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Rapid Identification of Enterovirulent *Escherichia coli* Strains using Polymerase Chain Reaction from Shrimp Farms

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Abstract: Although, *Escherichia coli* is widely distributed in the environment, only a small percentage is pathogenic to humans. The most commonly encountered are those belonging to the Enterotoxigenic (ETEC), Enteroinvasive (EIEC), Enterohaemorrhagic (EHEC) and Enteropathogenic (EPEC) subtypes. Aquaculture premises specially shrimp farm in tropical and subtropical countries largely susceptive to different types of *E. coli* strains. With the PCR system, an attempt was taken to identify the virulent *E. coli* in a rapid basis from water, sediment and live shrimp from different shrimp farms established in the shrimp production areas of southwest part of Bangladesh. The target genes chosen for this investigation included the PhoA, a housekeeping gene in all *E. coli* and thereafter the virulent genes LT1, LT1 and ST1 of ETEC, the VT of EHEC and EAE of EPEC, which were amplified with the primers designed for their specific genes. The restriction enzyme conformation and the gel electrophoresis bands showed the presence of *E. coli*, among which ETEC and EPEC groups were present in the environmental and biological samples of shrimp farms, brings up into the human health concern. The sanitation conditions amid farm were also investigated to find the link of pathogenic *E. coli*, which came into the result of less infection if the farm maintains improved sanitation. This study has clearly urged the exigency of periodical quick check of virulent *E. coli* with the versatile PCR system from brood management to post-harvest handling of shrimp.

Key words: Enterovirulent *E. coli*, shrimp infection, PCR

INTRODUCTION

Shrimp farming concentrated largely in tropical and subtropical countries for exporting, has experienced spectacular growth over recent decades. Currently, this sector in Bangladesh along with other countries is facing serious problems with microbial diseases mainly caused by opportunistic pathogens (Lundin, 1996). Total coliforms and fecal coliform have been widely accepted as indicators to the lack of sanitation over the years. The abundance of *E. coli*, however, has been reported recently to be more dangerous than that of coliforms (Fewtrell and Bartram, 2001). *E. coli*, the widely distributed pathogen in aquatic environment has been universally accepted as an indicator of fecal pollution because of its presence in high numbers in mammalian gut. Most of the *E. coli* strains are harmless, only a small percentage is pathogenic to humans though. To date, several types of enterovirulent *E. coli* have been recognized as the etiologic agents of various gastrointestinal infections in humans. There are several classes of enterovirulent *E. coli* (EEC) group responsible for waterborne diseases in human. They are: Enterohemorrhagic *E. coli* (EHEC) producing Shigatoxin, which makes bloody diarrhea, enterotoxigenic *E. coli* (ETEC) producing heat-stable or heat-labile enterotoxins, enteroinvasive *E. coli* (EIEC) causing bacillary dysentery via invasion of intestinal epithelial cells or by cytotoxin and enterotoxin and enteropathogenic *E. coli* (EPEC) having the mechanism of virulence 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

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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 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).

During the baseline survey, traditional (poorly managed and unhygienic periphery are observed) and improved traditional culture system were observed in most of the farms in this region, which might be susceptible to *E. coli* contamination. The enterovirulent strains of this pathogen may create enormous loss of the industry due to the unacceptability by international consumers. It is, therefore, highly prerequisite to detect whether any of enterovirulent strains of *E. coli* is present in the water, sediment and intestine of shrimp sample in the region in a reliable and rapid way.

The objective of this study was to address this safety issue through identifying the enterovirulent *E. coli* strains (ETEC, EHEC and EPEC) by a rapid detection method using PCR in the shrimp farms of southwest region of Bangladesh.

MATERIALS AND METHODS

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 (TS) and Improved Sanitation (IS) (Table 1). We sampled Water (W), Sediment (S) and Shrimp Intestine (Sh) from total 48 farms from different clusters viz., Bagerhat (B), Fakirhat (F) and Rampal (R). The farm ID was therefore set like '1(BIS)' meaning 'farm No. 1 of Bagerhat having Improved Sanitation'.

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°C. The Experiment was conducted at Genetics and Molecular Laboratory of Fisheries and Marine Resource Technology Discipline of Khulna University.

Bacterial strains and oligonucleotide primers: Six strain specific genes, alkaline phosphatase (PhoA), a housekeeping gene (present in all *E. coli*); the heat stable and head labile (LT1, LT2 and ST1) genes of ETEC; Verotoxin (VT) genes of EHEC and attachment and

Table 1: Criteria for traditional and improved sanitation of the shrimp farm

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, specially mollusk, which is widely used in these area.	No live feed used, only formulated feed used
Dirty premises	Clean and healthy environment

Table 2: Primer sequences and their position in the genes of pathogenic strains of *E. coli*

Target Gene	Primers	Sequence (5'-3')	Expected product size (bp)	Restriction digestion[a]	Accession No.
PhoA	PhoA-F	GTGACAAAAGCCCGGACACCATAAATGCCT	1371	150/506/715[b]	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	66/227/704[b]	M17894
	LT2-R	CAATAAAATCATCTTCGCTCATG			
VT	VT-F	GAACGAAATAATTTATATGTG	1240		M36727
	VT-R	CCTGATGATGGCAATTCAGTA			
EAE	EAE-F	GGAACGGCAGAGGTTAATCTGCAG	360	171/189[c]	M58154
	EAE-R	CGAAGCCATTTGCTGGGCGCTC			

[a]Restriction digestion was only done on the PCR products of the genes present in gel band, [b]Digested with *Alu*I, [c]Digested with *Mbo*II

Effacement (EAE) genes of EPEC were chosen to detect pathogenic strains in this study. Therefore, six pairs of specific primer (forward and reverse of each) were designed from aforementioned genes. The primer designation was done completely based on the verified sequences in NCBI (Accession numbers are in Table 2). With all the samples (water, sediment and intestine of shrimp), three pairs of PCR units (1 pair primer (only PhoA)×3 samples) were taken into investigation for each shrimp pond. The number of PCR operation for other primer sets, afterwards, depended on the presence of PhoA signal, while if positive, we proceeded on that sample.

Culture of bacteria and DNA Extraction: To enrich the quantity of bacterial cell, collected samples were incubated in 10 mL LB Broth overnight at 37°C in shaking incubator. DNA extraction was carried out from amplified bacterial cells using DNAZOL® Reagent (Invitrogen Life technologies, USA), ethanol and sodium hydroxide (Difco Laboratories, MI, USA).

Quantitative and qualitative determination of extracted DNA: Integrity of extracted DNA sample was observed by agarose gel electrophoresis (2% agarose gel containing 3 μL ethidium bromide, 6 V cm^{-1}, 1×TAE buffer). 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) against NaOH blank. The protocol used in this experiment was designed for a double-beam spectrophotometer. The DNA concentration (=A_{260}×50×500 as we use 2000 μL of 8 mM NaOH as blank cuvette whereas a mixture of 4 μL solubilized DNA and 1996 μL of 8 mM NaOH as sample cuvette) and purity (=$A_{260/280}$) was measured for further assessment.

PCR amplification and analysis of PCR products: Three microlitter of extracted DNA (ca. 150 ng) was used as a template in each PCR tube, to make 20 μL final volume, with 1 μL of primers (10 pmol), 2 μL of reaction mixture having 10× reaction buffer (20 mM Tris-HCl, 0.5 mM EDTA, 1 mM DDT, 100 mM KCl, pH 9.0, 15 mM $MgCl_2$), 2 μL dNTPs of 10 mM, 1 μL of 1 unit Top DNA Polymerase enzyme (Bioneer corporation, Baejeo, Korea) and rest amount of deionized water. Along with the sample DNA from shrimp ponds, we used a positive control (DNA extracted from highly polluted Mouri River, Khulna, Bangladesh) for PCR amplification.

PCR amplification was performed in C1000 Thermal Cycler (Bio-Rad Laboratories Inc, USA) (2 min at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 58°C (ramp 1°C sec^{-1}), 1 min at 72°C and final elongation time 10 min at 72°C).

Analysis of PCR products: After completion of the thermal cycling, a sample of 8 μL from each PCR products was analyzed by gel electrophoresis (2% agarose gel with 3 μL ethidium bromide, 6 V cm^{-1}, 1×TAE buffer). A specific band for PhoA (1371 bp position) was screened out on gel indicating the presence of *E. coli* in the sample. Then, the *E. coli* positive samples were taken to identify the specific banding pattern of enterovirulent strains.

Restriction enzyme analysis of PCR products: To expedite rapid conformation of the identity of the PCR-amplified products, restriction enzyme analysis was accomplished. Nucleotide sequences of the target gene fragments (obtained from the GenBank/NCBI databases) were searched for restriction enzyme recognition sites by online RestrictionMapper (http://www.restrictionmapper.org). The *Hinc*II, *Alu*I and *Mbo*II (Bioneer corporation, Deajeon, Korea) enzymes were found to be most suitable since their recognition sequences are present within six of the PCR-amplified products, yielding *Alu*I and *Mbo*II digestion products of different sizes as indicated in

Table 2. PCR products were purified from agarose gel (2%) following electrophoresis using the AccuPrep® PCR Purification Kit (Bioneer, Deajeon, Korea) before restriction analysis.

RESULTS

DNA extraction, quantification and PCR amplification: The overnight bacteria culture ensured a good amount of DNA (Fig. 1), which represents bacterial genomic DNA from each sample collected from shrimp farms. After confirming the DNA quality with 0.6-1.0 of $A_{260/280}$ in spectrophotometer, approximately 50 ng mL^{-1} sDNA was used for PCR amplification with strain specific primers.

PCR analysis of shrimp farm samples: In the study area, 60.42% shrimp farm was somehow contaminated (in water, sediments or shrimp or both in some cases) with *E. coli* regardless whether it was virulent or not. Among them, 56.25% (9 out of 16) in Bagerhat while 53.33% (8 out of 15) in Fakirhat and 70.59% (12 out of 17) in Rampal (Table 3) contamination was confirmed by pure culture of bacteria, PCR and gel electrophoresis. In the gel photographs (Fig. 2), the indication of the efficiency of PhoA primers was confirmed by the positive signal of Positive control (PC). Even the less intensity of correct amplicon (1371 for the PhoA) (e.g., shrimp sample of I(BIS) in Fig. 2b and 19(FTS) in Fig. 2f and water of 39(RTS) in Fig. 2r) was considered for the test of virulent strains thereafter along with available good and correct band signals (e.g., sediment sample of 18(FTS) of Fig. 2f and all samples of 41(RTS) and 42(RTS) in Fig. 2r).

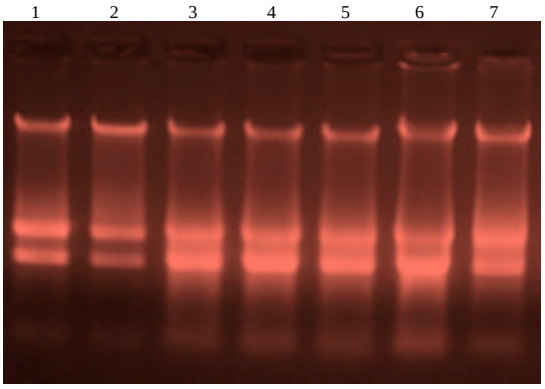

Fig. 1: DNA isolation from overnight cultured bacteria isolated from different samples of shrimp farms, Lane 1-7 represents various samples, DNA is visible in smearing as well as banding due to its high amount

Subsequently, the PCR amplification of specific genes and the specific size in gel band revealed the presence of the corresponding virulent *E. coli* strains (Fig. 3). Among 9 *E. coli* affected in cluster B, we found 5 (55.55%) farm containing enterovirulent *E. coli*, while in cluster F, we found 4 virulent among 8 contaminated farm (50%) and 9 among 12 contaminated farm in cluster R (75%) (Table 4).

Restriction enzyme of PCR: The specificity of the primers used in this investigation to detect pathogenic *E. coli* was

Table 3: Sources of *E. coli* from different shrimp farms

Source (Farm No.)	Cluster	Status of sanitation	*E. coli* (PhoA indicated) W	S	Sh
1	B	IS			+
2	B	TS	+		
3	B	IS			
4	B	IS			
5	B	IS			
6	B	TS	+		
7	B	TS	+	+	+
8	B	TS	+		
9	B	IS			
10	B	IS			
11	B	TS	+		
12	B	TS		+	
13	B	TS		+	+
14	B	IS	+		
15	B	IS			
16	B	IS			
17	F	TS	+		
18	F	TS		+	
19	F	TS		+	+
20	F	TS	+	+	+
21	F	TS		+	
22	F	IS			
23	F	IS			
24	F	IS			
25	F	IS			
26	F	TS	+		
27	F	TS		+	+
28	F	IS			
29	F	TS			
30	F	IS			
31	F	TS			+
32	R	TS	+	+	
33	R	IS	+		+
34	R	IS			
35	R	IS			+
36	R	TS	+		
37	R	TS	+		
38	R	IS			
39	R	TS	+		
40	R	TS	+	+	
41	R	TS	+	+	+
42	R	TS	+	+	+
43	R	TS			+
44	R	IS			
45	R	TS		+	
46	R	TS	+		
47	R	IS			
48	R	IS			

IS: Improved Sanitation, TS: Traditional Sanitation, B: Bagerhat, F: Fakirhat, R: Rampal, W: Water, S: Sediments, Sh: Shrimp intestine, +: Indicates the presence of the pathogens

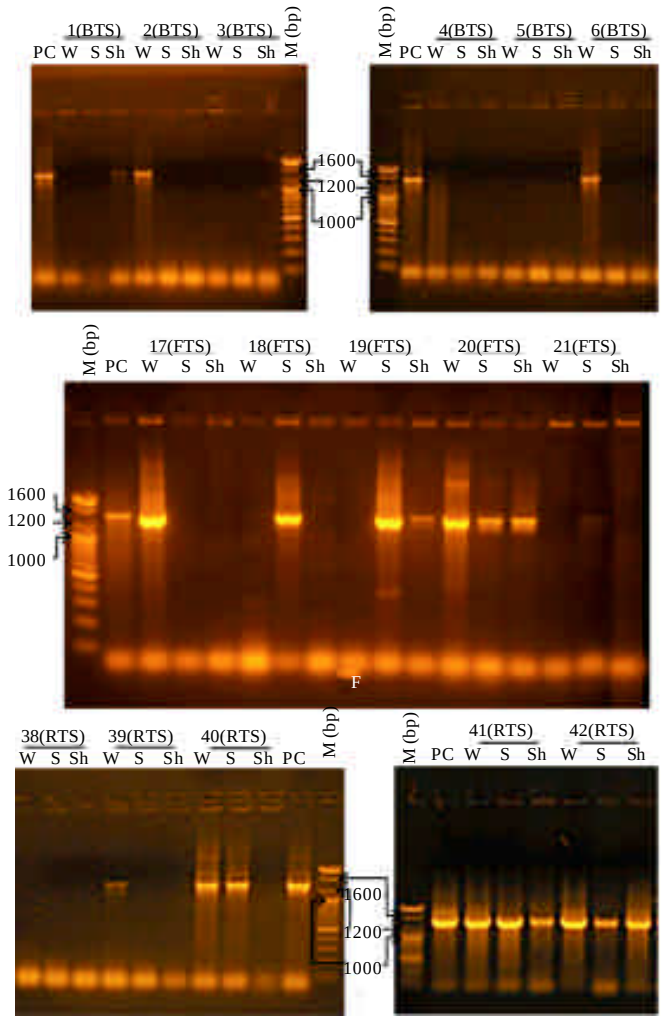

Fig. 2: Selective gel (2% agarose, 6 V/cm) snaps after PhoA amplified PCR indicate the presence of *E, coli* in different samples, PhoA binds on 1371 bp in DNA fragments in gel electrophoresis, In Cluster B (Bagerhat) 6 shown (available 16), Cluster F (Fakirhat) 5 shown (available 15), Cluster R (Rampal) 5 shown (available 17), Location and condition: BIS: Bagerhat-Improved Sanitation, BTS: Bagerhat-Traditional Sanitation, FTS: Fakirhat-Traditional Sanitation, RIS: Rampal-Improved Sanitation, RTS: Rampal Traditional Sanitation; The sample lane, W: Water, S: Sediments, Sh: Shrimp tissue, PC: Positive Control, M: Marker

verified with the restriction enzymes (*Alu*I for PhoA and LT2 and *Mbo*II for EAE). The fragment size of the digested PhoA in gel band (Fig. 4) was moderately consistent with the expected cut position (approx. 150 and 715 bp) calculated from computer-generated analysis (expected as 150/506/715) though a band on 560 bp (approxi.) appeared after gel electrophoresis. However, after digestion, the expected weak band (ca. 1300 bp) in lane D, PhoA is due to the undigested PCR products remained. Similar fragment was observed in the digested gel band of LT2 with *Alu*I as in the cut position of approx. 66, 227 and 704 though the 227 bp position was not exactly as it should be (Fig 3a). On the other hand, EAE was digested with *Mbo*II, which produced cut position 171 and 181 bp, as expected in the gel band though both are so close to get fused in the electrophoresis (Fig. 3b).

Traditional vs. improved sanitation: Expectedly, we have noticed that the improved sanitation brought less

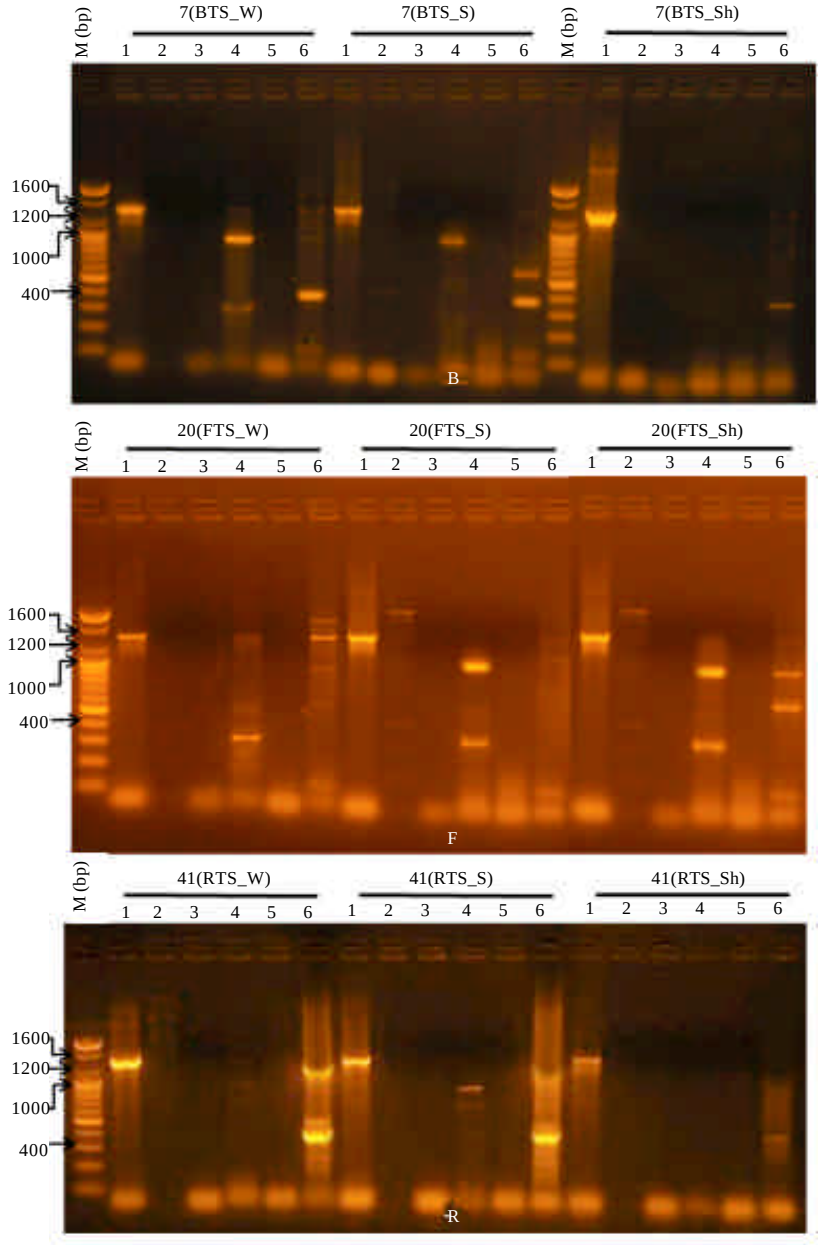

Fig. 3: Selective gel (2% agarose, 6V/cm) snaps after enterovirulent *E. coli* binding primer PCR indicates the presence of virulent strains in screened samples after PhoA (Table 3), Column 1: PhoA binds on 1371 bp, 2: ST1 absent (should bind on 638 bp), 3: LT1 absent(should bind on 725 bp), 4: LT2 binds on 997, 5: VT absent (should bind on 1240 bp), 6: EAE binds on 360 bp), M: Marker, In Cluster B (Bagerhat) 1 shown (available 9), Cluster F (Fakirhat) 1 shown (available 8), Cluster R (Rampal) 1 shown (available 12), Sample source: BTS_W: Bagerhat-traditional sanitation_Water, BTS_S: BTS_Sediment, BTS_Sh: BTS_Shrimp tissue, FTS_W: Fakirhat-Traditional sanitation_Water, FTS_S: FTS_Sediment, FTS_Sh: FTS_Shrimp tissue, RTS_W: Rampal-traditional sanitation_Water, RTS_S: RTS_Sediment, RTS_Sh: RTS_Shrimp tissue

contamination as we found only 14.29% farm (3 out of 21) maintaining improved sanitation were contaminated with *E. coli*, while 96.30% farm (26 out of 27) having traditional sanitation were contaminated with *E. coli*. EPEC group was found in one shrimp intestine (farm No. 35, Table 4) collected from one farm amid improved sanitation though.

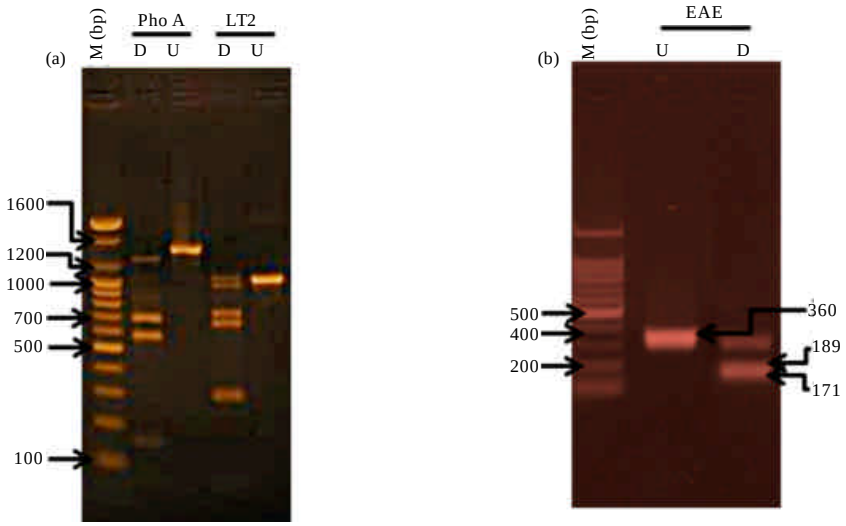

Fig. 4(a-b): Digestion of PCR products of PhoA and LT2 (a) with *Alu*I and EAE with *Mbo*II and (b), The expected cut position is 150/715 (PhoA), 66/704 (LT2) and 171/189 (EAE), D: Digested, U: Undigested

Table 4: Enterovirulent *E. coli* strains from the *E. coli* contaminated samples of shrimp farm

Source (Farm No.)	Cluster	*E. coli* (PhoA indicated)	Virulent strains of *E. coli* ST1	LT1	LT2	VT	EAE
1	B	Sh					
2	B	W					+
6	B	W					
7	B	W			+		+
		S			+		+
		Sh					+
8	B	W					+
11	B	W			+		
12	B	S					+
13	B	S					
		Sh					
14	B	W					
17	F	W					
18	F	S			+		
19	F	S					
		Sh					
20	F	W					
		S			+		
		Sh			+		
21	F	S					
26	F	W					+
27	F	S			+		
		Sh			+		
31	F	Sh					
32	R	W					+
		S					+
33	R	W					
		Sh					
35	R	Sh					+
36	R	W			+		+
37	R	W					
39	R	W					+
40	R	W					+
		S					+

Table 4: Continue

Source (Farm No.)	Cluster	*E. coli* (PhoA indicated)	Virulent strains of *E. coli* ST1	LT1	LT2	VT	EAE
41	R	W					+
		S			+		+
		Sh					+
42	R	W					
		S					
		Sh					
43	R	Sh			+		
45	R	S					+
46	R	W			+		

B: Bagerhat, F: Fakirhat, R: Rampal, W: Water, S: Sediments, Sh: Shrimp intestine, +: Indicates the presence of the strains

Water vs. sediment vs. shrimp intestine: Among 48 samples of water, 18 contains *E. coli*, among which only 11 were virulent (2 only ETEC group, 7 only EPEC and 2 both). On the other hand, 13 farms had *E. coli* in their sediments, among which 9 were virulent pathogens (3 ETEC, 4 EPEC and 2 both) and from the shrimp intestine, *E. coli* was found in 12 samples, among which only half of them were virulent (6 ETEC and 6 EPEC group).

ETEC vs. EHEC vs. EPEC: We have found only the presence of LT2 gene of ETEC group and EAE gene of EPEC as an enterovirulent pathogen. No verotoxin gene of EHEC was observed after PCR and gel electrophoresis. Among the 26 virulent *E. coli* contaminated samples, 8 contained only ETEC, 10 contained only EPEC, while 4 was contaminated with both the virulent group.

DISCUSSION

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*., 1992). 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. As alkaline phosphatase (PhoA) is housekeeping gene for *E. coli*, its positive signal on agarose gel, after PCR, confirms the presence of *E. coli* in the sample. Then, the *E. coli* positive samples were investigated for the identification of enterovirulent strains makes a rapid scenario of the samples in terms of pathogenic *E. coli*.

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 *et al*. (1999). There could be some artefacts in PCR and primers to get accurate band indicating the target strains. The application of restriction enzyme, however, can confirm the band in gel electrophoresis of selective primers after PCR products. Both PCR and restriction enzyme were performed in this investigation to eliminate the errors in rapid identification of pathogenic strains. As only PhoA, LT2 and EAE amplification were obtained after PCR, the restriction enzyme analysis was only done for those PCR products. For the fragment analysis, PhoA and other virulent genes of *E. coli*, Kong *et al*. (1999) used *Hinc*II enzyme, while we used *Alu*I for PhoA and LT2 and *Mbo*II for EAE. Among them, EAE was perfectly digested with two fragments (171/189 bp) (Fig. 3b), while PhoA and LT2 was cleaved with two expected cut position (150/715 and 66/704 bp, respectively) and another non-specific cut position in between (ca. 560 and 280 bp, respectively). Few unexpected bands could be appeared in the gel electrophoresis due to the undigested PCR products and enzyme weakness.

In the present experiment, there was no evidence of EHEC strain group in the sample farms. It might be due to the environmental variation of *E. coli* strain in the sampling area. Tave (1993) reported that every organism required a particular environment for growth and development, so, in this sense, the sampling area might be safer from the enterohemorrhagic *E. coli* strains. *E. coli*, however, responds frequently with the changing environment. We found only ETEC and EPEC pathogenic group, which is a causative agent of diarrhea in humans, rabbits, dogs, cats and horses (Todar, 2007). Karunasagar *et al*. (1999) claimed ETEC strains produce a cholera-like toxin called heat Labile Toxin (LT) and a second diarrhoeal toxin heat Stable Toxin (ST). As from the shrimp intestine along with water and sediments samples, LT2 gene was identified in this investigation, it is highly predictable to have cholera toxin, which can infect human intestine after consumption. Like ETEC, EPEC also causes diarrhea but the molecular mechanisms of colonization and etiology are different. EAE gene of EPEC has the mechanism of adherence to the intestinal mucosa causes a rearrangement of actins in the host cell, causing significant deformation due to "attachment and effacement" are likely the prime cause of diarrhea as well (Todar, 2007). The present result is supported by several authors (Kong *et al*., 1995; Lang *et al*., 1994; Paton and Paton, 1998; Ristaino *et al*., 1983), who conducted a various multiplex PCR protocols to simultaneously detect segments of different toxin genes of ETEC strains.

It is clear and expected that the improved sanitation attracts less *E. coli* as shrimp farm amid traditional sanitation (Table 1) had seven times more *E. coli* as much as that in improved sanitation. Karunasagar *et al*. (1999) claimed same objects such as hanging toilet, nearest cattle field as mentioned in the traditional sanitation to be the sources of *E. coli* in the aquaculture pond. Even the evidence of infection by the EPEC strain in one improved sanitized farm (Farm No. 35, Table 3, 4) explores some sources of contamination other than toilet, cattle dirty, canal water and live feeds. We can blame the source of

shrimp fry and brood, which are directly released in to aquaculture pond without this type of virulent pathogenic detection test.

CONCLUSION

Using versatile PCR analysis, we have shown, in this study, how the virulent *E. coli* strains can be identified in a rapid course. We have found two serious pathogenic groups of *E. coli* viz., ETEC and EPEC in the water, sediments and live shrimp, which remain undetected frequently through normal microbial test. The rapid identification of enterovirulent *E. coli* from shrimp farm was the first attempt in Bangladesh and we believe this investigation will give significant urge to save the shrimp industry as well as the human health concern. We also suggest that the samtation improvement is inevitable to keep the farm less infected with *E. coli*, along with the need of PCR screening of shrimp fry and brood before releasing into the aquaculture.

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