Research Article

Journal of Aquaculture and Technical Development

Quantification of the Coliform Bacteria and Detection of Enterovirulent *Escherichia coli* Strains Using Strain Specific genes in Shrimp Farms

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Abstract

Total coliform indicates the presence of Citrobacter spp., *Enterobacter* spp., *Escheriechia* spp., *Klebsiella* spp., *Salmonella* spp., *Shigella* spp. and *Vibrio* spp. In Bangladesh, traditional shrimp culture farms are highly susceptible to contamination with pathogenic bacteria because of poor sanitation near to these farms. The aim of the present study was to quantify total coliform and detect enterovirulent groups (EHEC, enterohaemorrhagic, EPEC, enteropathogenic and ETEC, enterotoxigenic) of *E. coli* in shrimp farms in winter and summer seasons at Khulna district of Bangladesh. All the farms were contaminated with coliform but *E. coli* was present only in 55% farms. In winter season, average number of coliform and *E. coli* in hygienic farms were 4.94×10^2 (cfu/mL) and nil whereas that in unhygienic farms were 8.17×10^2 (cfu/mL) and 4.15×10^2 (cfu/mL), respectively. However, in summer, the number of coliform and *E. coli* in hygienic farms were 8.26×10^3 (cfu/mL) and 8.51×10^2 (cfu/mL) whereas that in unhygienic farms were 1.11×10^4 (cfu/mL) and 4.52×10^3 (cfu/mL), respectively. Using cultural, biochemical and PCR techniques, 76 *E. coli* were identified. Among them, EPEC and ETEC groups were 22% and 29%, respectively whereas EHEC group was absent. The results of this study revealed that water contaminated in shrimp farms of Khulna district resulted from insufficient sanitation that represent unhygienic products.

Keywords: Coliform; *Escherichia coli*; PCR; Sanitation; Shrimp farm; Target gene

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Received Date: March 22, 2021

Accepted Date: April 1, 2021

Published Date: April 7, 2021

Citation: Sultana S, Sayeduzzaman, Shams FI, Hossain SJ, Sarower G (2021) Quantification of the Coliform Bacteria and Detection of Enterovirulent *Escherichia coli* Strains Using Strain Specific genes in Shrimp Farms. J Aqua Tech Deve 4: 006.

Introduction

Bangladesh is endowed with natural shrimp resources and shrimp farming has been recognized as a part of Blue Revolution for the geographic features of southwest coastal area [1,2]. In the early seventies, Bangladesh entered the world's export market for shrimp and since then this crustacean has suddenly become a very high-priced commodity. The shrimp sector has undergone dramatic changes in terms of area, production, and marketing [3]. Currently, shrimp industry is one of the most important contributors for economic sustenance and is the second largest export commodity of the country. Sometimes, the importing countries reject shrimp consignments claiming them unfit for consumption due to the presence of antibiotics, filth, unexpected foreign materials and pathogenic microbes such as *E. coli*, *Salmonella* sp., *Vibrio* sp. etc. The abundance of *E. coli*, however, has been reported to be more dangerous than that of coliforms [4,5].

Coliform and *E. coli*, which are widely distributed pathogens in aquatic environment have been universally accepted as an indicator of fecal pollution, because of their presence in high numbers in mammalian gut. Most of the *E. coli* strains are harmless, only a small percentage is pathogenic to humans [6,7].

To date, several types of enterovirulent *E. coli* have been recognized as the etiologic agents of various gastrointestinal infections in humans. The most commonly encountered are those belonging to the enterohaemorrhagic *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 [6].

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) [8], used widely has several disadvantages especially 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 [9] and thirdly, phenotypically MUG-negative *E. coli* [10], for instance, EHEC strains is not detected by this method [11]. For the specific detection of pathogenic ETEC and EHEC strains, therefore, several ELISA-based methods have been developed including detection of heat-labile LT [12], heat-stable ST1 [13] and ST2 [14] enterotoxins of ETEC; and verotoxins VT1 and VT2 of EHEC strains [15]. 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 [16].

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 [17] and EHEC strains [18]. Various multiplex PCR protocols to simultaneously detect segments of different toxin genes of enterovirulent strains of *E. coli* have also been developed viz., multiplex detection of *lt1/vt1/vt2* genes of ETEC and EHEC strains [19], *lt1/lt2* (ETEC) [20], *lt1/st1* (ETEC) [21], *elt/est* (ETEC), *eae* (EPEC), *ipaH* (EIEC) [22] and *lt/sth/stp* (ETEC), *stx1/stx2* (EHEC), and *eae/bfp* (EPEC) [23].

The enterovirulent strains of this pathogen may create enormous loss for the fishery 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 traditional and improved traditional shrimp farms in a reliable and rapid way. The objective of this study was to address this safety issue through enumeration of coliform quantification and enterovirulent *E. coli* detection in shrimp farms by PCR technique.

Materials and Methods

Study area and sampling

In this study, water samples were collected from 15 farms consisting of 6 hygienic and 9 unhygienic farms in winter (November to February) and from 16 farms consisting of 8 hygienic and 8 unhygienic farms in summer (May to August) at different sites of Khulna district. Using visual observation farms were categorized into unhygienic farm and hygienic farm. Criteria for unhygienic and hygienic farms are listed in Table 1. After collection, the water samples were brought to the laboratory carefully and preserved in the refrigerator for immediate use; however, for long term usage, samples were stored at 4° C.

Particulars	Unhygienic farm	Hygienic farm
Hanging toilet	Near to farms and/or water source.	Only concrete and closed toilet
Water source	River or canal	Ground water or rain water
Cow shed	Near to farms	No cow-shed
Grazing land	Near to farms	No grazing land
Fertilizer	Organic fertilizer	Inorganic fertilizer
Premises	Dirty	Clean and healthy

Table 1: Criteria for unhygienic and hygienic shrimp farm.

Counting of coliform and detection of *E. coli*

MacConkey agar was used to enumerate total coliform. A known quantity of collected aqueous samples was spread on MacConkey agar media and the plates were incubated at 37 °C for 24 hours. Lactose positive colonies showed red color on MacConkey agar plate and the number of coliforms was equal to the number of lactose positive colonies.

Then 5-10 red colonies on MacConkey agar plate were finally selected and isolated for detection of *E. coli.* The selected red colonies were subcultured onto nutrient agar until the pure cultures with homogenous colonies were obtained. Finally, for identification of *E. coli*, characteristics of isolated red colonies were studied using the following test-Eosine Methylene Blue (EMB) Agar Test [24], Lactose Fermentation Test, Methyl Red (MR) Test, Voges–Proskauer Reaction (VP) Test and Citrate Agar Test as described by [25]. When the selected red colonies were streaked on EMB plate, *E. coli* was identified with a characteristics green metallic sheen along the streak after a 24-48-hour incubation period. *E. coli* was also characterized by their ability to ferment lactose, positive for MR Test and negative for VP and Citrate utilization Test.

Culture of organisms and extraction of total DNA

Bacterial strains in water collected from 31 selected farms were cultured in 5 ml of Luria-Bertani broth (per litre: 10 g Bacto-tryptone, 5 g NaCl, 5 g yeast extract (Difco Laboratories, MI, USA) for overnight at 37°C. Cells were harvested by centrifugation at 14 000 rpm for 1 min and washed five times with sterile distilled water.

Total DNA extraction was carried out from amplified bacterial cells using DNAZOL® Reagent (Invitrogen Life technologies, USA), ethanol and sodium hydroxide (Difco Laboratories, MI, USA). The concentration and purity of DNA samples were determined from the ratio of absorbance at A_{260} and A_{280} (absorbance at 260 nm and 280 nm) using a spectrophotometer against NaOH blank cuvette. DNA sample containing cuvette was washed properly before loading next sample.

Target Genes and primer designation

The target genes chosen of this experiment were: alkaline phosphatase *phoa*, housekeeping gene (present in all *E. coli*); the heat-labile *lt1*, *lt2* genes and heat-stable *st1* genes of ETEC group; verotoxin *vt* gene of EHEC group and attachment and effacement eae gene of EPEC group.

Six pairs of specific primers were designed from gene sequence to amplify the target genes. The primer designation was done completely based on the verified sequences in the gene bank NCBI (Table 2). As a marker, 100 bp DNA sized was used to compare and ensure the size of PCR amplified product.

Groups	Target Gene	Primers	Product size (bp)	Accession no.
E. coli	Alkaline phosphatase (*phoA*)	Pho-F	1371	M13345
		Pho-R		
Enterotoxigenic (ETEC)	Heat-stable toxin 1 (*st1*)	ST1-F	638	M25607
		ST1-R		
	Heat-labile toxin 1 (*lt1*)	LT1-F	725	J01646
		LT1-R		
	Heat-labile toxin 2 (*lt2*)	LT2-F	997	M17894
		LT2-R		
Enterohemorrhagic (EHEC)	Verotoxin (*vt*)	VT-F	1240	M36727
		VT-R		
Enteropathogenic (EPEC)	Attachment and Effacement (*eae*)	EAE-F	1155	M58154
		EAE-R		

Table 2: Primers and expected size of PCR-amplified gene targets of pathogenic *E. coli*.

PCR Amplification and analysis of PCR products

A thermal cycler (C1000TM, BIO-RAD, USA) was used for PCR. The amplification program included an initial denaturation step at 94°C for 2 min and 35 cycles of denaturation at 94°C for 1 min, annealing at 58 °C for 1 min and final extension at 72°C for 1 min. The reactions were performed in a 0.2 ml PCR tube (Eppendorf, Humburg, Germany) in which 25µl reaction mixture containing 2µl extracted DNA sample (0.5µg/50µl), 2µl oligonucleotide primers (1µM), 5µl 5X green reaction buffer (1.5mM $MgCl_2$), 2µl dNTPs (0.2mM), 1µl *Top* DNA polymerase (1.25U) and 13µl de-ionized distilled water (Bioneer Corporation, Daejeon, Korea). PCR products were analyzed by gel electrophoresis in 2% agarose (Bioneer Corporation) containing 0.5

μg ml^{-1} ethidium bromide in TAE buffer. DNA bands were visualized by High Performance UV Transilluminator (UVP, CA, USA) and photographed using the DigiDoc-It 120 gel documentation system (UVP). 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).

Results and Discussion

In this study, 31 shrimp farms were selected to detect the occurrence of coliform and enterovirulent *E. coli*. The average number of coliforms in unhygienic farms was twofold higher than that in hygienic farms in winter season and these loads in both types of farms became highly increased in summer season (Table 3).

Season	Farms type	Average of total coliform (cfu/mL)	Average of total *E. coli* (cfu/mL)
Winter	Hygienic	4.94×10^2	Not detected
	Unhygienic	8.17×10^2	4.15×10^2
Summer	Hygienic	8.26×10^3	8.51×10^2
	Unhygienic	1.11×10^4	4.5×10^3

Table 3: Average number of total coliform and *E. coli* of 31 shrimp farms in two seasons.

All coliform contaminated water samples were subjected to investigation to detect the presence of *E. coli*. Among 31 shrimp farms, 17 farms (55% farms) showed contamination of *E. coli*. No contamination of *E. coli* was found in hygienic farms in winter, but found in unhygienic farms. Whereas, both hygienic and unhygienic farms were highly contaminated with *E. coli* (Table 3).

Following bacteria culture and DNA extraction, initially PCR analysis of 76 isolates of *E. coli* screened from 17 contaminated farms was carried out with a pair of primers targeted at *phoa* gene specific for *E. coli*. As shown in Figure 1

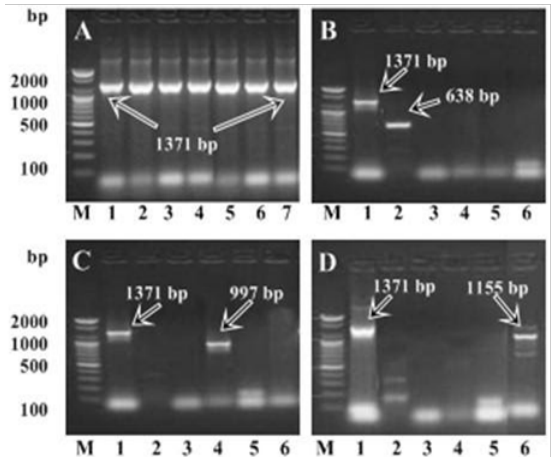

Figure 1: Electrophoretic analysis of PCR-amplified target genes from *E. coli* and its different strains obtained under optimal conditions of PCR. A, PCR-amplified phoa gene from *E. coli* isolates of contaminated farms. Lane M, 100 bp DNA marker; lane 1, positive control; lane 2 - 4, isolates from hygienic farms; lane 5 - 7, isolates from unhygienic farms. B, C and D, mobility of the different target genes is indicated on the right. Lane M, 100 bp DNA marker; lane 1, Pho-A amplimers; lane 2, ST1 amplimers; lane 3, LT1 amplimers; lane 4, LT2; lane 5, VT amplimers; lane 6, EAE amplimers. Gels are representative of independent experiments of 76 isolates.

A PCR bands corresponding to *phoa* genes were detected in seven representative isolates. The results indicated *E. coli* that is positive for this gene was present in all 76 isolates. Additional five pairs of oligonucleotide primers were designed in this study to simultaneously amplify a house-keeping as well as various virulence-associated genes of ETEC, EHEC and EPEC groups in a single tube. Seventy six isolates of *E. coli* screened from farms water were characterized by PCR and the results showed that *st*1 and *lt*2 toxin gene of group ETEC and *eae* virulence gene of EPEC group were present whereas toxin *lt*1 of group ETEC and *vt* verotoxin of group EHEC were totally absent among the isolates of *E. coli*. B-D shows the PCR bands of 1371 bp, 638 bp, 997 bp and 1155 bp which clearly indicated the presence of *phoA* gene, *st*1 gene, *lt*2 gene and *eae* gene, respectively (Figure.1 B-D). In both seasons, contamination of ETEC, EHEC and EPEC groups were not found in hygienic farms where as ETEC and EPEC were found to be contaminated in unhygienic farms.

Among the 76 isolated *E. coli*, EPEC and ETEC groups were 22% and 29%, respectively (Table 4).

Farms and colonies number	Farms			
	Winter		Summer	
	Hygienic	Unhygienic	Hygienic	Unhygienic
Farms examined	6	9	8	8
Contaminated farms with *E. coli*	nd	8	2	7
Isolated coliform colonies no.	60	90	80	80
E. coli colonies no.	nd	40	6	30
ETEC colonies no.	nd	11	nd	10
EHEC colonies no.	nd	nd	nd	nd
EPEC colonies no.	nd	9	nd	6

Table 4: PCR results summery of sampling farms in the winter and summer seasons.
nd: Not detected

EHEC strain was not detected in any farms that were contaminated with *E. coli*. This observation is consistent with previous reports claiming that enterotoxigenic *E. coli* strains that are *lt2*+ are rarely found [26], and so far, ETEC strains that are *lt2*+ have been isolated only in Brazil, Thailand and Bangladesh [27-29].

[29] were able to identify the virulent strains *lt2* genes of ETEC group and EAE gene of EPEC group from shrimp farms. In the current experiment, the PCR was performed without screening and removing detritus to avoid target loss [26] and to reduce extra time needed assuming that PCR is able to amplify target DNA if present in the sample. Overall, the PCR analyses confirmed that *st1*+, *lt2*+ (of ETEC origin) and *eae*+ *E. coli* strains were present in water of unhygienic farms. [30] claimed same objects such as hanging toilet, nearest cattle field as mentioned in the traditional sanitation to be the sources of *E. coli*. The result in this study revealed that the improved sanitation of shrimp farms attracted less *E. coli* than less sanitation ones.

In shrimp culture ecosystem, most of the bacteria play a negative role as they compete with shrimps for food and oxygen, causing stress and disease [31]. Generally, gram-negative bacteria were found to be the dominant forms in the shrimp culture ponds [32]. The presence of coliform indicates that water supply may be vulnerable to contamination by harmful microorganisms. *E. coli* is one of the members of total coliform that is found only in the intestines of mammals, including humans. Although only a small percentage of *E. coli* is pathogenic to humans, their presence in water indicates recent fecal contamination and may indicate the possible presence of disease-causing pathogens, such as bacteria, parasites and viruses. To better determine the health risks associated with exposure to pathogenic *E. coli* in the environment, the frequency at which pathogenic *E.*

coli occurs in the shrimp farms must be assessed. Our results above indicate the potential of the PCR assay as a versatile and efficient means to identify and differentiate pathogenic from non-pathogenic *E. coli* strains in shrimp farms. Conceivably, this method can also be used as a cost-effective means to determine the prevalence or frequency of occurrence of these organisms in diverse ecological niches by enabling rapid identification and typing of clinical and environmental isolates of ETEC, EHEC and EPEC strains.

Conclusion

The findings reported in this study describe a versatile, reliable and highly sensitive PCR for the rapid detection of ETEC, EHEC and EPEC strains of *E. coli* in shrimp farms. The preincubation of cells from water samples in LB broth for 8 h prior to PCR also greatly enhanced the detection sensitivity of the system for ETEC and EPEC strains. Overall, the data indicated that the PCR scheme is a potentially very useful and powerful technique for the microbiological assessment of shrimp farm hygienicity. This method would be particularly useful for the assessment of health risks that may be associated with exposure to ETEC, EHEC and EPEC pathogens, which this study has shown are commonly found in various samples of traditional shrimp farms which are highly susceptible for pathogenic bacterial contamination.

Declarations

Funding statement

This work was supported by Ministry of Education, Bangladesh with funds from USDA.

Competing interest statement

The authors declare no conflict of interest.

Ethical statement

This material is the authors' own original work, which has not been previously published elsewhere.

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