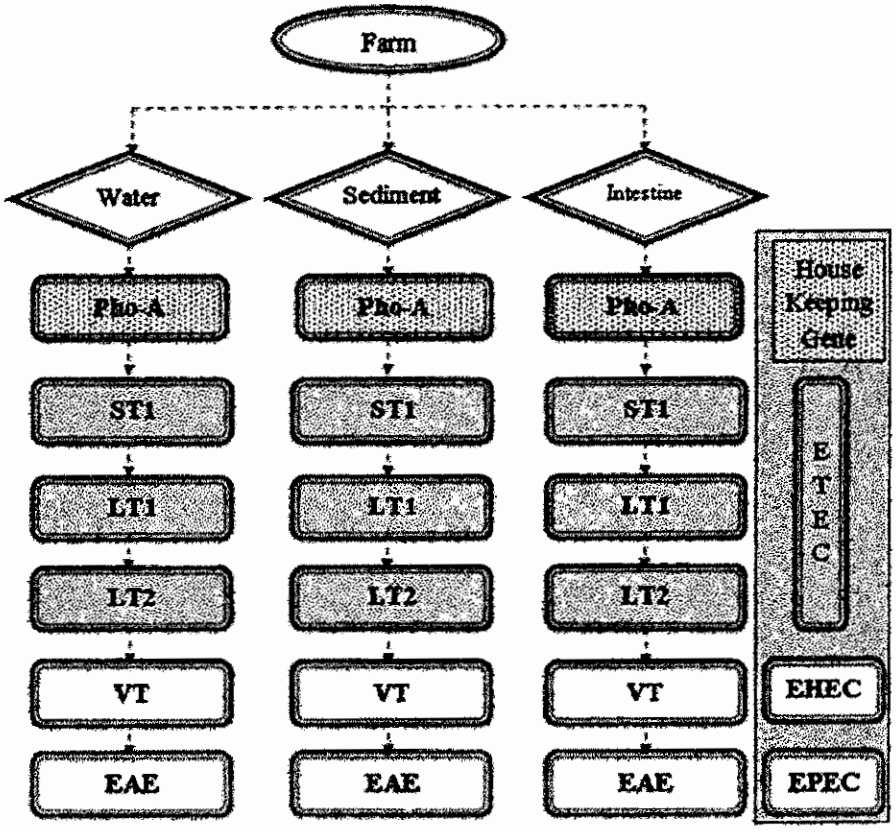


Figure 3.2: A schematic diagram for experimental units in each farm.

3.3 Sample collection

Water as well as sediment sample of each shrimp farm were collected from five different point of the farm by a pasteurized tube and was kept in a sterile 10 ml plastic vial. Vials were properly labeled according to the location of farm. Fresh shrimp (average body weight 29.39±2.21g) also collected from each farm which was kept in individual polythene bag with labeling followed by transferring to an icebox. As soon as possible, the shrimp containing icebox and the sample containing plastic vials were transported to Molecular Biology Laboratory, FMRT Discipline, Khulna University. The water and sediment containing vials were stored in the refrigerator at 4 °C temperature. Intestine of the collected shrimp was separated by dissecting the body and was kept each in 1.5 ml (Eppendrof) 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 Chemicals

From the beginning to the end of the experiment, a number of chemicals were used. Luria-Bertani (LB) broth (Difco Laboratories, MI, USA) was used for bacteria culture. The extraction kit, DNAzol reagent was the key component during DNA extraction which was collected from Invitrogen Life technologies, USA. Merck, Germany provided 100% Ethanol and solid Sodium hydroxide which was used for DNA wash and DNA solubilization respectively. For PCR sample preparation a set of chemicals containing 5x Green Reaction buffer, dNTP mixture, Go taq DNA polymerase enzyme and Enzyme dilution buffer for Go taq DNA polymerase from Promega, Madison, U.S.A. Di-ionized sterile distilled water was procured from Bioneer, Sheul, Korea. The PCR products were evaluated by agarose gel electrophoresis. The gel preparing materials, such as, Agarose powder, 50x TAE buffer,

Ethidium Bromide and gel loading buffer were also obtained from Bioneer, Sheul, Korea. In addition, a 100 bp DNA marker (Bioneer, Sheul, Korea) was used to compare the size of the sample DNA on agarose gel.

3.5 Media Preparation and culture of organism

The culture media was prepared by mixing 2g LB powder in 100 ml water and then it was autoclaved (121^0 C, 20 min), then 5 ml LB broth media was taken into a test tube where 100µl samples water, sediment and shrimp intestine separately were added and culture was carried out in a shaking incubator at 37°C overnight. Following overnight culture, the bacteria sample was used for DNA extraction (Ausubel *et al.*, 1990).

3.6 DNA Extraction

After overnight culture, bacterial DNA was extracted from the cultured sample. Numerous protocols are available for DNA extraction from animal tissue such as DNAzol isolation of total DNA, DEB treatment, DEB with RNAse treatment (Modified), Proteinase K isolation of total DNA, SDS isolation of total DNA. Among them use of DNAzol is a comperatively easy and standard procedure. This protocol was effective as well as straight forward comparing with other commercial kits (Siddiqui *et al*, 2008.)

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. The total isolation process is given below. Centrifuge machine (Vision Micro High Speed Refrigerated Centrifuge, Model no: VS-15000 CFN II, City, Country?) was used for centrifugation.

- At the very beginning of extraction process, 2 ml (1 ml in each tube) cultured sample was centrifuged at 10000 rcf for 5 minutes with a centrifuge machine (Vision Micro High Speed Refrigerated Centrifuge, Model no: VS-15000 CFN II). At the end of centrifugation, clear supernatant layer was removed by a pipette (1000 µl) and deposited plate layer was kept for further procedure.
- ***Lysis of cells:*** 500 µl DNAzol reagents were added to each tube of a sample pair. The plate was dissolved completely by gentle pipetting and the dissolved sample in pair tube was mixed up to keep into a single tube.
- ***Centrifugation:*** The dissolved cells were centrifuged for 10 min at 10,000 rcf at 4°C or room temperature. Following centrifugation, the resulting viscous supernatant was carefully transferred to a fresh tube. This step removes insoluble tissue fragments, RNA, and excess polysaccharides from the lysate/homogenate. This process was performed in order to minimize RNA carry-over into the DNA.
- ***DNA Precipitation:*** DNA from the lysate was precipitated by the addition of 0.5 ml of 100% ethanol per 1 ml of DNAzoL Reagent used for the isolation. Samples were mixed by inversion and store them at room temperature for 1-3 minutes. Then it was centrifuged for 10 minuts at 10000 rcf in cool temperature. DNA should quickly become visible as a cloudy precipitate. Carefully the supernatant was decanted leaving the DNA pellet near

the top of the tube. The tubes were placed upright for 1 min and was aspirated the remaining lysate from the bottom of the tubes.

- ***DNA Wash:*** The DNA precipitate was washed twice with 0.8-1.0 ml of 75% ethanol. At each wash, the DNA in ethanol suspended by inverting the tubes 3-6 times. The tubes were stored vertically for 0.5-1 min to allow the DNA to settle to the bottom of the tubes and remove ethanol by pipetting. At each wash, the DNA in ethanol was vortexed and was suspended by centrifugation for 10 minutes at 10000 rcf.

- ***DNA Solubilization:*** After removing the ethanol, the DNA was air dried by storing in an open tube for 5-15 seconds. (If the DNA is exposed to air for more than a few seconds, it will be much more difficult to dissolve.). If the DNA was not properly dried, it was kept on a heatblock at 55°C for 5 minuts. The DNA was dissolved in 8 mM NaOH by slowly passing the pellet through a pipette tip. Use of the 8 mM NaOH was assured full solubilization of the DNA precipitate. 100 µl of 8 mM NaOH was added to the DNA precipitate to dissolve. Then it was centrifuged for 3 minutes at 10000 rcf.

3.7 Qualitative determination of extracted DNA (Agarose gel electrophoresis)

Purity of extracted DNA sample was tested by agarose gel electrophoresis as described by Sambrook *et al.* (1989). A horizontal electrophoresis apparatus (Bioneer, Korea) was used for the electrophoresis process. 1.5% agarose gel was used. The gel was prepared with 150 ml 1x TAE buffer, 2.25 g agarose powder and 2 µl ethidium bromide. A constant volt of 100 V was passed through the gel, which was merged in sufficient 1x TAE buffer. A 100 bp marker was loaded along with the sample for the comparison of the size of DNA. Supply of power for electrophoresis was continued until DNA migrated near about the two-third of the gel towards the positive

electrode. DNA bands were visualized by UV illumination and photographed using the High Performance UV Transilluminators, (Ultra-Violet Products Ltd., UK).

3.8 Quantitative determination of extracted DNA (Spectrophotometric method determination of DNA concentration and Purity)

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

1) Double-stranded DNA concentration (C), µl/ml =Absorbance at $A_{260} \times 50 \times 500$;
2) Purity = Absorbance at A_{260} /Absorbance at A_{280}

3.9 Components for DNA amplification in PCR

Components used for producing PCR product for DNA amplification are given in table 3.1.

Table 3.1: Components of reaction mixture with their compositions and final concentration for 25 µl mixture.

Component	Final volume	Final concentration
1. ***Go Taq* Polymerase** (5 units/µl, *Go Taq* DNA Polymerase, Promega, U.S.A.)	1 µl	1 unit/25 µl
2. **5X green Reaction Buffer** (Tris; pH 8.5, 7.5 mM $MgCl_2$)	5 µl	1x (0.75mM$MgCl_2$)
3. **10 mM dNTP mixture**	2 µl	0.4 mM each dNTP
4. **Primers** (Pho, ST1, LT1, LT2, VT and EAE)	1 µl (Forward 0.5 µl + Reverse 0.5 µl)	10 pmol
5. **Template DNA**	2 µl	75-160 ng
6. **Di ionized water**	12 µl	

The enzyme is derived from bacteria (*E. coli*). It exhibits highest activity at pH 8.5 and 74 °C (Promega, U.S.A.). To prepare 1 unit *Go Taq* 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).

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

The target genes were chosen for this study were

- Alkaline phosphate (Pho-A) (present in all *E. coli*)
- The LT1, LT2 and ST1 genes of ETEC
- The VT verotoxin and EAE virulence genes of EHEC and EPEC respectively

Six pairs of specific primers were designed from above mentioned gene sequence. 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. The designed oligonucleotide primers (Both forward and reverse) named as, PhoA-F, PhoA-R, ST1-F, ST1-R, LT1-F, LT1-R, LT2-F, LT2-R, VT-F, VT-R, EAE-F and EAE-R were procured from Bioneer, Korea. Sequences of the six PCR primer pairs, their corresponding gene targets and size of expected amplification products were shown in Table 3.2.

Table 3.2: Primers and expected size of PCR-amplified gene targets of pathogenic *E. coli*.

Target Gene	Primers	Sequence (5′-3′)	Expected product size (bp)	Accession No.
PhoA	PhoA-F	GTGACAAAAGCCCGGACACCATAAATGCCT	1371	M13345
	PhoA-R	TACACTGTCATTACGTTGCGGATTTGGCGT		
ST1	ST1-F	CTTTCCCCTCTTTTAGTCAG	638	M25607
	ST1-R	TAACATGGAGCACAGGCAGG		
LT1	LT1-F	TTACGGCGTTACTATCCTCTCTA	725	J01646
	LT1-R	GGTCTCGGTCAGATATGTGATTC		
LT2	LT2-F	ATATCATTTTCTGTTTCAGCAAA	997	M17894
	LT2-R	CAATAAAATCATCTTCGCTCATG		
VT	VT-F	GAACGAAATAATTTATATGTG	1240	M36727
	VT-R	CCTGATGATGGCAATTCAGTA		
EAE	EAE-F	GGAACGGCAGAGGTTAATCTGCAG	1155	M58154
	EAE-R	CGAAGCCATTTGCTGGGCGCTC		

3.10 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 (Company, City, Country) 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 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 2 µl primer (Forward 1 µl + Reverse 1 µl), 5 µl 5x Reaction buffer, 2 µl dNTPs, 1 µl 1 unit Go taq DNA Polymerase enzyme as well. These all components were mixed in 13 µl de-ionized sterile distill water and thus a total of 25 µl PCR sample was prepared in a 0.2 ml PCR-tube (Eppendrof, Humburg, Germany) to operate in a thermal cycler.

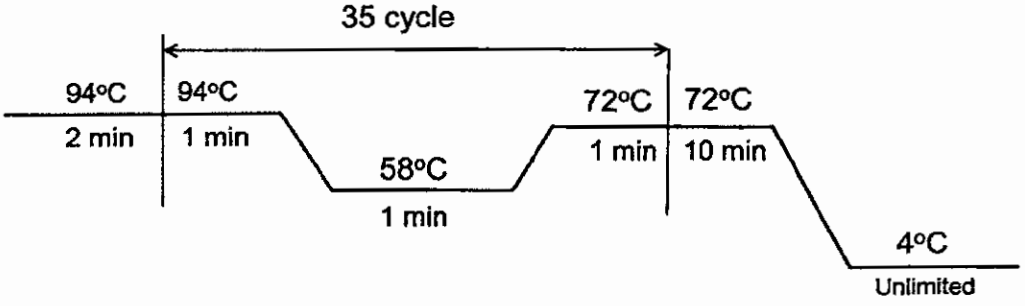


Figure 3.3: A schematic diagram of PCR cycle

In PCR running, control reactions consisted of:

1) Positive control: reaction mixtures with DNA extracted from Mouri river that always showed the positive response (Lab report, FMRT Discipline, Khulna University) if, all chemical and

2) Negative control: reaction mixtures with no template DNA.

3.11 Evaluation of PCR products

After completion of the thermal cycling, a sample of 10 µl from each PCR products was analyzed electrophoretically by running on a 1.5% agarose gel following the procedure described in the "Qualitative determination of extracted DNA (Agarose gel electrophoresis)" and the amplified product size was determined by comparison with a 100 bp DNA size marker.

During the evaluation of PCR products, at first, a specific band for alkaline phosphatase gene (1371 bp position) was screened out on gel to determine the presence of *E. coli* in the sample. Then, the *E. coli* positive samples were studied to identify the specific banding pattern of enterovirulent strains.

3.12 Restriction enzyme analysis of PCR products

To facilitate rapid conformation of the identity of the PCR-amplified products, restriction enzyme analysis was performed. Nucleotide sequences of the target gene fragments (fetched from the GenBank/EMBL data-bases) were searched for restriction enzyme recognition sites by computer using the MapSort software of the UWGCG DNA analysis package (Devereux et al., 1984). The *Alu*I enzyme was found to be most suitable since its recognition sequence is present within six of the PCR-amplified products, each yielding *Alu*I digestion products of different sizes as indicated in Table 3.3. PCR products were purified using the *AccuPrep*® Gel Purification Kit (Bioneer, Korea) before restriction analysis.

Table 3.3: Size of the digestion product of AluI restriction enzyme

Name of the gene	Sequence	Site Length	Overhang	Frequency	Cut Positions
Pho-A	AGCT	4	blunt	2	150, 715
ST1	AGCT	4	blunt	1	282
LT1	AGCT	4	blunt	3	258, 702, 718
LT2	AGCT	4	blunt	2	66, 704
VT	AGCT	4	blunt	1	301
EAE	AGCT	4	blunt	3	630, 771, 899

Procedure

At first the DNA band of agarose gel was cut out as small as possible and weighted in a clean 1.5 ml micro-centrifuge tube. Three volumes of gel binding buffer to 1 volume of the gel slice was added to the micro-centrifuge tube. The mixture was incubated at 60°C for 10 minutes. For the complete dissolving of gel slice the tube was vortex every 2-3 min. After dissolving the gel slice, the color of the mixture was checked as recommended yellow. If the color of the mixture was orange or red, 10 µl of 3M sodium acetate (pH 5.0) was added and mixed and he color turned into yellow. The mixture was transferred to the DNA binding column tube and centrifuged for 1 min. at 13,000 rpm. At this step the flow-through was vacated and the DNA binding filter column was re-assembled with the 2.0 ml collection tube. For the removal of salts and soluble impurities in the DNA binding column tube 500 µl of Washing Buffer was added to the DNA binding column tube and centrifuged for 1 min. at 13,000 rpm. Again the flow-through was vacated and the DNA binding filter column was re-assembled with the 2.0 ml collection tube. For more purification of DNA to removal of salts and soluble impurities in the DNA binding column tube another 500 µl of washing buffer was added to the DNA binding column tube and centrifuged for 1 min. at 13,000 rpm. After that flow-through was vacated and the DNA binding filter column was re-assembled with the 2.0 ml collection tube. For the removal of the residual ethanol the DNA binding filter column was dried by additional centrifugation at 13,000 rpm for 1 min transferred to the new 1.5 ml micro-centrifuge tube. For elution, 30 µl of elution buffer was added to the center of the DNA binding filter column, and waited for at least 1 minute at room temp. Finally the fragment DNA was eluted by centrifugation at 13,000 rpm for 1 min.

After completion of the cut of DNA using restriction enzyme, a sample of 8 µl from each primer products and 2 µl gel loading dye (Promega, Madison WI USA) was analyzed electrophoretically by running on a 1.5% agarose gel following the procedure described in the "Qualitative determination of extracted DNA (Agarose gel electrophoresis)" and the amplified product size was determined by comparison with a 100 bp DNA size marker.

Chapter Four

RESULTS

Chapter Four

Results

From the questioner surveyed during sample collection the information reveal that all farmers used river as a source of water but water in a few number of farms was screened for their merely treanment. Only in farm-1 and farm-2 of moheshkhali (Managed by BEXIMCO) the semi-intensive culture technique was practiced with water quality management and good hygienic condition. On the basis of the collected information directly from the farmer during sample collection, farms were categorized as hygienic farms and unhygienic farms (Table 4.1). Farm-1 and Farm-5 of Cox's Bazar Sadar; Farm-1 and Farm-3 of Chakaria and Farm-1, Farm-2 and Farm-4 of Moheshkhali was categorized as hygienic farms and rest were unhygienic farms.

Table 4.1: Physical and Environmental condition of selected farms

Area	Farm	Area(Acre)	Culture technique	Water source	Sanitation condition	Fry source	Grazing land/cattle shed
Cox's Bazar Sadar	Farm-1	1.66	Traditional	River	Moderate	Hatchery	No
	Farm-2	1.00	Traditional	River	Poor	Both Wild & Hatchery	Yes
	Farm-3	13.33	Traditional	River	Poor	Both Wild & Hatchery	No
	Farm-4	3.00	Traditional	River	Poor	Both Wild & Hatchery	Yes
	Farm-5	4.66	Traditional	River	Moderate	Hatchery	No
Chakaria	Farm-1	100	Traditional	River	Good	Hatchery	No
	Farm-2	120	Traditional	River	Poor	Both Wild & Hatchery	Yes
	Farm-3	60.00	Traditional	River	Moderate	Hatchery	Yes
	Farm-4	1.50	Traditional	River	Poor	Both Wild & Hatchery	Yes
	Farm-5	1.50	Traditional	River	Poor	Both Wild & Hatchery	Yes

Mohekhali	Farm-1	0.8	Semi-intensive	River	Good	Hatchery	No
	Farm-2	0.8	Semi-intensive	River	Good	Hatchery	No
	Farm-3	150	Traditional	River	Poor	Wild	No
	Farm-4	300	Traditional	River	Moderate	Hatchery	No
	Farm-5	150	Traditional	River	Poor	Both Wild & Hatchery	Yes

The extracted DNA was amplified by Polymerase Chain reaction (PCR). From the two step experiments (step 1: detection of *E. coli* bacteria with alkaline phosphatase (Pho-A) primer; step 2: diagnosis of positive samples containing bacteria whether virulent or not using other primers), area wise electrophoresis pattern are shown in different figure.

The concentration of DNA samples were determined from the absorbance at A_{260} (absorbance at 260 nm) using a double beam spectrophotometer and purity was calculated by using the formula mentioned previously. The final yield of purified DNA ranged from 425 to 775 µg/ ml (Table 4.2)

Table 4.2: Absorbance value of 260 nm and 280 nm wave length, corresponding ratio and DNA concentration of different samples.

List of farm and sample			A_{260}	A_{280}	A_{260} / A_{280}	DNA conc. µl/ml
Cox's Bazar SadarUpazila	Farm 1	Water	0.026	0.029	0.89	650
		Sediment	0.018	0.022	0.82	450
		Intestine of shrimp	0.021	0.022	0.95	525
	Farm 2	Water	0.019	0.025	0.76	475
		Sediment	0.021	0.029	0.72	525
		Intestine of shrimp	0.020	0.024	0.83	500
	Farm 3	Water	0.025	0.029	0.86	625
		Sediment	0.021	0.028	0.75	525
		Intestine of shrimp	0.017	0.027	0.63	425
	Farm 4	Water	0.022	0.023	0.96	550
		Sediment	0.023	0.025	0.92	575

by PCR. During electrophoresis a 100 bp marker was used. Under UV condition all *E. coli* positive samples band on a specific location which is 1371 base pair. The electrophoresis patterns of the samples are shown in Fig. 4.1.

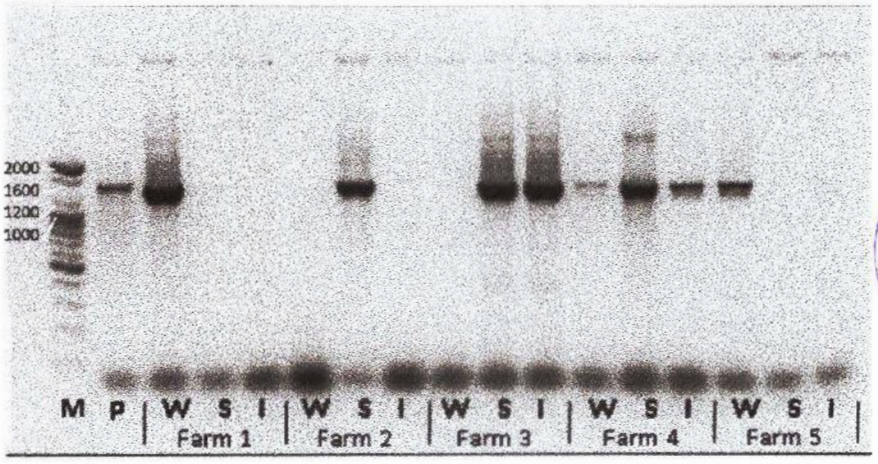
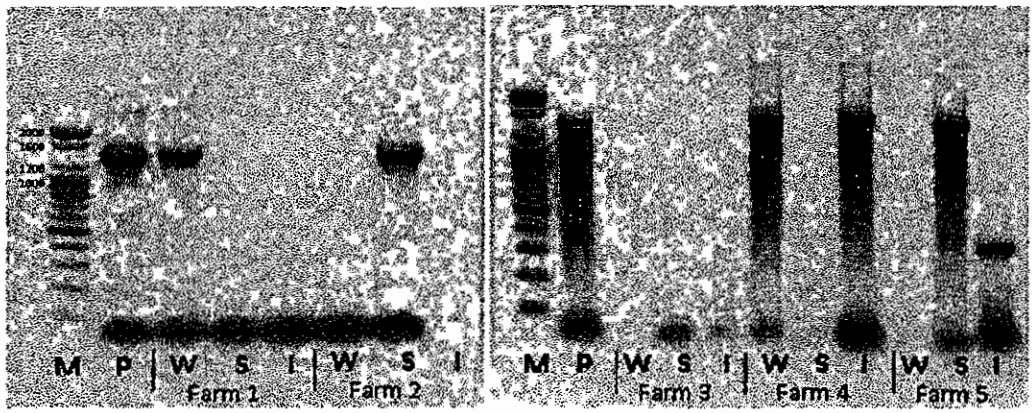


Figure 4.1: UV visualization of total genomic DNA extracted from the five shrimp farm samples of Cox's Bazar Sadarupazila following electrophoresis in a 1.5% agarose gel. Lane M and P represented 100 bp DNA ladder or marker, positive control respectively. In case of other lanes, W, S, I symbolic lane represent water sample, sediment samples and Intestine of shrimp respectively.

4.1.2 Chakaria Upazila

All 15 extracted DNA samples (5 water, 5 sediment and 5 shrimp intestine) from 5 farms of Chakaria upazila were amplified by PCR. The electrophoresis patterns of the samples are shown in Fig. 4.2.



(a) (b)

Figure 4.2: UV visualization of total genomic DNA extracted from the five shrimp farm samples of Chakaria upazila following electrophoresis in a 1.5% agarose gel. Electrophoresis pattern of farm-1 and farm-2 are shown in figure 4.2 (a) and farm-3, farm-4 & farm-5 are in figure 4.2 (b). In both figures fast lane marked as M stands for Marker or ladder and lane 2 represent as P is for positive control. Water, Sediment and Intestine of shrimp template containing lanes are shown as W, S, I respectively with farm number.

4.1.3 Moheshkhali Upazila

In the present experiment, similar banding pattern was also found for the samples collected from 5 different farms on Moheshkhali upazila. Under UV condition all *E. coli* positive samples showed band on 1371 base pair. The electrophoresis patterns of the samples are shown in Fig. 4.3.

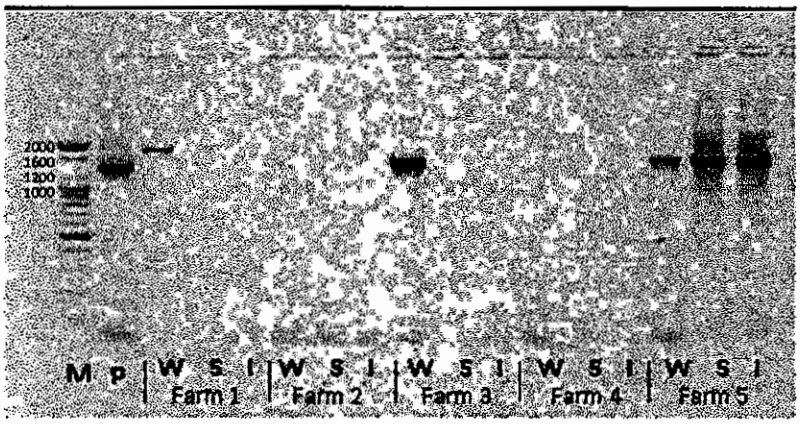


Figure 4.3: UV visualization of total genomic DNA extracted from the five shrimp farm samples of Moheshkhali upazila following electrophoresis in a 1.5% agarose gel. Lane M and P represented 100 bp DNA ladder or marker, positive control respectively. In case of other lanes, W, S, I symbolic lane represent water sample, sediment samples and Intestine of shrimp respectively.

From the first step of the experiment, several samples showed positive banding withAlkaline Phosphatase (Pho-A) primer resulting the presence of *E. coli* bacteria. In Cox's Bazar Sadar area about 8 samples showed positive result for the presence of *E. coli* and in Chakaria and Moheshkhali are 5 and 4 samples respectively.They are listed in the figure 4.2.

Table 4.3: Presence of any *E. coli* bacteria with Alkaline phosphatase (Pho-A) primer

	Farm 1			Farm 2			Farm 3			Farm 4			Farm 5		
	Water	**Sedimen**	**Intestine**	**Water**	**Sedimen**	**Intestine**	**Water**	**Sedimen**	**Intestine**	**Water**	**Sedimen**	**Intestine**	**Water**	**Sedimen**	**Intestine**
Cox's Bazar sadar	√	×	×	×	√	×	×	√	√	√	√	√	√	×	×
Chakaria	√	×	×	×	√	×	×	×	×	√	×	√	×	√	×
Moheshkhali	×	×	×	×	×	×	×	√	×	×	×	×	√	√	√

Here, √ represents Presence of *E. coli* and × represents absence of *E. coli*

4. 2. Detection of virulent strain of *E. coli* bacteria with all six gene primer

The *E. coli* positive samples were farther tasted with other virulent primers to identify that whether the bacteria is virulent strain of that species or not. The samples showed negative response to presence of *E. coli* were not considered for the all primer tasted because the absence of the Alkaline Phosphatase (Pho-A) means the zero probability of having *E. coli*.

4.2.1 Cox's Bazar Sadar Upazila

From the 15 samples of 5 different farms of Cox's Bazar Sadar upazila, 8 samples showed positive mark to *E. coli* bacteria presence. They were Water of farm -1, farm-4 and farm-5; Sediment of farm -2, farm-3 and farm-4; and the shrimp intestine of farm-3 and farm-4. These samples were further tested with all six pairs of oligonucleotide primers to simultaneously amplify an Alkaline Phosphatase (Pho-A) and various virulence-associated

genes of ETEC, EHEC and EPEC strains. In all primers tests, Water of farm-1 & farm-5 and shrimp intestine of farm-3 & farm-4 showed no virulent strain *E. coli*. Only the Alkaline Phosphatase (Pho-A) showed distinct band under UV light electrophoresis shown in figure 4.4.

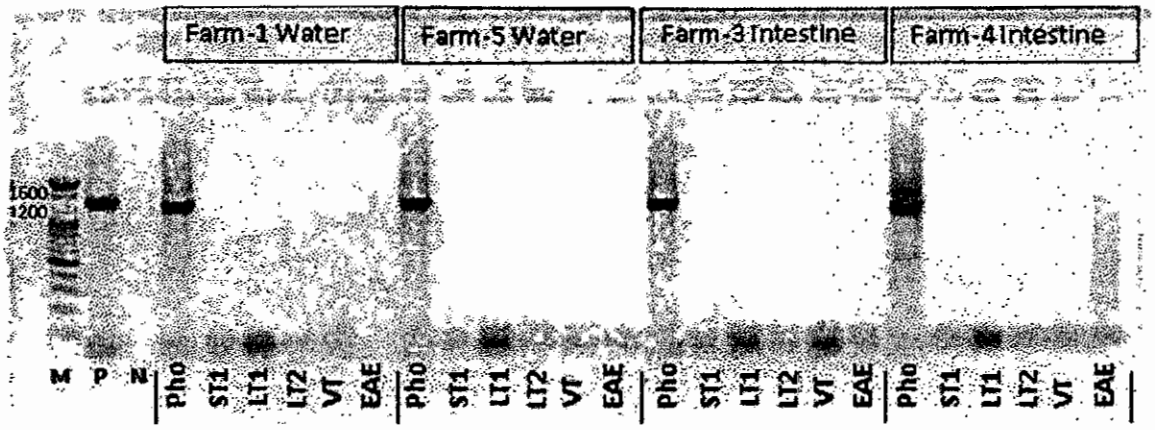


Figure 4.4: UV visualization of Water of farm-1 & farm-5 and shrimp intestine of farm-3 & farm-4 Cox's Bazar Sadar upazila following electrophoresis in a 1.5% agarose gel. Here, M, P and N denoted lane represent the 100 bp marker, positive control and negative control respectively.

In case of sediment of farm-2 & farm-3 and water & sediment of farm-4 several bands of target genes were found on electrophoresis gel which indicated the presence of *E. coli* of entero-pathogenic group. The electrophoresis pattern under UV light visualization is shown in figure 4.5.

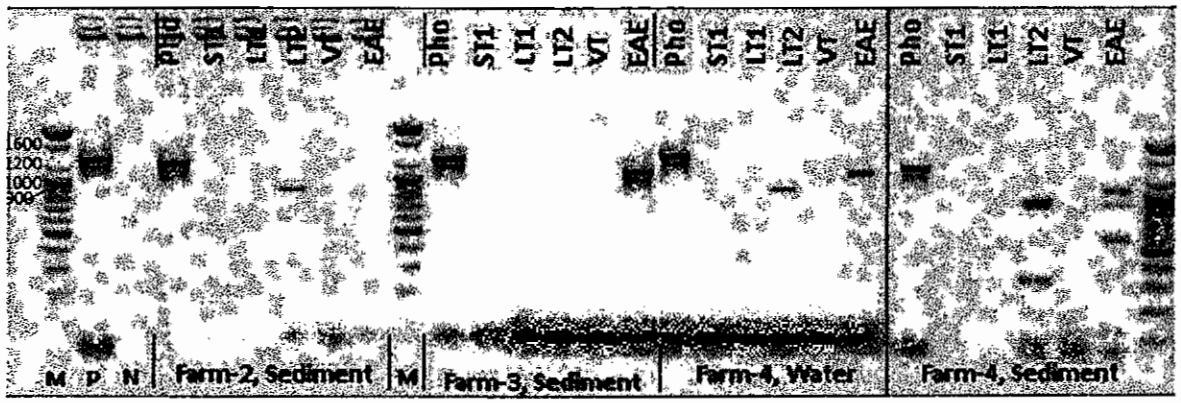


Figure 4.5: UV visualization of Sediment of farm-2 & farm-3 and water & sediment of farm-4 of Cox's Bazar Sadar upazila following electrophoresis in a 1.5% agarose gel. Here, M, P and N denoted lane represent the 100 bp marker, positive control and negative control respectively.

4.2.2 Chakaria Upazila

In the present study, out of 15 samples of 5 farms from Chakaria upazila about 5 samples showed positive response to Alkaline Phosphatase (Pho-A) primer, samples werethen tasted further for diagnosis of the presence of virulent strain of *E. coli*.

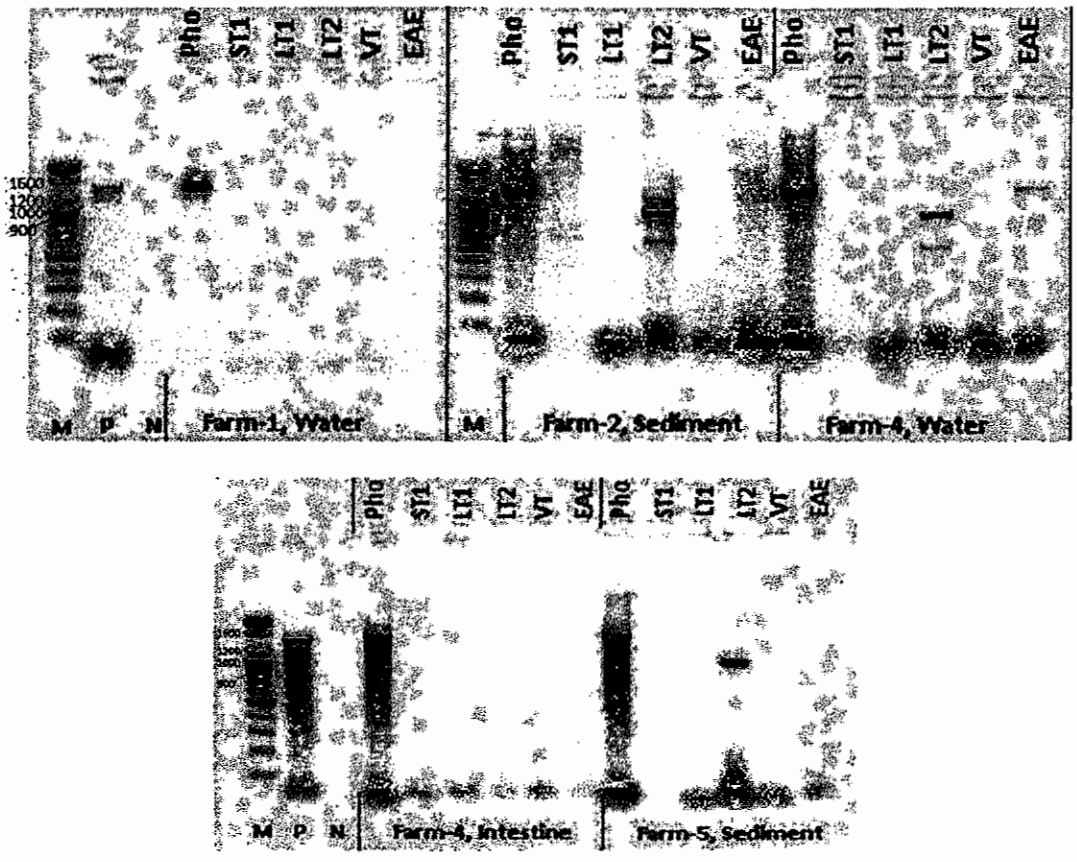


Figure 4.6: UV visualization of Intestine of farm-4 and sediment of farm-5 of Chakaria upazila following electrophoresis in a 1.5% agarose gel.M, P and N denoted lane represent the 100 bp marker, positive control and negative control respectively.

4.2.3 Moheshkhali Upazila

From 5 farms of moheshkhali upazila sediment of farm-3 and water, sediment & shrimp intestine respond to the Alkaline Phosphatase as Pho-A primer. In case of the screening of virulent strain of *E. coli* of those 4 samples only in the water of farm-3 respond to LT2 primer. Electrophoresis pattern of the screening is shown in figure 4.5.

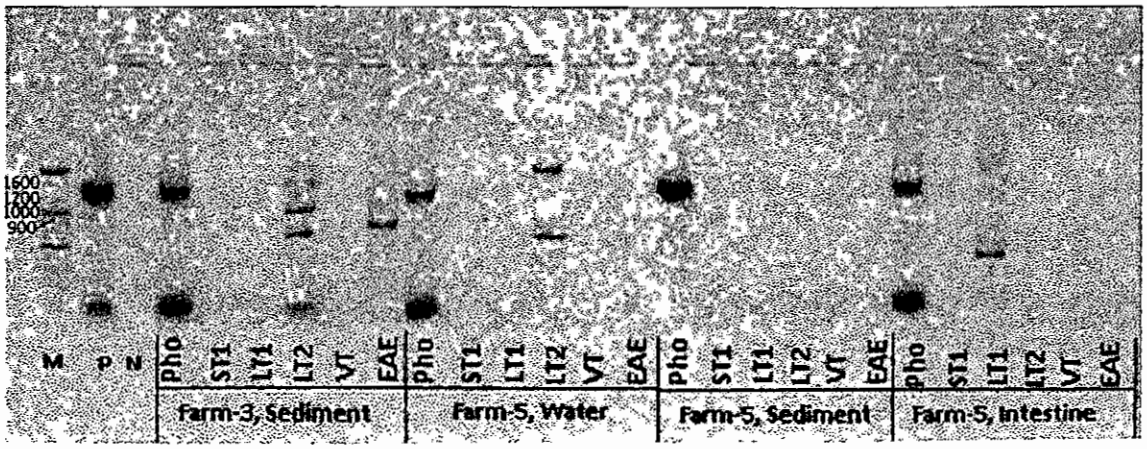


Figure 4.7: UV visualization of Sediment of farm-3 and Water, sediment& intestine of farm-5 of Moheshkhali upazila following electrophoresis in a 1.5% agarose gel.Here, M, P and N denoted lane represent the 100 bp marker, positive control and negative control respectively.

4.3 Restriction enzyme analysis

The specificity of the primers used in this investigation to detect pathogenic *E. coli* was verified with the restriction enzymes *Alu*I. The *Alu*I restriction site was in 150 and 715 bp position in PCR product produced from Pho-A gene. The fragments size following the digestion with *Alu*I enzymes were found approximately 150 bp, 565 bp, and 656 bp that were exactly went with expected size (Fig. 4.8). Hence this result concluded that the PCR product turned out with primers Pho-A-Forward and Pho-A-Reverse was Pho-A gene. However, after

digestion, the expected weak band (ca. 1300 bp) in lane D, Pho-A is due to the undigested PCR products remained. Similarly the *Alu*I restriction site was in 66 bp and 704 bp position in PCR product produced from LT2 gene (fragment sizes of approx. 66 bp, 293 bp and 638 bp) and 630 bp, 771 bp and 899 bp position in PCR product produced from EAE gene (fragment sizes of approx. 128 bp, 141 bp, 256 bp and 630 bp). In case of EAE gene as expected in the gel band though 128 and 141 bp are so close to get fused in the electrophoresis.

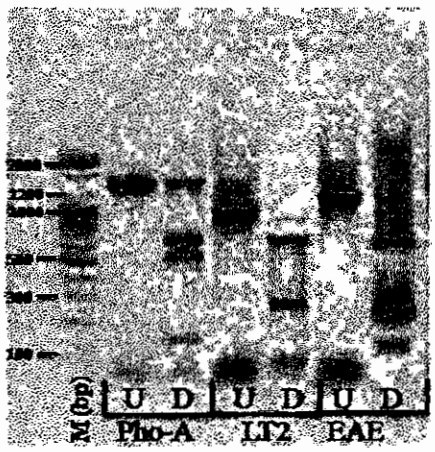


Figure 4.8: UV visualization of restriction enzyme analysis of PCR products using *Alu*I enzyme in a 1.5% agarose gel electrophoresis. M, lane represents the 100 bp marker; U, lane non-restricted DNA band and D, digested DNA band.

Overall summery of the experiment observed by electrophoresis after amplification of targeted DNA by PCR is shown in the table 4.3

Table 4.4: Overall summery of the experiment result

Enteroviruet Groups	Target genes	Farms in hygienic condition																					
		Cox's Bazar Sadar						Chakaria						Moheshkhali									
		Farm-1			Farm-5			Farm-1			Farm-3			Farm-1			Farm-2			Farm-4		
		W	S	I	W	S	I	W	S	I	W	S	I	W	S	I	W	S	I	W	S	I
Alkaline Phosphatase	Pho-A																						
ETEC	ST1																						
	LT1																						
	LT2																						
EHEC	VT																						
EPEC	EAE																						

Entero. Groups	Target genes	Farms in unhygienic condition																					
		Cox's Bazar Sadar									Chakaria									Moheshkhali			
		Farm-2			Farm-3			Farm-4			Farm-2			Farm-4			Farm-5			Farm-3			Farm-5	
		W	S	I	W	S	I	W	S	I	W	S	I	W	S	I	W	S	I	W	S	I	W	S	I
Alkaline Pho.	Pho-A																								
ETEC	ST1																								
	LT1																								
	LT2																								
EHEC	VT																								
EPEC	EAE																								

Here, represents absence of *E. coli*

Represents Presence of *E. coli*

Represents Presence of virulent strain of *E. coli*

Chapter Five

DISCUSSION

Chapter Five

Discussion

The aim of this work was to detect the enterovirulent *E. coli* strains (ETEC, EHEC and EPEC) by a rapid detection method using Polymerase Chain Reaction (PCR) in the shrimp farms of Cox's Bazar Region.

Fecal coliforms normally reside in the intestinal tract of warm-blooded animals. One of the most common groups of fecal coliform bacteria is *E. coli*. The fecal category contains both pathogen (disease-causing) and nonpathogenic bacteria. The presence of fecal coliforms is indicative of fecal contamination and of the potential presence of enteric pathogens (disease causing organisms which originate in the digestive system), especially bacterial pathogens. (EFS, 2003)

Fecal wastage of human, cattle, swine, horses, dogs, chickens, turkeys etc are common source of *E. coli* bacteria to contaminate water as well as aquatic organisms (Bitton, G. 2005). Various selective pressures in these environments favor the development, persistence and dissemination of robust strains, some of which may be resistant to antimicrobial agents (Schroeder *et al.*, 2002a, b). During the time of sample collection and baseline survey, cattle fields were observed near the farms as well as rivers, which are the main sources of water in shrimp farms. In the time of sample collection, some ring slab toilets as well as some hanging toilets were also observed. So, these could be the possible source of *E. coli.* in the farms.

Diagnostic screening for the presence of *E. coli.* strains by PCR starts with extraction of the DNA materials to be amplified. Representative samples are of primary importance in the

analysis of pathogens. In the present experiment, the PCR was performed without screening and removing detritus to avoid target loss (Tengs *et al.,* 2000) and to reduce extra time needed assuming that PCR is able to amplify target DNA if present in the sample. In the present experiment, a single-step DNA extraction solution DNAzol was used to extract total genomic DNA from different samples. 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 *E. coli.* strains 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.*,1996).

For PCR detection of general strains of *E. coli*, a pair of primers targeted at the *E. coli* alkaline phosphatase (Pho-A) housekeeping gene (Chang *et al.,* 1989), which has been previously demonstrated to be specific for *E. coli* (Kong *et al.,* 1995) were used. The remaining 5 pairs of primers were targeted at the LT1 (Leong *et al.,* 1995), LT2 (Pickett *et al.,* 1989) and ST1 enterotoxin genes of ETEC; the VT1 (Paton *et al.,* 1998) and VT2 verotoxin genes of EHEC; and the EAE virulence gene of EHEC and EPEC strains (Lightner, 1998). The VT primers which were targeted at two different stretches of DNA sequences within the VT1 and VT2 genes that share 100% sequence similarity were expected to amplify both or either of the genes from the DNAs of the corresponding EHEC strains. Similarly, the EAE primers which were targeted at two DNA domains that are highly conserved in the EAE genes of EHEC and EPEC strains were expected to amplify the targeted gene fragment from DNAs of either of those strains.

In the present study, the screening by PCR was done in two steps. At first, a specific band for alkaline phosphatase gene (1371 bp position) was screened out to determine the presence of *E. coli.* in the sample during the evaluation of PCR products. As alkaline phosphatase (PhoA) is the house keeping gene for *E. coli.*, its banding pattern on gel confirms the presence of *E. coli.* in the sample. Then, the *E. coli.* positive samples were studied for the identification of enterovirulent strains.

In the present study, from the 5 farms of Cox's Bazar Sadar Upazila the presence of various enterovirulent *E. coli.* strains were observed in farm-2, farm-3 and farm-4. At the fast step of screening a specific band for alkaline phosphatase gene (1371 bp position) was observed in the water of farm-1 & farm-5, sediment of farm-2, farm-3 & farm-4 and Shrimp intestine of farm-3 & farm-4 (Figure 4.1). The electrophoretic patterns of the PCR products using the six pair primer sets are shown in figure 4.4 and figure 4.5 that represents the 997 bp and 1155 bp banding pattern indicates the presence of LT2 of ETEC in sediment of farm-2 and water of farm-4 and EAE of EPEC in the sediment of farm-3 and water & sediment of farm-4 sample. In other samples, no any virulent strain of *E. coli* was found. Only banding pattern found in 1371 bp indicates presence of bacteria but no any virulent strain. The presence of this enteropathogenic *E. coli.* strains might be due to the contaminated water source from nearby farms or narrow canals carrying water from river. Supplied feed in the improved traditional culture system as well as the grazing cattle also may be a source of contamination. The pathogen is a causative agent of diarrhea in humans, rabbits, dogs, cats and horses (Toder, 2007)

From the sample collected from Chakaria upazila, among five farms only farm-3 shows negative response to the targeted housekeeping gene Alkaline Phosphatase (Pho-A) which means there was no presence of any *E. coli* in the water or sediment or intestine of shrimp of that farm. In case of the other farms, water of farm-1 & farm-4; sediment of farm-2 & farm-5 and shrimp intestine of farm four shows a specific band on 1371 bp in electrophoretic pattern ensure the presence of *E. coli* Bacteria shown in the figure 4.2. The electrophoretic patterns of the PCR products using the six pair primer sets are shown in figure 4.6 and figure 4.7 of *E. coli* positive samples of Chakaria region. The electrophoretic represents the 1371 bp, 997 bp and 1155 bp banding pattern indicates the presence of LT2 of ETEC in sediment of farm-2 & farm-5 and water of farm-4 and EAE of EPEC in the water of farm-4 sample. During sampling, it was observed that a cow market named "Ilishia cow market" is very close to the farm-4 and the deep tubewell water used for the washing of hand after the call of nature is directly connected to the farm. Overall poor management system, unhygienic environment was observed in that region which might be the major reason for the presence of these virulent strains. Karunasagar *et al.* (1999) reported cattle, sheep, pigs and chicken are thought to be the reservoir of ETEC and the zoonotic transmission of pathogen attains from animal to man is thought to be occur. For aquaculture products this could be a problem wherever animal dung/ waste is used to fertilize culture ponds. It is similar to the work of Kong *et al.* (1995) who conducted a various multiplex PCR protocols to simultaneously detect segments of different toxin genes of ETEC strains have also been developed. Similarity is also found with the experiment of Lang *et al.* (1994) and Paton and Paton (1998), who evaluated a very sensitive multiplex PCR system for the rapid detection and typing of ETEC and EHEC strains of *E. coli.* in the aquatic environment.

Among the three upazila of Cox's Bazar district, comparatively less infection of *E. coli* was found in the Moheshkhali region. Sediment of farm-3 & farm4 and sediment & intestine of farm-5 shows band on 1371 bp in electrophoretic pattern resulting the presence of *E. coli* bacteria shown in the figure 4.3. Among them only in the sediment of farm 3 sample respond to the LT2 of ETEC gene shown in figure 4.8. No other virulent strain found in the *E. coli* positive samples. In case of farm-1, farm-2 and farm-4 there was no any infection of the targeted bacteria. Among the five farms in farm-1 and farm-2 semi-intensive culture system is practiced by BEXIMCO Fisheries Limited. Comparatively good and hygiene environment was observed during sampling and baseline survey.

The present result is supported by Ristaino *et al.* (1983), Kong *et al.* (1995), Lang *et al.* (1994) and Paton and Paton (1998) who conducted a various multiplex PCR protocols to simultaneously detect segments of different toxin genes of ETEC strains have also been developed, for example, multiplex detection of LT1/LT2. It is also similar to the work of Pickett *et al.* (1989) and Victor *et al.* (1991) who observed the use of the polymerase chain reaction (PCR) and gene probe technology has provided rapid and highly sensitive methods for the specific detection of pathogenic ETEC.

In the present study, in case of ETEC only heat-labile enterotoxin (LT) strain was observed in the sample farms. No heat-stable (ST) ETEC strain was found. It may be for the reason of world wide abundance pattern of the specific strain. According to WHO (2009), the heat-stable toxin (ST) is an 18-amino acid-long, highly folded peptide which also causes disruption of chloride channels in the cell and secretory diarrhoea. LT is expressed in about 66% of ETEC strains, either alone or in combination with ST, and thus is significantly

responsible for the worldwide disease burden of ETEC. There was also no evidence of EHEC strain group in the sample farms. It might be occurs due to the environmental variation of *E. coli.* strain in the sampling area. Tave (1993) reported every organism requires a particular environment for growth and development, so, in this sense, the sampling area might be safer from the enterohemorrhagic *E. coli.* strains; since *E. coli.* frequently responses with the changing environment.

To better determine the health risks associated with exposure to pathogenic *E. coli.* in the environment, the frequency at which pathogenic *E. coli.* occurs in the environment must be assessed. Currently, there is no biochemical or serological markers to facilitate rapid identification and typing of ETEC and EPEC variants that possess different polymorphic forms or combinations of these virulence genes. The results above indicate the potential of the PCR assay to analyze the habitat infaction with pathogenic and non-pathogenic *E. coli.* strains in shrimp farm samples. Conceivably, the method can also be used as a cost effective means to determine the prevalence or frequency of occurrence of these organisms in diverse ecological niches by enabling rapid identification and typing of clinical and environmental isolates of ETEC and EPEC strains. The PCR protocol exhibited specificity and high sensitivity towards *E. coli.* since no product was generated when the same primer set was used to amplify negative control sample. A single step PCR with sample having clinical signs of disease is sufficient to amplify enough *E. coli.* specific DNA to be visualized following electrophoresis (Tan *et al.*, 2001), has been supported by the present study as a DNA band from a single step PCR product was only observed . However, detecting enterovirulent *E.coli* strains with obvious sign of infection is of no significance as far as viral quarantine is concerned. Since, the present experiment was intended to establish the protocol that could be routinely applied to detect

bacteria in various aquatic inhabitant of shrimp farm in all life stage as brood to offspring, present experiment purposively select both apparently healthy shrimp and apparently infected shrimp. Here the route of infection may be various as because the farmer have no data on the brooders, larvae they used, food, water and plankton.

The nature of *E. coli.* entrovirulent strains outbreaks and mortality pattern has become most unpredictable and health management effort has become chance. Effective prevention and control methods are urgently needed to control the spread of the bacterial disease. Knowledge on the presence of bacteria through diagnostic PCR in post larvae intended for stocking in ponds will greatly help the farmer to understand the risk associated with stocking infected larvae and appropriate management measures for their culture. To this end, the methodological details presented in this thesis for assessment of *E. coli.* using PCR is not dependent on mortality rate and clinical signs that will aid farm managers in decision making in culture management, perhaps allowing proactive steps to be taken to reduce losses when bacterial strikes. The results suggest that a PCR screening of enterovirulent *E.coli* strain infection for shrimp brood and larval stocks and other potential bacteria carriers could be an effective tool in fight against the diseases that has almost devastated the shrimp industry of the country.

Chapter Six

CONCLUSION

Chapter Six

Conclusion

By analyzing the habitat infection of penaeid shrimp with enterovirulent strains of *E. coli*, it could be argued that, the sampling area is infected with enterotoxin (ETEC) and enteropathogenic (EPEC) strains of enterovirulent *E. coli*. This can affect the quality and regularity of production as well as the quantity of harvest. As shrimp culture practice and shrimp processing industries to export shrimp worldwide increased significantly in last two decades, habitat infection with pathogenic bacteria is a major concern now a days. It is necessary to find out the specific causative agent of bacterial infection in shrimp habitat to develop the shrimp sector of Bangladesh. In the field of shrimp disease diagnosis out of different molecular biological techniques, PCR based techniques are beginning a new era of excellence to ensure the disease controlled crop production.. Further research, however, is required to enable high quality validation and trial of these methods as well as diagnosis of the infection of other pathogenic bacteria.

Chapter Seven

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Chapter Seven

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Chapter Eight

APPENDICES

Chapter Eight

Appendix

Appendix-A

Questioner Form (*E.coli*)

1. Location : Farm size:
2. Management Typey: Govt. Private Lease NGO
   Hiring Partnership
3. Culture Technique : Traditional, Improve Trad'nl, Semi intensive
4. Water source :
5. Sanitation facility : Poor Medium Good
6. Fry source :
7. Fertilizer : a. Organic
   b. Inorganic :
8. Feed type :
9. Diseases Problem :
10. Disease symptom:
11. Antibiotic used :
12. Chemicals Used :
13. Production/ yrs :
14. Stocking of Fry /yrs:

Appendix B

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 1.5% agarose gel containing 2 µl ethidium bromide in 150 ml 1x TAE buffer. Supply of power for electrophoresis was continued until DNA migrated near about the two-third of the gel towards the positive electrode. DNA size was determined by comparison with a DNA size marker (100 bp DNA ladder, Bioneer, Korea). DNA bands were visualized by UV illumination and photographed using the Gel Doc 1000 documentation system (Bio-Rad).

Materials

1. Electrophoresis buffer; TAE buffer (40 mM Tris- Acetate & 1mM **EDTA,** pH 8.0);
2. Agarose powder;
3. Ethidium bromide (NOTE: Ethidium bromide is a known mutagen and thus handled as a hazardous chemical — hand gloves were used while handling);
4. Gel loading buffer (BlueJuiceTM Gel Loading Buffer: 65% (w/v) sucrose, 10 mM Tris-HCI (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 2.25 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. 2 µl Ethidium bromide was added to the solution and mixed well;
   e. The solution was poured into the gel casting tray containing sample comb and were allowed to solidified at room temperature; (Note: with decreasing temperature gel will be solidify rapidly thus step 1.4 to1.6 was done quickly without consuming high time.

f. After gel has solidified, the comb was removed, using care not to rip the bottom of the wells.

g. The gel could be stored at -4°C for further use.

3. The gel, still in its plastic tray, was inserted horizontally into the electrophoresis chamber and was covered by adding approximately 450 ml TAE buffer;
4. 4 µl of the extracted DNA sample was mixed with 2 µl of loading buffer on a parafilm and were poured into a sample wells. By maintaining sample's serial number, this was done for every sample separately. To avoid cross-contamination, separate micro-tips were used for every sample.
5. Approximately 2 µl of colored DNA size marker was poured into first sample wells on gel ;
6. The electrophoresis chamber was covered with the lid;
7. The power supply was switched on, only volt was fixed at a constant value of 50, the machine was run until DNA migrated to the two-third of the gel towards the positive electrode (DNA was usually blue colored based on loading buffer);
8. After 25 to 30 minutes The power supply was switched off and the lid of the electrophoresis chamber was removed;
10. The gel, still in its plastic tray, was removed from the electrophoresis chamber and placed on an ultraviolet (UV) transilluminator.
11. The UV transilluminator was switched on, DNA banding pattern was visible;
12. The resulting banding pattern was captured by using the digital camera and software.

Appendix-C

Spectrophotometric method determination of DNA concentration and purity:

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

Materials

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

Procedure

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

1. Spectrophotometer was warmed up before use.
2. Spectrophotometer's software was opened
3. 2000 µl of 8 mM NaOH was taken in blank cuvette;
4. DNA sample (4 µl solubilized DNA + 1996 µl of 8 mM NaOH) was taken in another cubette.
5. Resulting data against each absorbance was found on the program's page.
6. DNA sample containing cubette was washed properly before loading next sample.
7. A list of data of the samples against two absorbance was found and saved.
8. The machine was turned off before the program was closed .
9. Calculation;
   a) Double-stranded DNA concentration (C), µg/ml =Absorbance at A_{260} ×50×500;
   b) Purity = Absorbance at A_{260} /Absorbance at A_{280}

Appendix-D

Description of Ladder:

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

Application: Size confirmation of DNA fragments.

Features:

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

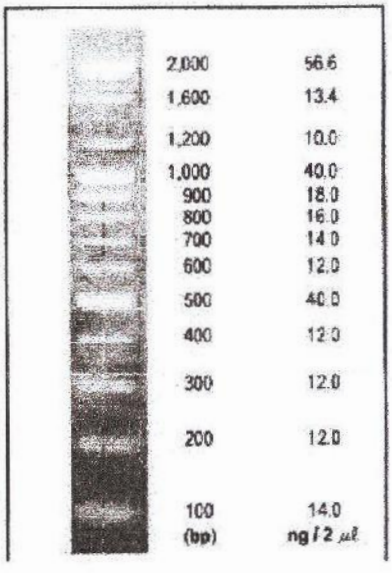

Fig-8: 1% agaros gel strained with Ethidium Bromide

Photo Gallery

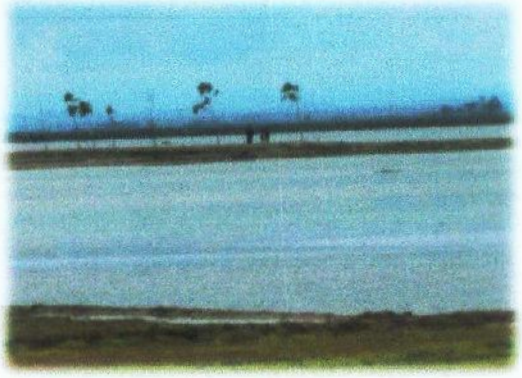

Figure: Farm-2, Moheshkhali

Figure: Open toilet nearby farm-4, Cox's Bazar Sadar

Figure: Grazing cattles nearby farm-4 of Chakaria

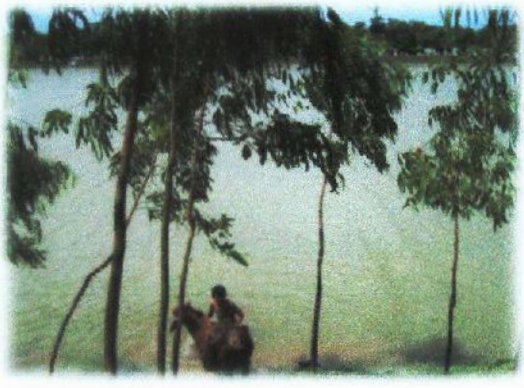

Figure: Washing of cattles in farm-4 of Chakaria

Figure: Sample collection

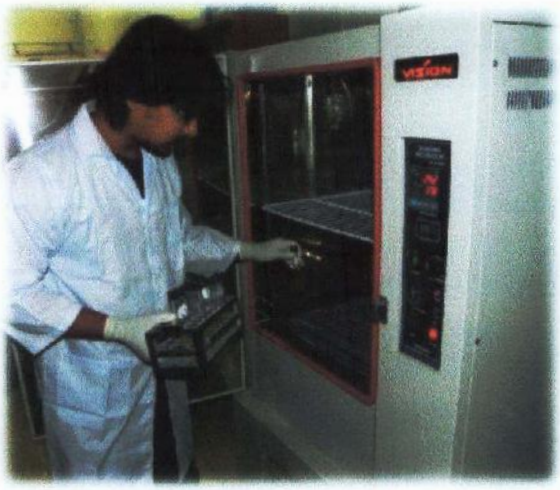

Figure: Overnight culture of bacteria

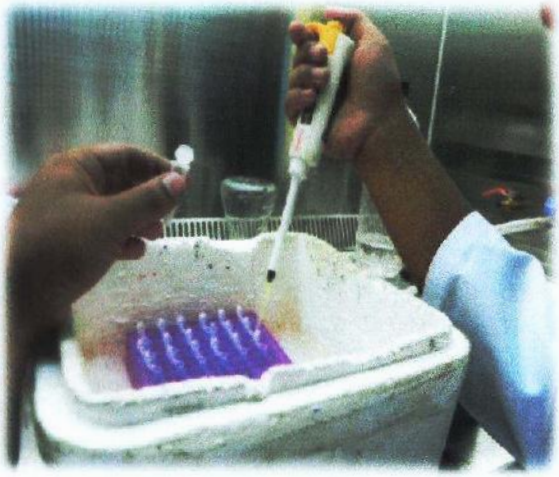

Figure: Preparation of the sample mixture

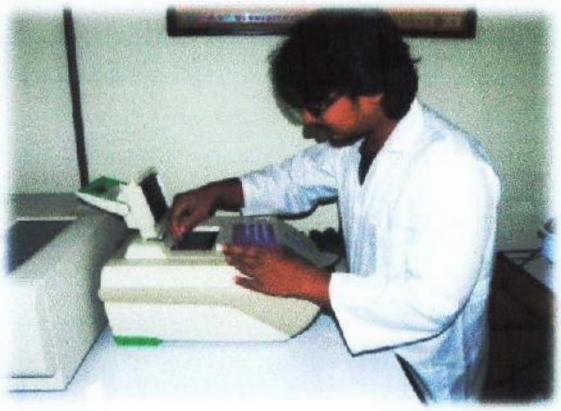

Figure: Sample mixtures placed on the thermal cycler

Figure: Gel electrophoresis to evaluate PCR product.