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POLYMERASE CHAIN REACTION TECHNIQUE FOR RAPID CHECK OF VIRULENCY OF ESCHERICHIA COLI FROM SHRIMP FARMS

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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). However, PCR efficiency depends on the primer design, target samples and environmental factors. The aim of the present study was therefore to detect enterovirulent strains of E. coli in the four different shrimp farms of Dumuria, Khulna, Bangladesh. Water, sediment and shrimp were examined from each farm. Six pairs of oligonucleotide specific primers were used for the amplification and thereby detection of target genes viz. the alkaline phophatase (phoA), the heat-labile toxin1 (hlt1), heat-labile toxin2 (hlt2), heat-stable toxin1(hst1), 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 using DNA markers. The results revealed the presence of phoA gene, hlt1 and hlt2 genes of ETEC in the shrimp and water samples of two different farms. At the same time, vt gene of EHEC was found especially in the shrimp body. One farm was completely free of EEC strains, which might be due to sanitation. Moreover, enterovirulent E. coli strains were absent in the sediment of all of the experimental farms. These findings of the study suggest that the PCR method is a highly sophisticated and useful tool to analyze the dynamics of fecal bacteria in shrimp farms.

Key words: *Escherichia coli*, Enterovirulent, Shrimp, Primer, PCR.

1. INTRODUCTION

Shrimp farming is very popular and economically feasible in many tropical and subtropical countries; however susceptible of pathogenic diseases [1,2]. *Esherichia coli* is one of the most common pathogen in aquaculture premises. *E. coli* is widely distributed in the aquatic environment and has been universally accepted as an indicator of faecal pollution [3]. Most of the *E. coli* strains are harmless, but only a small number is pathogenic to other organisms. Several classes of these enterovirulent *E. coli* (EEC) cause diseases in human as well, which are somehow waterborne. They are: enterohemorrhagic *E. coli* (EHEC) producing Shigatoxin (causes bloody diarrhea), enterotoxigenic *E. coli* (ETEC) producing heat-stable or heat-labile enterotoxins, enteroinvasive *E. coli* (EIEC) producing cytotoxin and enterotoxin (causes bacillary dysentery), and enteropathogenic *E. coli* (EPEC) having the mechanism of virulence unrelated to the excretion of typical *E .coli* enterotoxins [4].

The detection of the virulency of any pathogen is a prerequisite to remediate them. Considering this context, several methods have already been developed to detect *E. coli* 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 [5]. With the new era of molecular tools, PCR and gene probe technology have provided rapid and highly sensitive methods for the specific detection of pathogenic ETEC [6] and EHEC strains [7]. Various multiplex PCR protocols to simultaneously detect segments of different toxin genes of EEC strains have also been developed viz., multiplex detection of *lt*1/*vt*1/*vt*2 genes of ETEC and EHEC strains [8], *lt*1/*lt*2 (ETEC) [9], *lt*1/*st*1 (ETEC) [10], *elt*/*est* (ETEC), *eae* (EPEC), *ipaH* (EIEC) [11] and *lt*/*sth*/*stp* (ETEC), *stx*1/*stx*2 (EHEC), *eae*/*bfp* (EPEC) [12]. In 2013, Roy et al. [2] studied several shrimp farms to test the virulency of *E. coli* present in water, sediments and shrimp flesh

from Bangladesh. They, claiming this as the first attempt using molecular tool for the rapid detection of pathogenic bacteria from shrimp farm in this promising shrimp producing country, reported that ETEC and EPEC group of *E. coli* was present in both biotic and abiotic sample of shrimp farm. Indeed, the prevailing poor sanitation and drainage facilities near the shrimp farms might provide higher susceptibility to be contaminated with pathogenic *E. coli*. If water is contaminated with any pathogenic or virulent *E. coli* strains, those strains will ultimately enter into the shrimp body. This might create an adverse impact on shrimp health and even it may cause death of shrimp.

After Roy and his colleagues' [2] research to identify these pathogenic bacteria, our view, as an extensive attempt, is to select geographically different shrimp farms to fine-tune the exact identification of any pathogenic or virulent agent, which is the prerequisite for taking preventive measures to solve the prevailing problems in Bangladesh. In this context, PCR technique was modified by redesigning the used primer by the former authors [2] to detect the different enterovirulent strains of *E. coli* from shrimp farms.

2. MATERIALS AND METHODS

2.1. Study Area and Sampling

How the farm and premises conditions influences on the *E. coli* contamination was one of the objectives of this study. During collection of samples, therefore, we set several criteria to mark the farm as traditional sanitation and improved sanitation (Table 1). Shrimp, water and sediment samples were collected from four different locations (Dumuriasadar = F_1, Gutudia = F_2, Kharnia = F_3 and Sobhana = F_4) in Khulna district, Bangladesh.

Preparation of samples in sterile condition and separate instrument and time ensured its status of not being secondary and cross contamination, followed by storing at 4 ºC. The Experiment was conducted at Genetics and Molecular Laboratory of Fisheries and Marine Resource Technology Discipline of Khulna University.

Table 1 Criteria for traditional and improved sanitation of the shrimp farm [2]

Traditional sanitation	Improved sanitation
Hanging toilet is very close to farm or at the canal or rivers which is the main water source of the farm.	No hanging toilet, only concrete and closed toilet if necessary.
Connection of sewerage line with the canal, which is the water source of the farm.	Water source is ground water or rain water.
Cow-shed and grazing land very close to farm.	No cowshed or grazing land nearby farm.
Excessive and continuous use of cow dung as fertilizer or for plankton growth.	No direct and excess use of cow dung or no use at all.
Live feed, especially mollusk, which is widely used in these areas.	No live feed used, only formulated feed used.
Dirty premises.	Clean and healthy environment.

2.2. Experimental Design

Water, sediment and shrimp samples were collected at first from four shrimp farms designed as F_1, F_2, F_3 and F_4. One positive (P) control (water of Khulna University Lake, which is proved to have *E. coli* [2]) and one negative (N) control (absent DNA template into PCR tube) were taken for the experiment. Six pairs of designed oligonucleotide primers (APHO-F, APHO-R; HST1-F, HST1-R; HLT1-F, HLT1-R; HLT2-F, HLT2-R; VT-F, VT-R and EAE-F, EAE-R) were used for the amplification and then detection of six different target genes. 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. The fourth step involved the amplification of target gene by PCR technology. In the final step, Gel electrophoresis was performed to detect enterovirulent *E. coli* strains by observing clear bands.

2.3. Bacteria Culture and DNA Extraction

Two grams Luria-Bertani (LB) broth was added with 100 ml of distilled water followed by the autoclaving at 121 ºC for 20 minutes. Autoclaved 5 ml LB broth and 100 µl samples, which may contain environmental bacteria, were then mixed followed by placing on shaking incubator (VS-8480SL, Korea) at 37 ºC with 200 rpm for overnight to enrich *E. coli* culture.

DNA extraction was carried out from amplified bacterial cells grown in culture media using DNAZOL® Reagent (Invitrogen Life technologies, USA), ethanol and sodium hydroxide (Difco Laboratories, MI, USA).

2.4. DNA Integrity, Concentration and Purity

To check the integrity of extracted DNA, agarose gel electrophoresis technique was applied (2% agarose gel containing 3 µl ethidium bromide, 6 V/cm, 1X TAE buffer) [2]. DNA bands were visualized and photographed by high performance UV transilluminators (Ultra-Violet Products Ltd., UK). 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) againstNaOH blank. The protocol used in this experiment was designed for a double-beam spectrophotometer. The DNA concentration ($=A_{260}\times50\times500$) purity ($=A_{260/280}$) was measured for further assessment [2].

Table 2: List of different primers and expected size of target genes of pathogenic *E. coli*.

Target Gene	Primers	Sequence (5'→3')	Product Size (bp)	Accession No.
Alkaline phosphatase	APHO-F	AAGCCCGGACACCATAAATGCCTGTTCTGG	903	M13345
	APHO-R	GGTTGGTACACTGTCATTACGTTGCGGATT		
Heat-stable toxin1	HST1-F	TCTGTCTTTCCCCTCTTTTA	175	M25607
	HST1-R	TTAATAACATGGAGCACAGG		
Heat-liable toxin1	HLT1-F	CGGCGTTACTATCCTCTCTATGT	275	J01646
	HLT1-R	TTTGGTCTCGGTCAGATATGTGA		
Heat-liable toxin2	HLT2-F	ATATCATTTTCTGTTTCAGCAAA	720	M17894
	HLT2-R	CAATAAAATCATCTTCGCTCATG		
Verotoxin	VT-F	TAACGAAATAATTTATATGTGG	520	Z36901
	VT-R	TGATGATGGCAATTCAGTAAA		
Attachment & Effacement	EAE-F	CGGCAGAGGTTAATCTGCAGAGTG	360	M58154
	EAE-R	GCCATTTGCTGGGCGCTCATCA		

2.5. Target Genes

The target genes chosen of this experiment were: Alkaline phosphate (*phoA*), housekeeping gene (present in all *E. coli*); the *hlt*1, *hlt*2 and *hst*1 genes of ETEC; the *vt* of EHEC and *eae* virulence genes of EPEC. Six pairs of specific primers were chosen from gene sequence (Table 2) to amplify the target DNA fragments of genes. To compare the size of double stranded DNA from 100 to 2,000 base pairs, 100 bp designed DNA markers were used. The DNA marker consists of 13 double stranded DNA fragments ranging in sizes from 100 to 1,000 (Bioneer, Korea).

2.6. Target Gene Amplification in Thermal Cycler

At first, the concentrations of all the extracted DNA samples were adjusted. The reactions were performed in a 20 µl reaction mixture containing 1 µl DNA sample (having 20-25 ngof template DNA), 2 µl (10 pmole/µl) oligonucleotide primers (Bioneer, Korea), 2 µl 10X reaction buffer, 2 µl 10 mM dNTPs mixture, 2 µl Taq DNA polymerase (1 unit) and 11 µl de-ionized distilled water. The reaction mixtures were then placed in a DNA thermal cycler (C1000^{TM}, BIO-RAD, USA) for PCR. 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. A positive control, constituting highly *E. coli* contamination and a negative control, having no DNA template in the reaction mixture were kept. After the completion of thermal cycling, 8 µl of each PCR products was analyzed electrophoretically by running through a 2% agarose gel and the amplified product size was determined by comparing with a 100 bp DNA size marker.

3. RESULTS AND DISCUSION

Extracted and PCR amplified DNA samples of bacterial cells showed the clear band patterns during gel

electrophoresis. The average A_{260}/A_{280} ratio of the extracted DNA materials was 0.56±0.22 which is 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 µg/ml with the highest yield being from shrimp intestine samples of F_2 farm and the lowest from water samples of F_1 farm. Agarose gel electrophoresis of the PCR samples revealed the presence of some *E. coli* strains in each farm (Figures 1-–4).

In the samples of F_1 farm, 903 bp, 275 bp and 520 bp bands were found that clearly indicates the presence of housekeeping *phoA* gene, *hlt*1 gene of ETEC group and *vt* gene of EHEC group. *phoA* and *hlt*1 genes were found in both the water and shrimp intestine but the *vt* gene was detected only in the shrimp intestine. All of the tested enterovirulent strains were absent in sediment samples of F_1 farm.

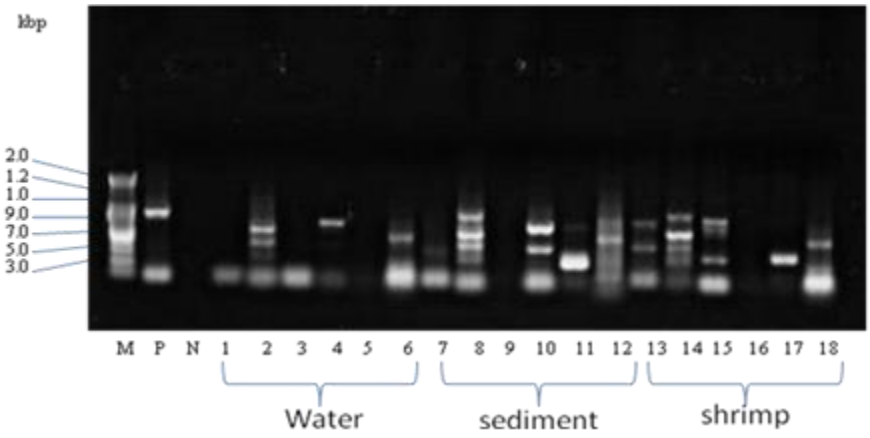

Figure 1: UV visualization of PCR products of F_1 farm. Lanes 1, 7, 13 are for *phoA*; 2, 8, 14 for *hst*1; 3, 9, 15 for *hlt*1; 4, 10, 16 for *hlt*2; 5, 11, 17 for *vt*; 6, 12, 18 for *eae* genes. Lane M, P and N are for 100 bp DNA marker, positive control and negative control, respectively.

In both the water and shrimp intestine samples of F_2 farm, 903 bp bands (*phoA* gene) was detected and 520 bp bands (*vt* gene) was found only in the shrimp intestine samples (Figure 2). All of the tested EEC groups were also absent in the sediment samples of F_2 farm. In the samples of F_3 farm, 903 bp (housekeeping *phoA* gene) and 720 bp bands (*hlt*2gene of ETEC group) were detected. These genes were detected only in the water samples (Figure 3) and the tested enterovirulent *E. coli* strains were absent in shrimp and sediment samples of F_3 farm. In all of the water, shrimp and sediment samples of F_4 farm, there was no evidence for the presence of any *E. coli* strains while agarose gel electrophoresis was performed (Figure 4).

Electrophoresis analysis of the PCR products using six pairs of primers revealed the presence of 903 bp, 275 bp, 520 bp and 720 bp DNA bands in the water and shrimp samples of four different shrimp farms (F_1 F_2, F_3 and F_4).

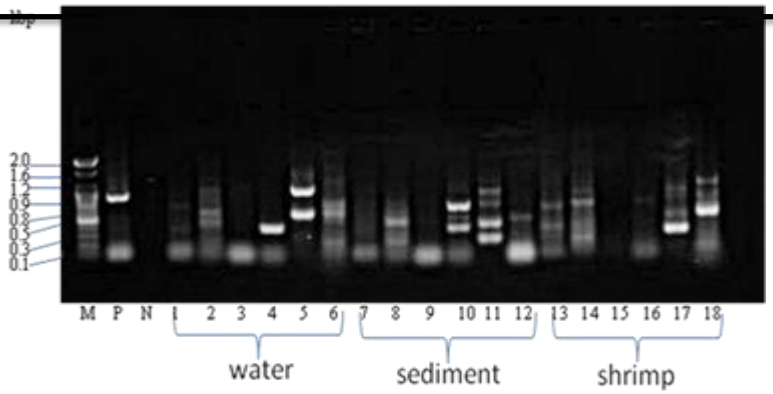

Figure 2: UV visualization of PCR products of F_2. Lanes 1, 7, 13 are for *phoA*; 2, 8, 14 for *hst*1; 3, 9, 15 for *hlt*1; 4, 10, 16 for *hlt2*; 5, 11, 17 for *vt* and 6, 12, 18 for *eae* genes.

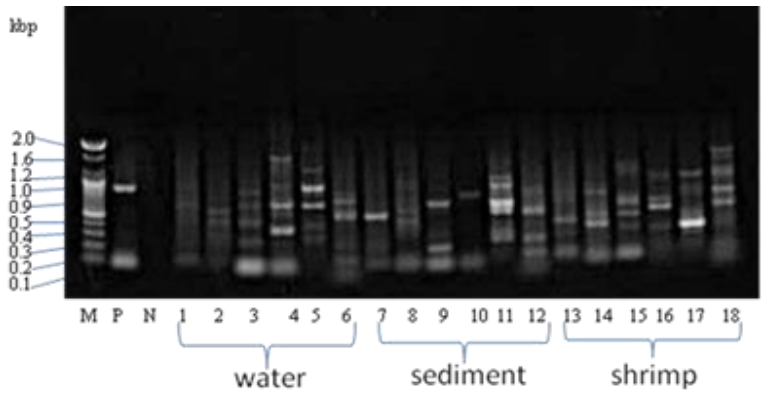

Figure 3: UV visualization of PCR products of F_3.Lanes 1, 7, 13 are for *phoA*; 2, 8, 14 for *hst*1; 3, 9, 15 for *hlt*1; 4, 10, 16 for *hlt*2; 5, 11, 17 for *vt* and 6, 12, 18 for *eae* genes.

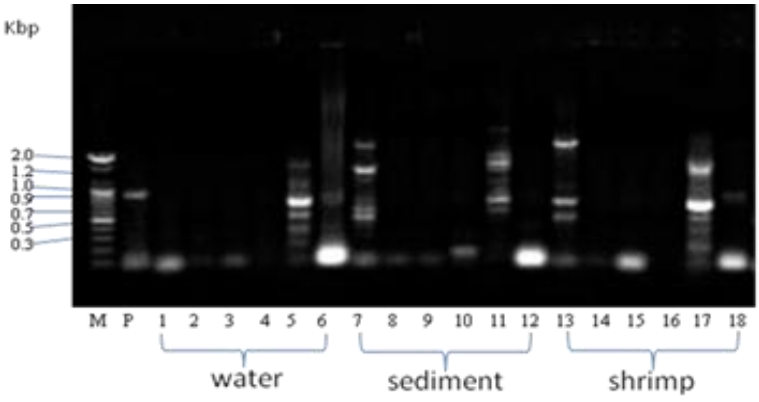

Figure 4: UV visualization of PCR products of F_4. Lanes 1, 7, 13 are for *phoA*; 2, 8, 14 for *hst*1; 3, 9, 15 for *hlt*1; 4, 10, 16 for *hlt*2; 5, 11, 17 for *vt* and 6, 12, 18 for *eae* genes.

These band patterns clearly indicate the presence of four different strains of *E. coli* in the shrimp farms. The detected four different genes were housekeeping *phoA* gene, *hlt*1 gene and *hlt*2 gene of ETEC group and *vt* gene of EHEC group. All the tested enterovirulent strains were absent in sediment samples of all four farms as well as in the water, sediment and shrimp samples of F_4 farm. *E. coli* strains of ETEC group were detected in both water and shrimp intestine samples but the EHEC strain was present only in the samples of shrimp intestine. This may be because of transmission from live feed sources (zooplankton, mollusk etc.) and some may contain this bacterial load in intestine from post larval stage as most of the post larvae are collected from nature for culture. It is confirmed that the each *E. coli* strain contains at least one virulence gene [10, 13, 14]. As enterovirulent *E. coli* strain was present only in water, not in shrimp and sediment; this simply indicates the presence of lower amount of *E. coli* in the water of F_3 farm. Absence of different enterovirulent strains in F_4 farm axiomatically indicates very good water management practice and hygienic condition maintenance in and around the farm which was noticed during sample collection. Similar hygienic influences were also reported by Roy *et al*. [2].

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 (as in case of F_3 farm), 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 Khulna University Lake. This is because this lake acts as the main dumping site of the University. Results of the present study support the study of Kong *et al*. [4] 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*. [8], Paton and Paton [13] also reported various multiplex PCR protocols to simultaneously detect segments of different toxin genes of ETEC and EHEC strains. Pickett *et al*. [15] and Wakabayashi and Wakabayashi [16] 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. While the previous concurrent study [2] revealed the presence of ETEC and EPEC group, we, taking geographically different locations, found EHEC instead of EPEC. Even with the redesign of primer sequences (Table 2) from Roy *et al*. [2], we have found these enterovirulent *E. coli*. by applying PCR for rapid identification of target genes. So, the present study suggests for the better water management practice in all aspects of shrimp culture

4. CONCLUSION

The present study was successful in detecting some enterovirulent strains of *E. coli* from the shrimp farms of Khulna, 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 practiced 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. This fine-tune conclusion after Roy *et al.* [2] recommended the use of PCR to detect pathogenic strains in every stage of aquaculture from brood management to harvested crops.

5. ACKNOWLEDGMENTS

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