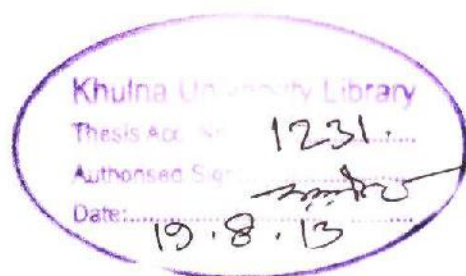


**THE USE OF PCR TO AID IN THE RAPID IDENTIFICATION OF
ENTEROVIRULENT *Escherichia coli* IN SHRIMP FARMS**



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December, 2012

THE USE OF PCR TO AID IN THE RAPID IDENTIFICATION OF ENETROVIRULENT *Escherichia coli* IN SHRIMP FARMS

A Thesis By

Md. Sayeduzzaman

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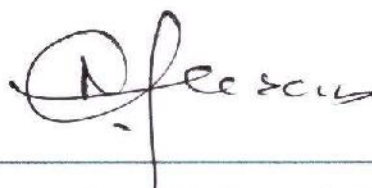
Khulna, Bangladesh



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OF ENTEROVIRULENT *Escherichia coli* IN SHRIMP FARMS**

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December, 2012

DECLARATION

The Use of PCR to Aid in the Rapid Identification of Enterovirulent *Escherichia coli* in 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.

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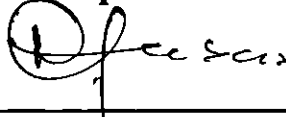


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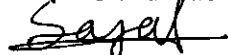


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Dedicated to

My Beloved Parents



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Abstract

Polymerase Chain Reaction (PCR) technique allows the detection of the most enterovirulent *Escherichia coli* strains: enterotoxigenic (ETEC), enteroinvasive (EIEC), enterohaemorrhagic (EHEC) and enteropathogenic (EPEC). The prospective shrimp sector is facing a threat of disease. However, the study on the virulence of *E. coli* in shrimp habitat is still scarce. The aim of the present study was to detect enterovirulent strains of *E. coli* in the ten different shrimp farms which was categorized on the basis of unhygienic (UF₁, UF₂, UF₃, UF₄, UF₅ and UF₆) and hygienic (HF₁, HF₂, HF₃ and HF₄) condition of farms in Kalapara, Patuakhali, Bangladesh. Water, sediment and shrimp samples were examined from each farm. Six pairs of oligonucleotide primers were used for the amplification and thereby detection of target genes viz. the alkaline phosphatase (Pho-A), the heat-labile toxin1 (LT1), heat-labile toxin2 (LT2), heat-stable toxin1 (ST1), verotoxin (VT) and attachment and effacement (EAE) virulence genes. Finally, PCR products were analyzed and detection of different strains was confirmed by agarose gel electrophoresis and restriction enzyme analysis with AluI enzyme. The results revealed the presence of EPEC groups were common in most of the samples of shrimp farms having unhygienic condition. EPEC was present in water of UF₁ and UF₆. Sediment of UF₂ and shrimp of UF₆ showed EPEC positive. Whereas ETEC groups were found in shrimp of UF₂ and UF₆ and also water and sediment of farm UF₅. *E. coli* was absent in UF₃ and harmless *E. coli* was present in UF₄. In case of farms in hygienic condition, EEC groups was absent in HF₂, HF₃ and HF₄ where harmless *E. coli* was observed in water and shrimp of HF₁. 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. This knowledge will help in production of *E. coli* free shrimp and rapid detection of *E. coli* polluted shrimp in processing industry.

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List of Abbreviations

Abbreviation	Elaboration
μ l	Micro liter
bp	Base pair
CFU	Colony Forming Unit
EAE	Attachment and Effacement
EEC	Enterovirulent <i>Escherichia coli</i>
EHEC	Enterohaemorrhagic <i>Escherichia coli</i>
EIEC	Enteroinvasive <i>Escherichia coli</i>
EPEC	Enteropathogenic <i>Escherichia coli</i>
ETEC	Enterotoxigenic <i>Escherichia coli</i>
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

1.1 Background

The shrimp industry is the fastest growing food producing industry and meets the demand for fish and shellfish resulting decline in capture fisheries. The health of fish and microbiological safety of aquaculture products depend on the microbiological quality of the production environments that may cause diseases. This environment can also affect the microbial quality of the fish and ultimately the processed product (NACA/FAO, 2000).

Shrimp farming practiced largely in tropical and subtropical countries for exporting, has experienced spectacular growth over recent decades. In the 2010-11 fiscal years, the total production of shrimp is 7.82% of total fish production and about 239460 mt. in which 52.05% is from culture basis (DoF, 2012). Bangladesh has earned 3568.2 cores Tk. by exporting 54891 mt. frozen shrimp in the 2010-11 fiscal years (DoF, 2012). The Shrimp industry is the second largest export commodity of the country. Of all the exportable agro-based primary commodities, shrimp is by far the most important. It alone contributes more than 70% to the total export earnings from all the agro-based products, including tea, raw jute, vegetables, fruit, etc. (Karim, 2004).

The main cultured species is the tiger shrimp, *Penaeus monodon* (locally known as bagda chring) of which the scientific name is *Penaeus monodon* (about 77% of export earning of shrimp sector). It is a marine shrimp and is cultivated in brackish water. There is a lot of demand in the international market for shrimp and Bangladesh is blessed with an environment that is congenial for shrimp production. Shrimp farming is practiced in the coastal region of Bangladesh. Shrimps are cultured in about 2.20 lack hector of the coastal region and the livelihood of 12 lack people directly or indirectly in shrimp farming (DoF, 2012).

The major shrimp producing districts are Bagerhat, Satkhira, Pirojpur, Khulna, Patuakhali, Cox's Bazar and Chittagong. But shrimp farming is a crucial to the fisheries department of Bangladesh now-a-days, because of risk of mortality in different stages. In Bangladesh disease is one of the major factors that affect shrimp production. It is reported that Bangladesh lost 45.3% of production in 1996, leading to a reduction in foreign income of 42.3% from shrimp exports from 13% of extensive and 74% of semi-intensive shrimp farms (Chowdhury, 1997). In aquatic environments, diseases in fishes and shrimps are caused by opportunistic pathogens (Lundin, 1996).

Bacterial and Viral diseases have most serious losses occurred. Unlike fish, shrimp lack an antigen/antibody system and so cannot be vaccinated in the way fish can be (Roach, 1999). Prevention is the only cure. Hence, disease is inevitable in these uncontrollable culture models. 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, 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).

A wide range of other viral, bacterial and protozoan diseases results from the contamination of water with human faeces, leading to outbreaks of Cholera, Typhoid and Diarrhea. Kidney and cardiovascular diseases have also been found to be water related (Sanni, 2009).

Currently, aquaculture industry in Bangladesh and other parts of the world specially southeast region has been facing serious problems due to microbial diseases. Thus, health management is of major importance in aquaculture. Species from *Vibrio* are the most

common bacterial pathogens causing some of the most serious diseases, growth retardation and sporadic mortalities in Penaeid shrimp (Lavilla-Pitago *et al.*, 1998; Vandenberghe *et al.*, 1998). Freshwater contaminated by fecal material from men and animals may contain a large variety of human pathogenic microorganisms. For years, total coliforms and fecal coliform were the most widely used indicators but, more recently, the abundance of *Escherichia coli* has been shown to be more related to the sanitary risk than that of coliforms (Fewtrell and Bartram, 2001).

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 high 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 humans. 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 aetiological agents of various gastrointestinal infections in humans. 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 the detection 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) (Edberg *et al.*, 1990), used widely has several disadvantages specially in relation to its lack of specificity. Firstly, it does not distinguish pathogenic from nonpathogenic strains of *E. coli*. Secondly, some other strains of *Salmonella*, *Shigella* and *Yersinia* can be detected by splitting MUG (Bettelheim, 1992) and thirdly, phenotypically MUG-negative *E. coli* (Chang *et al.*, 1989), for instance, EHEC strains is not detected by this method (Doyle and Schoeni, 1984). For the specific detection of pathogenic ETEC and EHEC strains, therefore, several ELISA-based methods have been developed including detection of heat-labile LT (Ristaino *et al.*, 1983), heat-stable ST1 (Carroll *et al.*, 1990) and ST2 (Urban *et al.*, 1990) enterotoxins of ETEC; and verotoxins VT1 and VT2 of EHEC strains (Downes *et al.*, 1989). However, some of the tests are known to have variable sensitivities, so depends on the relative levels of gene expression of the target gene products under selective culture conditions, makes disadvantage of rendering unculturable cells non-detectable (Roszak and Colwell, 1987).

The Polymerase Chain Reaction (PCR), on the other hand, has seen numerous recent applications in pathogen detection and shrimp pathology research. PCR is an *in vitro* method for selective, repeated duplication of a specific segment of DNA. The development of nucleotide sequencing methods, the storage of this information in a computer researchable database, invention of automated oligonucleotide synthesis methods, discovery of thermostable DNA polymerase have all contributed to the rapid implementation and widespread use that PCR enjoys today (Karunasagar *et al.*, 1999), for detecting pathogen directly even in a uncultured manner (McKeever and Rege, 1999; Wagener, 1997).

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 visible by gel electrophoresis (Perkins and Martin, 1999).

In recent years, the use of 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 detect segments of different toxin genes of ETEC and EHEC strains, have also been developed. For instance, 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, LT1/VT1/VT2 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).

In this study, I used a versatile multiplex PCR method that is capable of simultaneously detecting up to seven different virulence genes known to be found in various combinations in ETEC, EHEC and EPEC strains of pathogenic *E. coli*.

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. 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. 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).

Although, the shrimp farms in Bangladesh are highly subjected to the contamination of domestic sewages. In Bangladesh disease is one of the major factors that affect shrimp production. Traditionally cultured shrimp farms in Bangladesh are highly susceptible to be contaminated with such pathogenic *E. coli* strains because of lack of proper sanitation near these farms. This reason is more than enough to have virulent *E. coli* strains contamination in shrimp, water and sediment in the farms of kalapara upazilla.

1.2 Objective of the study

The objective of the study was to detect the enterovirulent *E. coli* strain specific gene with PCR.

Chapter- Two

Literature Review

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.

Ristaino *et al.* (1983) stated that the specific detection of pathogenic ETEC and EHEC Strains which produce different types of enterotoxins, several ELISA-based methods has been developed. They include ELISAs for the detection of heat-labile LT. Same procedure was followed by Carroll *et al.* (1990) to detect heat-stable STI, Urban *et al.* (1990) to detect heat-stable STII enterotoxins of ETEC and Downes *et al.* (1989) to detect verotoxins VT1 and VT2 of EHEC strains.

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.

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.

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.

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; LTII/VTII/VTII genes of ETEC and EHEC strains and VT1/ VT2/HlyA/EAE-A/Rfb0111/Rfb0157 genes of EHEC.

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.

Hanninen and Hirvela-Koski (1997) 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.

Wakabayashi and Wakabayashi (1999) evaluated *Flavobacterium*, *F. columnare*, has been studied by RFLP analysis of the small subunit rRNA gene, revealing many gene groups that all share the same phenotype.

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 invasins *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 *aggR*-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.

Garcia-Sanchez *et al.* (2007) evaluated the role of wild artiodactyls as reservoirs of *Escherichia coli* O157:H7 for livestock and humans. *Retroanal mucosal* swabs samples from 206 red deer (*Cervus elaphus*), 20 roe deer (*Capreolus capreolus*), 6 fallow deer (*Dama dama*) and 11 mouflon (*Ovis musimon*) were screened. Polymerase chain reaction (PCR) was used to detect the presence of genes coding O157 and H7 antigens and the virulence factors verocytotoxin, intimin and enterohaemolysin. Three *E. coli* O157:H7 isolates were obtained from red deer (1.5%). Two of them showed inability to ferment sorbitol and lack of β -D-glucuronidase (GUD) activity, however, the other strain investigated was an atypical sorbitol-fermenting *E. coli* O157:H7 with GUD+ activity.

Acc 1251

Chapter- Three

Materials and Methods

3.1 Profile of the study area

The Experiment was conducted at Molecular Biology Laboratory of Fisheries and Marine Resource Technology (FMRT) Discipline of Khulna University. Shrimp farms were situated at kalarapara Upazilla under Patuakhali district. The criteria of farm selection were at first base line survey through questionnaire (Appendix-E). Shrimp, water and sediment samples were collected from ten different shrimp farms at Kalapara of Patuakhali. The farms were categorized as hygienic (HF) and (UF) on the basis of some criteria such as culture technique, water source, sanitation facility, feed, fry source and presence of grazing land or cattle shade. However, the farms that had hygienic condition were located at Khapuara and Shibli hatchery under kalapara upazilla. The unhygienic condition was found in those farms that were located at Chakamuia, Mithaganj, and Latachapli of the same upazilla.

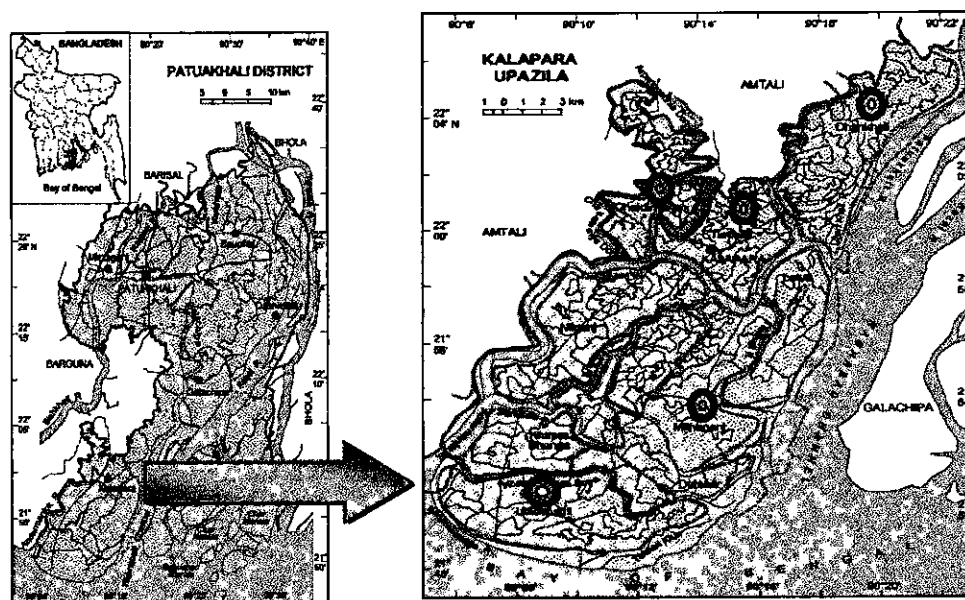


Fig 3.1: Sampling area at kalapara upazila ; the circle indicates the approximate location of sampling farm

3.2 Experimental design

Water, sediment and shrimp samples were collected at first from ten shrimp farms designed as UF₁, UF₂, UF₃, UF₄, UF₅ and UF₆ from unhygienic farms category and HF₁, HF₂, HF₃, and HF₄ from hygienic farm category. One positive (P) control (water of Moure River) was taken for the experiment. The second step involved the extraction of DNA from the bacterial cell. In third step, qualitative and quantitative analyses of extracted DNA were done by using agarose gel electrophoresis and spectrophotometric analysis respectively. The fourth step involved the amplification of target gene by PCR technology; this step involved in two phases, at first phase, all samples were checked for the presence of alkaline phosphatase (Pho-A) and only *E. coli* positive samples were further checked for the presence of enterovirulent strains in next phase. In the final step, Gel electrophoresis was performed to detect enterovirulent *E. coli* strains by observing clear bands through comparing with a 100 bp DNA ladder as well as restriction enzyme analysis was performed for rapid conformation of the identity of the PCR-amplified products. The target genes were chosen for this investigation included: Alkaline phosphatase (Pho-A), housekeeping gene (present in all *E. coli*); the LT₁, LT₂ and ST₁ genes of ETEC; the VT and EAE virulence genes of EHEC and EPEC, respectively and six pairs of oligonucleotide primers were used named such 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.

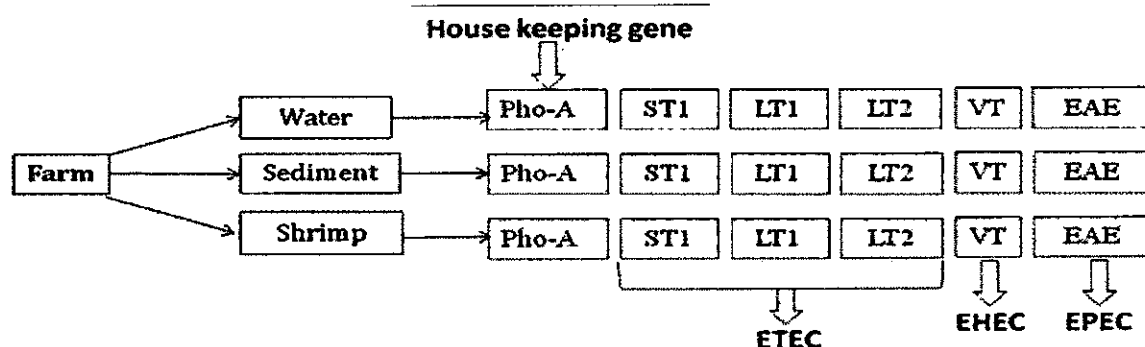


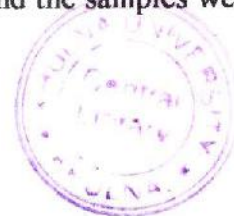
Fig-3.2: Samples and primer used in each farm

3.3 Sample collections

Both water and sediment sample were collected from five different point of each shrimp farm by a pasteurized tube and were kept in a sterile 10 ml plastic vial. Fresh shrimp, *Penaeus monodon* (average body weight 21 ± 5.14 g) was also collected from each farm which was kept in individual polythene bag that was kept in ice box. As soon as possible, the plastic vials containing sample were transported to Molecular Biology Laboratory, FMRT Discipline, and Khulna University. After collection, samples were brought to the laboratory and preserved at 4°C temperature. Intestine of the collected shrimp was separated by dissecting the body and was kept each in 1.5 ml (Eppendorf) 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.

3.4. Preparation of media and organisms culture

For media preparation, 2g Luria-Bertani (LB) broth was added with 100 ml of distilled water and the mixture was autoclaved at 121°C for 20 minutes. Then 5ml autoclaved LB broth and 100 µl samples were mixed in a test-tube which was then placed in a shaking incubator (VS-8480SL, Korea) at 37° C with 200 rpm (rotation per minute) for overnight to culture. The overnight culture provided huge amount of bacteria and the samples were ready to use for the preparation of DNA extract.



3.5 DNA Extraction:

The DNA isolation was done by using DNA_{zol} reagent (Genomic DNA Isolation Reagent) followed by the protocols of Ausubel *et al.*, (1990) and further optimized by Islam, (2006). DNA_{zol} reagent is a complete and ready-to-use reagent for the genomic DNA isolation from small or large volume of solid and liquid samples that normally combines both reliability and efficiency with simplicity of the isolation protocol. The procedure was

completed within 30 minutes with 70-100% of DNA recovery. 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 μ l) and deposited plate layer was kept for further procedure. About 500 μ l 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. 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 removed 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 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 stored them at room temperature for 1-3 minutes. Then it was centrifuged for 10 minutes 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. 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. 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 minutes. 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.

3.6 Qualitative and quantitative determination of extracted DNA

3.6.1 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 µl ethidium bromide (EtBr) 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. A.

3.6.2 Spectrophotometric method determination of DNA concentration and Purity

The concentration of DNA samples was 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 cuvette. 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), $\mu\text{l/ml} = \text{Absorbance at } A_{260} * 50 * 500;$
- 2) Purity = Absorbance at $A_{260} / \text{Absorbance at } A_{280}$

3.7.0 Description of target genes and primers:

3.7.1 Target genes:

The target genes were chosen for this investigation included: Alkaline phosphate (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. Six pairs of specific primers were designed from above mentioned gene sequence (Table- 3.1).

3.7.2 Oligonucleotide primers:

The target genes chosen of this experiment were: Alkaline phosphate (Pho-A), housekeeping 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. Six pairs of specific primers were chosen from gene sequence (Table 3.1) to amplify the target DNA fragments (genes). To compare the size of double stranded DNA from 100 to 2,000 base pairs, 100 base pairs designed DNA markers were used. The DNA marker consists of 13 double stranded DNA fragments ranging from 100 to 1,000 (Bioneer, Korea).

Table 3.1: Primers and expected size of PCR-amplified gene targets of pathogenic *E. coli*

Enterovirulent groups	Target Gene	Primers	Product size (bp)	Accession No.
House keeping gene	1. Alkaline phosphatase (PhoA)	Pho-F	1371	M13345
		Pho-R		
Enterotoxigenic (ETEC)	2. Heat-stable toxin 1 (ST1)	ST1-F	638	M25607
		ST1-R		
	3. Heat-labile toxin 1 (LT1)	LT1-F	725	J01646
		LT1-R		
Enterohemorrhagic (EHEC)	5. Verotoxin (VT)	VT-F	1240	M36727
		VT-R		
Enteropathogenic (EPEC)	6. Attachment and Effacement (EAE)	EAE-F	1155	M58154

3.8.0 Target gene amplification by Polymerase Chain Reaction (PCR):

At first, the concentration of all the extracted DNA samples was adjusted. The reactions were performed in a 0.2 ml PCR tube (Eppendorf, Hamburg, Germany) in which 25µl reaction mixture containing 2µl DNA sample, 2µl oligonucleotide primers, 5µl 5X green reaction buffer, 2µl 10mM dNTPs mixture, 1µl Taq DNA polymerase and 13µl de-ionized distilled water. The 0.2 ml PCR tube containing reaction mixtures were then placed in a DNA thermal cycler (C1000™, BIO-RAD, USA) for polymerase chain reaction (Fig. 3.3).

Table 3.2: The component of reaction mixture and their compositions in 25 µl reaction mixture together with final concentration for the PCR.

Component	Final volume	Final concentration
1. Go Taq DNA Polymerase* (5 units/µl)	1 µl	1.25 unit
2. 5X Green Reaction Buffer (Tris; pH 9.0, 15 mM MgCl ₂)	5 µl	1x
3. 10 mM dNTP mixture (each dNTP	2 µl	1.0 mM

	2.5 mM)	
4. Primers (Pho, ST1, LT1, LT2, VT and EAE)	2 μ l (Forward 1 μ l + Reverse 1 μ l)	1.0 μ M
5. Template DNA	2 μ l	0.25 μ g/25 μ l
6. Di- ionized water	13 μ l	

*To prepare 1 unit *Taq* DNA polymerase, it was 4 times diluted with di-ionized water.

The PCR conditions for target DNA amplification were: initial extended step of denaturation at 94°C for 2 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, primer annealing at 58°C for 1 minute and elongation at 72°C for 1 minute. While running PCR reactions, a positive control was also kept. The positive control constitutes highly *E. coli* contamination which ensured all the chemicals in the PCR samples worked efficiently

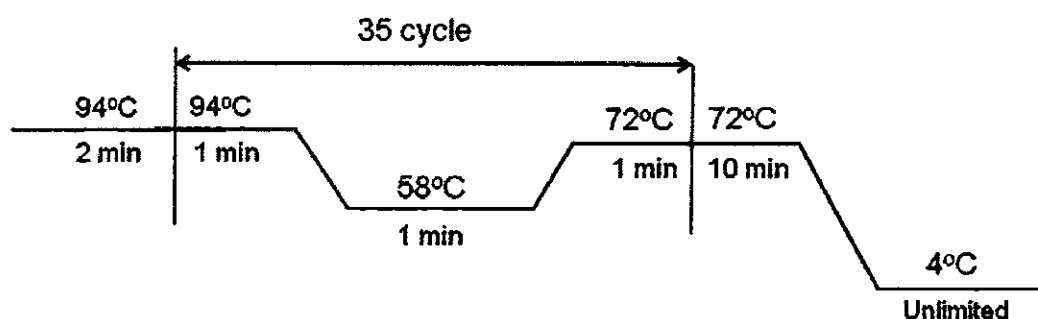


Figure 3.3: A schematic diagram of PCR cycle

3.9. Evaluation of PCR products:

3.9.1 Gel electrophoresis

After completion of the thermal cycling, a sample of 10 μ l from each PCR products was analyzed electrophoretically by running on a 2% 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.

3.9.2 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 *AluI* enzyme was found to be most suitable since its recognition sequence was present within six of the PCR-amplified products, each yielding *AluI* 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	Frequency	Cut Positions
Pho-A	AGCT	2	150, 715
ST1	AGCT	1	282
LT1	AGCT	3	258, 702, 718
LT2	AGCT	2	66, 704
VT	AGCT	1	301
EAE	AGCT	3	630, 771, 899



The purification was performed according to the following 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 the 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 temperature. 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 μ l from each primer products and 2 μ l gel loading dye (Promega, Madison WI USA) was analyzed electrophoretically by running on a 2% 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

Result

Farms were categorized in two groups, hygienic farm (HF) and unhygienic farm (UF). The hygienic farm located at kalapara and kuakata. In this farm water is collected from deep tube well artificial feed is used. On the other hand water source is river that is directly connected to the human drainage system and cow dung or poultry litter was used to increase the natural feed in the unhygienic farm. On the basis of the collected information, directly from the farmer during sample collection, environmental and physical parameters are given in table.

Table 4.1: Environmenral and physical condition of the farms

Area	Farm	Size (Acre)	Culture technique	Water source	Sanitation facility	Fry source	Grazing land/cattle shed	Feed
Kalapara	HF1	0.5	Semi-intensive	Deep tube well	Good	Hatchery	No	Artificial
	HF2	0.5	Semi-intensive	Deep tube well	Good	Hatchery	No	Artificial
Kuakata	HF3	0.25	Semi-intensive	Deep tube well	Good	Hatchery	No	Artificial
	HF4	0.40	Semi-intensive	Deep tube well	Good	Hatchery	No	Artificial
Chakamuia	UF1	100	Traditional	River	Good	Both Wild & Hatchery	Yes	Natural
	UF2	150	Traditional	River	Poor	Both Wild & Hatchery	Yes	Natural
Dhankhali	UF3	200	Traditional	River	Good	Both Wild & Hatchery	Yes	Natural
	UF4	50	Traditional	River	Poor	Hatchery	Yes	Natural
Mithaganj	UF5	120	Traditional	River	Poor	Both Wild & Hatchery	Yes	Natural
	UF6	150	Traditional	River	Poor	Both Wild & Hatchery	Yes	Natural

Extracted and PCR amplified DNA samples of bacterial cells showed the clear band patterns during gel electrophoresis. The A_{260}/A_{280} ratio of the extracted DNA materials was within 1. Thus, spectrophotometric analysis revealed the satisfying purity of extracted DNA samples from all the experimental shrimp farms. The final yield of DNA ranged between 0.16 - 0.91 $\mu\text{g/ml}$ with the highest yield being from water sample of UF₂ farm and the lowest yield from shrimp sample of HF₁ farm.

Table4.2: Spectrophotometric data for the evaluation of DNA purity.

List of farms and samples		A_{260}	A_{280}	A_{260} / A_{280}	DNA conc. $\mu\text{g/ml}$
HF ₁	Water	0.02	0.028	0.71	0.50
	Sediment	0.012	0.018	0.67	0.30
	Intestine of shrimp	0.006	0.010	0.6	0.16
HF ₂	Water	0.009	0.012	0.75	0.23
	Sediment	0.008	0.015	0.53	0.20
	Intestine of shrimp	0.013	0.024	0.54	0.33
HF ₃	Water	0.009	0.02	0.45	0.23
	Sediment	0.01	0.018	0.56	0.25
	Intestine of shrimp	0.019	0.028	0.68	0.48
HF ₄	Water	0.016	0.021	0.76	0.4
	Sediment	0.012	0.018	0.67	0.30
	Intestine of shrimp	0.010	0.016	0.63	0.25
UF ₁	Water	0.008	0.015	0.53	0.20
	Sediment	0.009	0.013	0.69	0.23
	Intestine of shrimp	0.018	0.028	0.64	0.45
UF ₂	Water	0.036	0.048	0.75	0.91
	Sediment	0.009	0.015	0.6	0.23
	Intestine of shrimp	0.018	0.028	0.64	0.45
UF ₃	Water	0.009	0.015	0.6	0.23
	Sediment	0.018	0.028	0.64	0.45
	Intestine of shrimp	0.008	0.015	0.53	0.20
UF ₄	Water	0.031	0.042	0.74	0.76
	Sediment	0.018	0.028	0.64	0.45
	Intestine of shrimp	0.009	0.013	0.69	0.23
UF ₅	Water	0.02	0.028	0.71	0.50
	Sediment	0.008	0.012	0.67	0.20
	Intestine of shrimp	0.006	0.010	0.6	0.16
UF ₆	Water	0.009	0.02	0.45	0.23
	Sediment	0.01	0.018	0.56	0.25
	Intestine of shrimp	0.019	0.027	0.70	0.48

During Pho-A testing, 1371bp band pattern was found which clearly indicates the presence of *E. coli* in that samples of farms whether hygienic condition or in unhygienic condition (Fig: 4.1, Fig: 4.2, and Fig: 4.3).

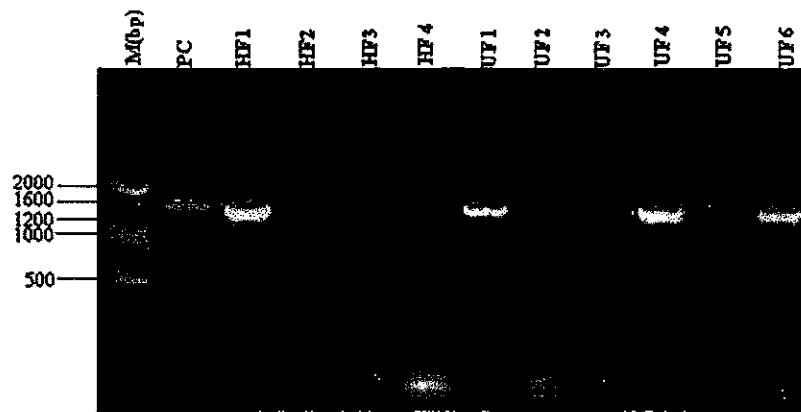


Fig 4.1: UV visualization of water samples following electrophoresis in selective gel (2% agarose and 6 V/cm). The PCR indicates the presence of *E. coli* in **HF1, UF1, UF4, UF5 and UF6** of water sample under UV light in a selective gel. M and PC indicate 100 bp marker and positive control.

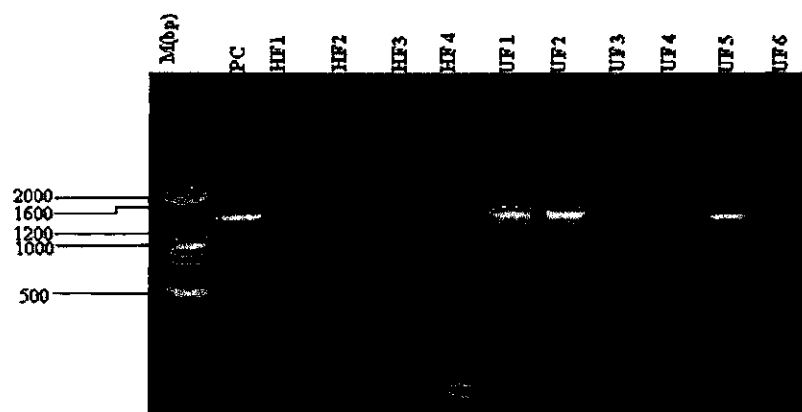


Fig 4.2: UV visualization of water samples following electrophoresis in selective gel (2% agarose and 6 V/cm). PCR indicates the presence of *E. coli* in **UF1, UF2 and UF5** of sediment sample. M and PC indicate 100 bp marker and positive control.

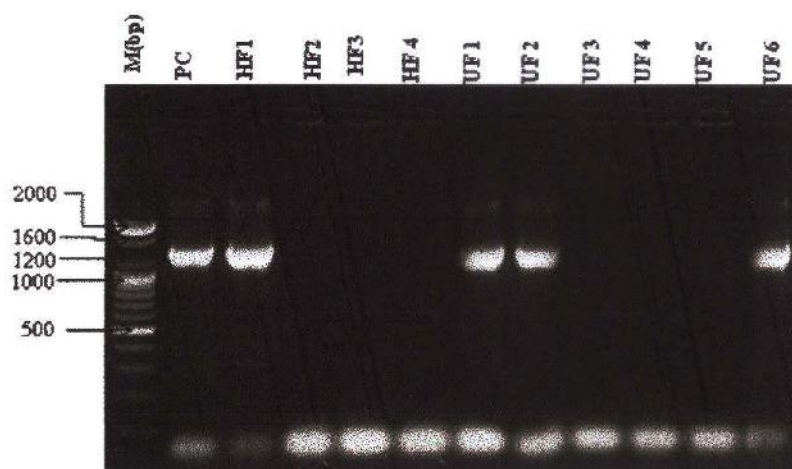


Fig 4.3: UV visualization of water samples following electrophoresis in selective gel (2% agarose and 6 V/cm). The PCR indicates the presence of *E. coli* in HF1, UF1, UF2 and UF6 of shrimp intestine sample. M and PC indicate 100 bp marker and positive control.

In the second phase, the Pho-A positive samples were checked by all enterovirulent primers. Among, the enterovirulent *E. coli* strains, enteropathogenic group (EAE) was common in most of the samples of farms in unhygienic condition including water of farms UF₁ and UF₂ sediment and shrimp of farm UF₂, all samples (water, sediment and shrimp) of farm UF₃ but in case of UF₆ HPEC group (EAE) was present in both sample of water and shrimp intestine. Whereas, heat stable enterotoxin (ST1) was found in shrimp of farm UF₂ and sediment of farm UF₅. Heat labile LT1 of ETEC group was found in sample, water and sediment of UF₅. Heat labile (LT2) of ETEC group was found only in the shrimp of UF₆. No enterovirulent groups were found pho-A positive water of farm UF₄ and sediment and shrimp of UF₁ of farm UF₅ (Figure 4.3, 4.4, 4.5 and 4.6). Farms in hygienic condition (HF) were free of *E. coli*



except HF₁ but there were no enterovirulent strains in the Pho-A positive sample, water and shrimp of HF₁.

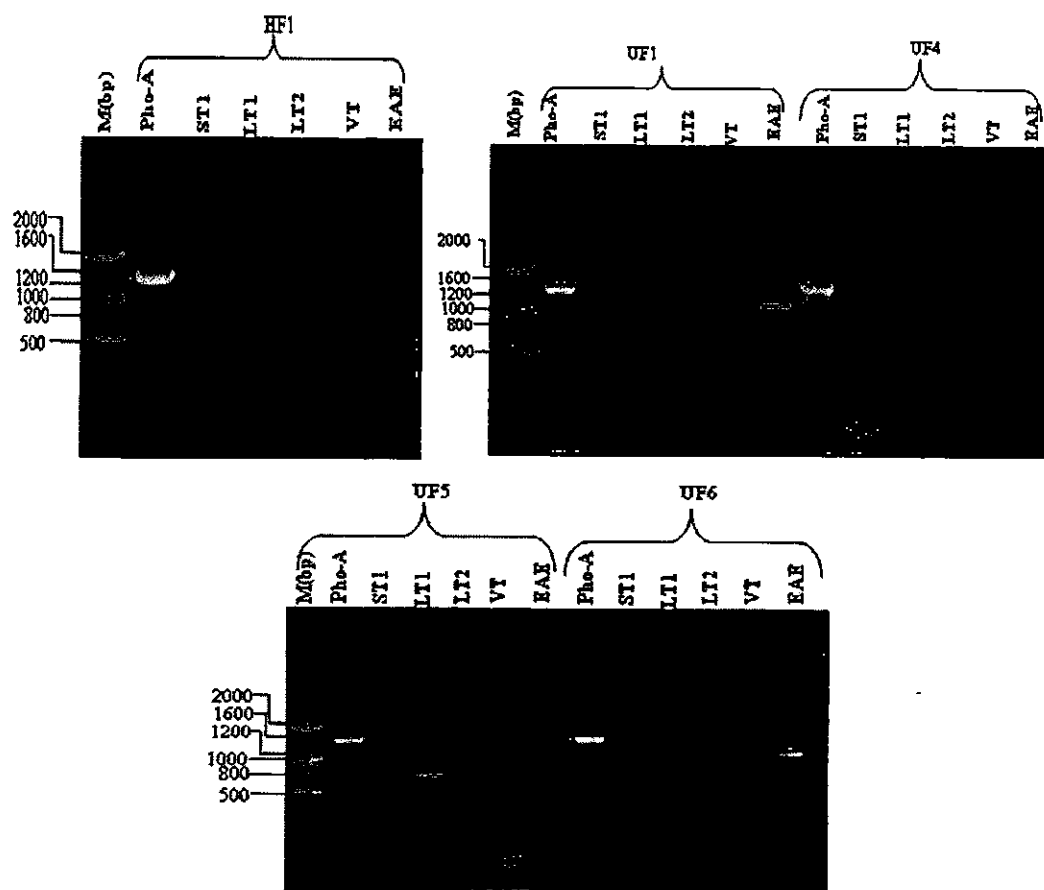


Fig 4.4: UV visualization of water samples following electrophoresis in selective gel (2% agarose and 6 V/cm). PCR indicates the absence of virulent strains in screened water samples of HF₁ and UF₄ but EPEC group is found in UF₁ and UF₆. ETEC group is found in UF₅ of water. M and PC indicate 100 bp marker and positive control.

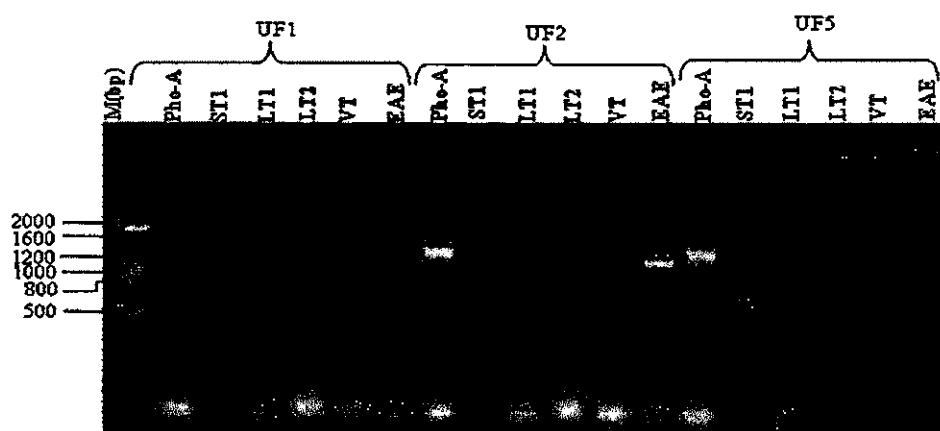


Fig 4.5 UV visualization of water samples following electrophoresis in selective gel (2% agarose and 6 V/cm). The PCR indicates the absence of virulent strains in screened sediment samples of UF1 but EPEC and ETEC group are found in UF2 and UF5 respectively. M and PC indicate 100 bp marker and positive control.

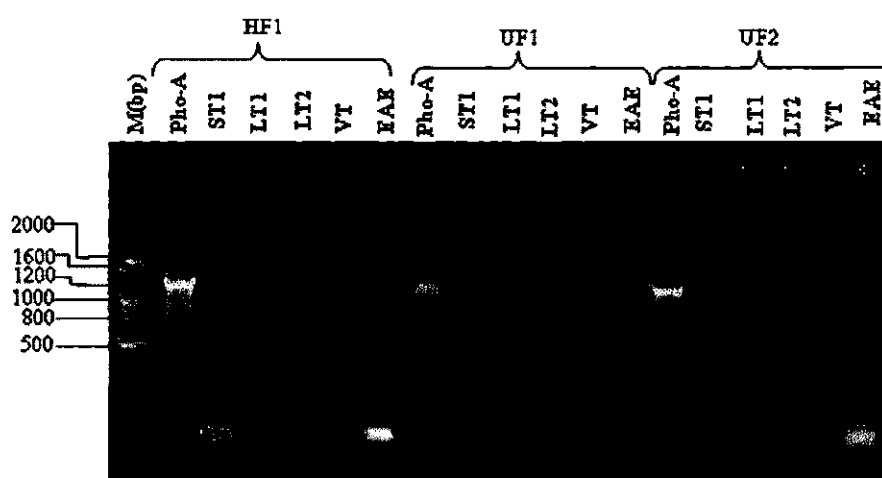


Fig 4.6 UV visualization of water samples following electrophoresis in selective gel (2% agarose and 6 V/cm). The PCR indicates the absence of virulent strains in screened shrimp samples of HF1 and UF1 but ETEC group is found in UF2. . M and PC indicate 100 bp marker and positive control.

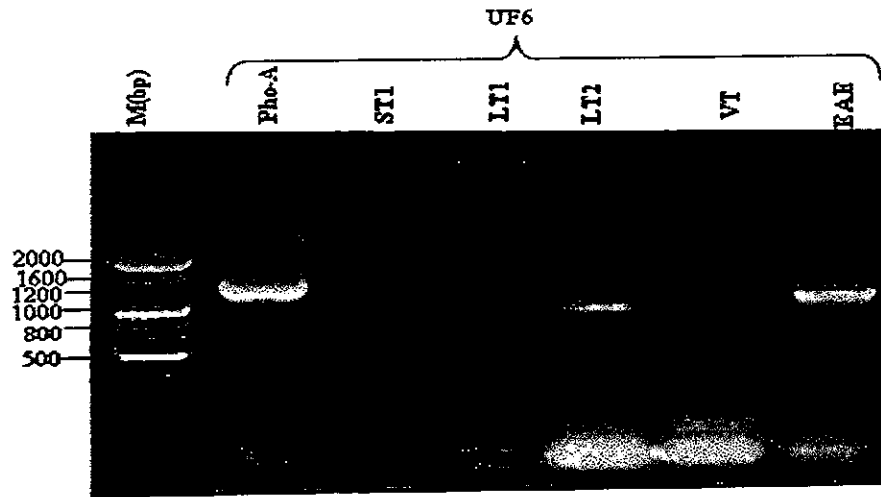


Fig 4.7 UV visualization of water samples following electrophoresis in selective gel (2% agarose and 6 V/cm). PCR indicates the presence of ETEC and EPEC group are found in sample of UF6. M and PC indicate 100 bp marker and positive control.

Restriction enzyme analysis

and verify the specificity of the DNA observe during gel electrophoresis. Restriction enzyme was performed. To confirm the identity of the amplimers, restriction enzyme was done. The *AhaI* restriction site was in 150 and produced from Pho-A gene. The fragments size following and approximately 150 bp, 565 bp, and 656 bp that (Fig 4.8). Hence this result concluded that the PCR product was Pho-A-R of Pho-A gene. The *AhaI* restriction fragments were approximately observed in the fragments were found in LTI primer that's

cut position is 258,702 and 718 bp in *AluI* restriction enzyme. The EAE amplimer from EHEC was also confirmed by *AluI* digestion which produced four fragment sizes of approx. 128, 141, 256 and 630 bp. Similarly, In the LT2- specific amplimer was cleaved into three fragment sizes of around 66, 293 and 638 bp. These fragments also matched with the cut position, 67 and 704 bp, using *AluI* restriction enzyme. So this this result concluded that the PCR product turned out with primers of LT2 gene.

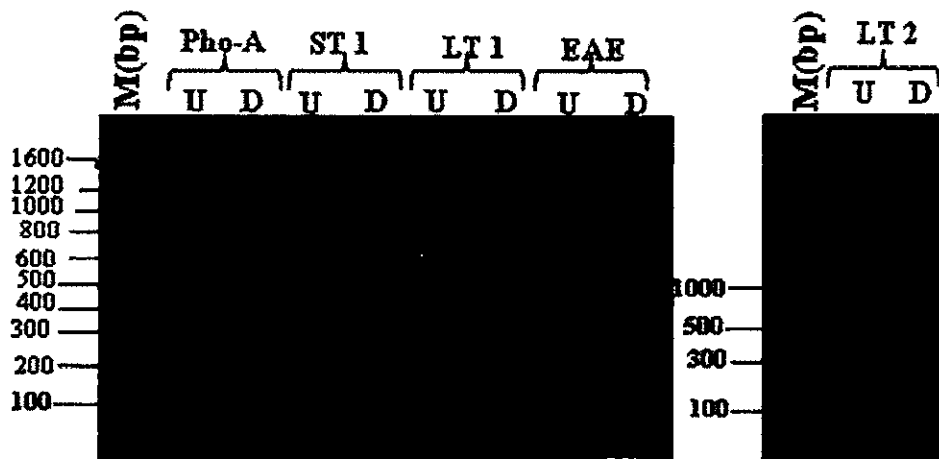


Figure 4.8: Selective gel (2% agarose, 6V/cm) snaps after estriction enzyme analysis of PCR products using *AluI* enzyme. M denoted lane represents the 100 bp marker; U and D denote lane represents the uncut DNA band and Digested DNA band of specified primer.

Summery of the result was shown in Table 4.3

Table 4.3: Summery of the results

Enterovirulent Group	Target genes	Farms in Hygienic condition												Farms in unhygienic condition																	
		HF1			HF2			HF3			HF4			UF1			UF2			UF3			UF4			UF5			UF6		
House keeping gene	Pho-A	w	s	I	w	s	I	w	s	I	w	s	I	w	s	I	w	s	I	w	s	I	w	s	I	w	s	I	w	s	I
		+		+													+	+	+	+	+		+			+	+		+	+	
ETEC	ST1																														
	LT1																														
	LT2																														
EHEC	VT																														
EPEC	EAE																														

W=Water, S=Sediment and I=Shrimp Intestine.

 = Only Pho-A present,  = Enterovirulent *E.coli* Present.

Chapter- Five

Discussion

Escherichia coli is a Gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are harmless, but some strains can cause serious food poisoning in humans (Vogt and Dippold, 2005). The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K2 (Bentley and Meganathan, 1982) and by preventing the establishment of pathogenic bacteria within the intestine. *E. coli* and related bacteria constitute about 0.1% of gut flora, and fecal-oral transmission is the major route through which pathogenic strains of the bacterium cause disease. Cells are able to survive outside the body for a limited amount of time, which makes them ideal indicator organisms to test environmental samples for fecal contamination (Feng et al., 2002).

The sensitive versatile multiplex PCR has provided the direct indication of the presence of the pathogenic *E. coli* in the various sources of the shrimp farm. We have sampled water, sediments and live shrimp since the robust strains of *E. coli* can develop, persist and disseminate by the favor of the environmental conditions, leads to the resistance to antimicrobial agents (Schroeder *et al.*, 2002a,b).

In this experiment, the PCR was performed without screening and removing detritus to avoid target loss (Tengs *et al.*, 2000) and to reduce time consumption assuming that PCR is able to amplify target DNA if it is present in the sample. Efficient methods of sample treatment are needed to exploit fully the potential of the PCR technique to detect pathogens due to the presence of PCR inhibitors (Rossen *et al.*, 2002). Either Lysozyme, proteinase K, detergents by

centrifugation and filtration or use of proteinase K and organic solvents (phenol, chloroform and iso-amyl alcohol for deproteinizing cell) has been suggested to make PCR efficiency (Kroll, 1993; Lo *et al.*, 1996). Guanidinium isothiocyanate, however, the main ingredient of DNAZOL[®], a strong denaturant of proteins is capable of dissolving cytoplasmic and nuclear membranes, thus is talent to solubilize all cellular components, allows selective precipitation of DNA in the presence of alcohol as well as improves the release of DNA into solution (Cox, 1968). Therefore, DNAZOL[®] used in this investigation provides reliability and efficiency with simplicity of the isolation protocol suitable for handling large number of samples in a course

The Primers for the target genes were designed such as way that the expected size of PCR products could be different, which ultimately facilitates the discrimination of banding of gel electrophoresis in a rapid way, as also done by Kong and his colleagues (1999).

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. On the other hand, some farmers used live feeds (mainly molluscs) or some other applied excess cowdung in their farms. So, these could be the possible source of *E. coli* in the farms (Karunasagar *et al.*, 1999). In the study, the unhygienic environment and poor management practices were found in some shrimp farms which played key roles to categorize the farms into two groups. Shrimp farms having unhygienic environment and poor management were apparently infected and farms having hygienic environment and good management were uninfected.

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 detection experiment was performed in two steps. At first, all the samples (water, sediment and shrimp) were checked through *pho-A* primer in a thermal cycler. 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*, it was available in both of studied apparently infected and uninfected farm category. In second step, only the *E. coli* positive samples were further tested by six pair primers to identify the presence of any enterovirulent groups in different samples.

Electrophoresis analysis of the PCR products using six pairs of primers revealed the presence of 1371 bp (Pho-A), 638 bp (ST1), 725bp (LT1), 997 bp (LT2) and 1155 bp (EAE) DNA bands in the water, sediment and shrimp samples of five different apparently infected shrimp farms (UF₁ UF₂, UF₄, UF₅ and UF₆). These band patterns clearly indicate the presence of four different enterovirulent strains of *E. coli* in the shrimp farms.

Among the enterovirulent groups, EPEC was common in UF₁, UF₂ and UF₆ farms whereas, no enterovirulent groups were evident in the samples of UF₄. Briefly, EPEC was found in

water of UF₁ and UF₆ as well as sediment and shrimp of UF₂ and UF₆ respectively. The presence of this enteropathogenic *E. coli* strains might be due to the poor sanitation system or contaminated water source from nearby farms or narrow canals which were observed during sampling. The pathogen is a causative agent of diarrhea in humans, rabbits, dogs, cats and horses (Toder, 2007). Like ETEC, EPEC also causes diarrhea, but the molecular mechanisms of colonization and etiology are different. EPEC lack fimbriae, ST and LT toxins, but they utilize an adhesin known as intimin to bind host intestinal cells. This virotype has an array of virulence factors that are similar to those found in *Shigella*, and may possess a shiga toxin. Adherence to the intestinal mucosa causes a rearrangement of actin in the host cell, causing significant deformation. EPEC cells are moderately invasive (i.e. they enter host cells) and elicit an inflammatory response. Changes in intestinal cell ultrastructure due to "attachment and effacement" are likely the prime cause of diarrhea in those afflicted with EPEC (Toder, 2007).

The evidence of ETEC was found in the shrimp samples of UF₂ and UF₆ and also found in the samples of water and sediment of UF₅. Poor management system, unhygienic environment was observed during sampling that might be the major reason for the presence of these virulent strains. In addition, a cow-shed and grazing ground was very close to the farm UF₅, and the cowdung was used as fertilizer; therefore, there might be a chance of enterovirulent *E. coli* contamination. 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. ETEC strains produce a cholera-like toxin called heat labile toxin (LT) and a second diarrhoeal toxin heat stable toxin (ST) (Karunasagar *et al.*, 1999).



In the present experiment, there was 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. In this sense, the sampling area might be safer from the enterohemorrhagic *E. coli* strains; since *E. coli* frequently responses with the changing environment.

In the study of farms in hygienic condition category, only 1371 bp DNA band pattern (pho-A) was observed on electrophoresis gel in water and shrimp of HF₁.and shrimp samples of HF₁ after first round (Pho-A) PCR test and no enterovirulent groups was evident after second round (all primers) PCR test. HF₁ was a farm of hatchery this house keeping gene may come from their parents. In some farms (HF₂, HF₃ and HF₄), deep tubewell water was used for culture to avoid contamination of various pathogens from nearest river water.

It is out of the ordinary to note that different *E. coli* strains are present in water and shrimp intestine but not in the sediment, also in water but not in shrimp and sediment, these may be because of the source of water and fry, hanging toilet, live feed, poor management system etc. There was a huge load of different *E. coli* strains in the positive sample that was the water samples of Moure river. This is because this lake acts as the main dumping site of the Khulna city. Results of the present study support the study of Kong *et al.* (1999) who stated the effectiveness of very sensitive multiplex PCR system for the rapid detection and typing of ETEC and EHEC strains of *E. coli* in the aquatic environment. Lang *et al.* (1994), Paton and Paton (1998) also reported various multiplex PCR protocols to simultaneously detect segments of different toxin genes of ETEC and EHEC strains. Pickett *et al.* (1989) and Wakabayashi and Wakabayashi (1999) observed the polymerase chain reaction (PCR) and gene probe technology as highly sensitive methods for the specific detection of pathogenic

ETEC. The results of the present study clearly indicate the potentiality of the PCR assay to identify and differentiate virulent *E. coli* strains in shrimp farms.

Chapter-Six

Conclusion

The present study was successful in detecting some enterovirulent strains of *E. coli* from the shrimp farms of Patuakhali, Bangladesh. The findings of the research clearly indicate the absence of enterovirulent *E. coli* strains in the farms having good water and feeding management with proper hygiene maintenance around the shrimp farm. Shrimp culture has been practicing in the coastal districts of Bangladesh in an uncontrolled way and this area is also increasing; thereby having comparatively lower production from this sector. Thus, the overall management system of shrimp culture must be developed immediately in Bangladesh in order to avoid the contamination of various pathogenic microbes in aquaculture farms as well as to increase the production performance.

Chapter Seven

References

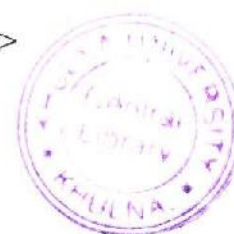
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Chapter Eight

Appendix

Appendix-A

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.

Appendix-B

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 (Spectrophotometer, 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 μ l of 8 mM NaOH was taken in blank cuvette;
4. DNA sample (4 μ l solubilized DNA + 1996 μ l of 8 mM NaOH) was taken in another cuvette.
5. Resulting data against each absorbance was found on the program's page.
6. DNA sample containing cuvette 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), μ l/ml = Absorbance at A_{260} *50*500;
 - b) Purity = Absorbance at A_{260} /Absorbance at A_{280}



Table- 8.1: Spectrophotometric data for the evaluation of DNA purity.

Appendix-C

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 μ l/5 mm lane width
- Typical Loading Number of Lanes: 125 - 166 (5 mm lane width)
- Range (bp): 100 - 2,000
- Number of Bands: 13

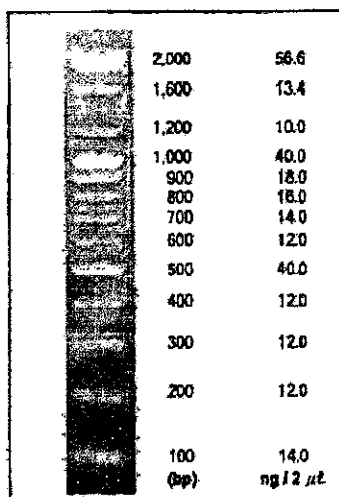


Fig-8: 1% agaros gel strained with Ethidium Bromide

Appendix-E

Questioner Form for Shrimp farm survey

- | | | | | |
|---------------------------|----------------|------------|----------|------------------|
| 1. Location : | Farm size: | | | |
| 2. Management By: | Govt. | Private | Lease | NGO Hiring |
| 3. Culture Type : | Mono cult, | Poly cult, | Trad'nl, | Improve Trad'nl, |
| | Semi intensive | | | |
| 4. Water source : | | | | |
| 5. Hygienic condition : | 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: | | | | |



Figure: Open toilet nearby shrimp farm in Chakamuia



Figure : Water and Sediment Sample collection



Figure: DNA extraction



Figure: DNA Centrifugation



Figure: Spectrophotometric Analysis



Figure: Gel Electrophoresis .



Figure: Installation of samples in PCR



Figure: Observing bands under UV transilluminator