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Occurrence of Pathotypes of *Escherichia coli* in Aquatic Environment

B. Koteswar[1]*, A. Devivaraprasad Reddy[2], G. Ravi[3], and Indrani Karunasagar[2]

[1]National Institute of Fisheries Post Harvest Technology and Training,
Vishakhapatnam, Andhra Pradesh, India
[2]Department of Fisheries Microbiology, College of Fisheries, Mangalore, Karnataka, India
[3]Fisheries Research Station, PVNR Telangana Veterinary University, Palair - 507 157, India
*Corresponding author

A B S T R A C T

Escherichia coli are recognized indicators of faecal contamination. However, this perspective has changed with the ability of some strains of *E. coli* to cause human illness. The pathogenic *E. coli* strains can cause a variety of diseases, including diarrhoea, dysentery, and haemolytic uremic syndrome, bladder and kidney infections. Different strains are usually associated with different diseases; this versatility of *E. coli* strains is due to the different strains possessing unique virulence genes. Though *E. coli* contamination of tropical seafood is quite common, the distribution of different pathogenic types in seafood is not studied in detail except for Shiga toxin producing *E. coli*. The objective of this study was identification of the pathotypes, if any, associated with them. *E. coli* was isolated from several fresh and frozen finfish samples, molluscs, cephalopods and crustacean shellfishes, and water and sediment samples. Among the different aquatic environment and seafood samples examined, only two squids and one flat fish sample was found positive for the presence of the virulence gene, *eaeA*. These three samples yielded atypical EPEC (*eaeA*+, *bfp*-, *stx*-), while being negative for other virulence genes by PCR. Other samples though positive for *E. coli,* did not possess the virulence genes. The results highlight the presence of some of the pathotypes of *E. coli* in seafood samples and hence are recognized as a potential threat to human health.

Introduction

The genus *Escherichia* is a member of the Enterobacteriaceae family and *E. coli* is the most common aerobic organism in the intestinal tract of man and warm-blooded animals. Most of the *E. coli* strains are harmless commensals that colonize the gastrointestinal tract and probably play important roles in maintaining intestinal physiology. *E. coli* plays a crucial role in food digestion by producing vitamin K from undigested material in the large intestine.

An important member of the normal intestinal micro biota of humans and other mammals, *E. coli* has also been widely exploited as a cloning host in recombinant DNA technology (Kaper *et al.,* 2004). However, some strains of *E. coli* are pathogenic and can cause diarrheal disease. *E. coli* strains are differentiated based on a serotyping scheme involving O (somatic), H (flagellar) and K (capsular) antigens. Pathogenic *E. coli* is divided into specific groups depending on virulence,

clinical symptoms and distinct O: H antigens. The important groups are: enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffuse-adhering *E. coli* (DAEC), enteroaggregative *E. coli* (EAggEC) and entero hemorrhagic *E. coli* (EHEC), or Shiga toxin producing *E. coli* (STEC) (Donnenberg., 2005). Several *E. coli* pathotypes have been implicated in diarrheal illness, a major public health problem worldwide, with over two million deaths occurring each year (Kosek *et al.,* 2003). Besides being the major cause of human urinary tract infections, *E. coli* has been linked to cause many other diseases like pneumonia, meningitis and traveller's diarrhea. Pathogenic strains of *E. coli* can cause severe diarrhea in all age groups by producing a powerful endotoxin. Treating *E. coli* infections with antibiotics may actually place the patient in severe shock that could possibly lead to the death. The main source of *E. coli* infections have been faecal contaminated water and contaminated food handlers. Outbreaks by pathogenic *E. coli* have mostly involved undercooked ground beef and raw milk, however, poor hygiene, cross contamination by food handlers or dirty water may transfer the organism. Also, such strains may accumulate in filter feeding bivalves cultured in contaminated waters. Though the *E. coli* is not indigenous to the aquatic environment, it may survive and even multiply in warm tropical waters (Rhodes and Kator, 1988; Jimenez *et al.,* 1989) and thus also isolated from presumed unpolluted waters.

Materials and Methods

Sample collection

Various aquatic samples which included twenty two finfish, thirty shellfish, six water and three sediment samples were collected in sterile polythene bags from fish landing centre and fish ponds in Mangalore. Sewage water was collected from sewage treatment plant. Sediment samples were collected from fish ponds and also from a region in Mangalore where sewage water is discharged into the sea. Water samples from sea and freshwater ponds were collected in sterile polythene bags and brought to the laboratory for further analysis

Enrichment

25 g of each sample (finfish, shellfish, frozen fish,) was taken and homogenized with 225 ml of physiological saline in a homogenizer for 5 min. 1 ml each of homogenized samples was then inoculated into two tubes containing 9 ml each of *E. coli* broth. One tube was incubated at 37 °C and the other at 44.5 °C for 24 hr in a water bath. The same procedure was repeated with sediment sample.

A suspension was prepared by mixing 25 g sediment with 225 ml of physiological saline and then inoculated as above. In the case of water and ice (melted), 1 ml of the sample was directly inoculated to two tubes containing 9 ml of E.C broth. As detailed above, one tube was incubated at 37 °C and the other at 44.5 °C for 24 hr.

Selective plating

One loopful of culture from the enrichment broth was streaked onto three selective agar plates *i.e.* Sorbitol- MacConkey agar, MacConkey and EMB agar plates. The plates were incubated at 37 °C for 24 hr. Colourless colonies, reddish colonies and colonies showing metallic sheen that developed on Sorbitol- MacConkey agar, MacConkey agar and Eosine methylene blue agar respectively, were picked and subjected to further biochemical tests for the identification of *E. coli*

Biochemical tests

Biochemical tests like gram's straining, indole test, Methyl Red-Voges Proskauer test (MR-VP) and Simmon's citrate test were done for the identification and confirmation of *E. coli.* Using molecular methods for identifying pathotypes of *E. coli*.

PCR for confirmation of *E. coli* pathotypes

Extraction of DNA

Test cultures were grown overnight in 3 ml Luria Bertani broth. 50 µl aliquot of this culture was suspended in 450 µl of 1 x TE buffer (pH 8.0) and lysed at 98°C for 15 min in hot bath, followed by snap cooling on ice. The cell debris was settled by centrifugation at 10,000 rpm for 5 min and the supernatant was used for PCR amplification. All the PCR reactions were performed in PTC 200 thermal cycler (M.J. Research Inc., USA).

PCR amplification

PCR reaction was carried out in 30 µl reaction mixture containing10X buffer (100 mM Tris-HCl, pH 8.3, 20 mM $MgCl_2$, 500 mM KCl, and 0.1% gelatin), 200 mM of dNTPs (dATP, dTTP, dGTP, dCTP), 10 pmol each of forward and reverse primers and 1.0 unit of Taq DNA polymerase. The buffer for Taq DNA polymerase, dNTPs and Taq poly-merase was obtained from Bangalore Genie, Bangalore. In a sterile PCR tube 22.1µl of sterile distilled water, 3 µl of 10X assay buffer, 0.6 µl of 200 mM dNTP mix, 2 µl of each primer (forward and reverse) (10 pmol/ml), 0.3µl of Taq polymerase and 2 µl of template DNA solution was taken. Amplification was carried out in a thermocycler, (MJ-Research, USA) and programmed for required number of cycles of amplification. Each cycle consisted of denaturation, annealing, and extension. The programme included an initial denaturation at 94 ºC for 5 min, and a final delay at 72 ºC for 5-10 min. The gene cycling condition for the above mentioned genes of the various pathotypes of *E. coli* is given in table 1.

Detection of amplified DNA

PCR amplified product were resolved by electrophoresis in a 1.5 % agarose gel to visualize the amplicons. The agarose gels were prepared in 1X TAE buffer. When the molten agarose had cooled to below 65ºC, ethidium bromide was added to a final concentration of 0.5 µg/ml and the gel was cast. 20 µl of the amplicon was mixed with 4 µl of 6X loading buffer and loaded into the wells. 100 bp ladder (Bangalore Genei, Bangalore) was used as a molecular weight marker. Electrophoresis was carried out at 80-120 volts and the bands were visualized under UV light. The gels were photographed using the GelDoc documentation system (Herolab, Weisloch, Germany).

Results and Discussion

A total of sixty-six samples comprising of fish, shrimps, clams, mussels and squids collected from fish landing centre and markets in Mangalore were analyzed. Water and sediment samples were collected from fish ponds in the campus of College of Fisheries, Mulky estuary and seawater was from Mangalore coast. Sewage samples were collected from a sewage treatment plant in Yekkur, Mangalore. All samples were analyzed for the presence of pathotypes of *E. coli*. Finfish samples comprising sardines, mackerels, eel, flat fish, anchovies and croaker were analyzed. Among these finfish samples, *E. coli* was isolated from all varieties except eel wherein only one sample was analyzed. Out of 22 finfish samples studied, *E. coli* was observed in 18 samples. The shellfish samples comprised clams, mussels,

shrimps, squid and oysters. 27 out of 30 shellfish samples were positive for *E. coli*. Six water samples were analyzed (3 from fishpond, 2 seawater samples, and 1 from sewage outfall near the fish pond on the campus). All the water samples were positive for *E. coli.* All the three sediment samples analyzed for the presence of *E. coli* were positive. Three frozen fish (mackerels) samples were analyzed for the presence of *E. coli* and only one was positive for *E. coli.* Two ice samples from landing centre were analyzed for the presence of *E. coli* and both were positive.

Detection of pathotypes of *E. coli* by PCR

Aquatic environmental samples and the isolates obtained from them were subjected to PCR to detect the presence of *stx*2 gene of EHEC, *eaeA* gene of EHEC and EPEC, *lt* and *st* gene of ETEC, *ipaH* gene of EIEC, *daaE* gene of DAEC, *aafII* gene of EAEC. Three (one flat fish and two squid samples) of 66 samples were positive for *eaeA* (common for EHEC and EPEC). The gel picture showing amplification of 384 bp fragment of *eaeA* gene in three isolates is presented in figure 1. The percentage of this genotype was 4.54 %. None of the samples and their respective isolates was positive for other pathotypes of *E. coli* genes by PCR.

Traditionally, *E. coli* has been used as an indicator organism for the presence of other pathogenic bacteria in water and food. More than 200 serotypes of *E. coli* are categorized under at least six pathogenic types- enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enterohaemorrhagic *E. coli* or Shiga toxin-producing *E. coli* (EHEC or STEC), enteroinvasive *E. coli* (EIEC) and enteroadherent/ aggregative *E. coli* (EAEC/EaggEC), diffusely adherent *E. coli* (Donnenberg., 2005). Some *E. coli* strains can

cause a variety of diseases, including diarrhea, dysentery, hemolytic uremic syndrome and bladder and kidney infections. Infections due to Shiga toxin- producing *E. coli* (STEC) can result in severe bloody diarrhea (hemorrhagic colitis) and life-threatening complications such as hemolytic uremic syndrome (HUS) (Fischer *et al.,* 2001). Different strains are usually associated with different diseases. This versatility of *E. coli* strains is due to different strains having acquired different sets of virulence genes (Teophilo *et al.,* 2002). Though *E. coli* contamination of tropical seafood is quite common, the distribution of different types in seafood is less studied except for Shiga toxin-producing *E. coli* (Karunasagar *et al.,* 2005). Incubation temperature has a profound effect on growth of some pathotypes of *E. coli*. Though 44.5ºC is the selective temperature for growth of faecal coliforms and *E. coli,* circumstantial evidence suggests that some strains of pathogenic *E. coli* like EHEC O157: H7 are unable to grow at 44.5ºC (Varnam and Evans, 1991). Tusharamani (2001) showed that for the isolation of *E. coli*, incubation of broth at 44.5ºC would be important. The incidence of *E. coli* was higher at 44.5ºC compared to 37ºC. This could be due to the overgrowth of *E. coli* by other coliforms at 37ºC. The results of this study showed that incidence of *E. coli* was higher at 37 ºC compared to 44.5ºC. The incubation temperature of 44.5ºC would be selective for *E. coli.*

In the present study showed the maximum occurrence of *E. coli* in seafood samples analyzed. Kumar *et al.,* (2001) reported 78% incidence of *E. coli* in fish and 70 % in clam and 100% in oyster. Among 66 samples analyzed, 57 were presumptively identified as positive for *E. coli* by conventional methods. In this study, the incidence of *E. coli* was 86.6 %, which can be comparable with results obtained by Kumar *et al.,* (2001).

Primer sequences used for detection of enterohaemorrhagic *E. coli*

Gene	Primer sequence (5' – 3')	Size of the product (bp)	References
*stx*1and *stx*2	GAACGAAATAATTTATATGT TACTGACATTGTTAGTTT	900	Lin *et al.,* 1993
*stx*2	CCATGACAACGGACAGCAGTT CCTGTCAACTGAGCAGCACTTTG	779	Fagan *et al.,* 1999
eae A	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	384	Paton and Paton, 1998
stx common	GAGCGAAATAATTTATATGTG TGATGATGGCAATTCAGTAT	518	Yamasaki *et al.,* 1996

Primer sequences used to detect enterotoxigenic *E. coli*

Gene	Primer sequence (5' – 3')	Size of the product (bp)	References
Lt	GGCGACAGATTATACCGTGC CCGAAATTCTGTTATATATGTC	696	Schultz, 1994
St	TTAATAGCACCCGGTACAAGCACG CTTGACTTCAAAAGAGAAAATTAC	147	Olsvik and Strockbine,

Primers sequences used for detection of enteropathogenic *E. coli*

Gene	Primer sequence (5' – 3')	Size of the product (bp)	References
bfp	AAT GGT GCT TGC GCT TGC GC GCC GCT TTA TCC AAC TGGTA	324	Gunzberg *et al.,* 1995
eae A	GACCCGGCACAAGCATAAGC CCACCTGCAGCAACAAGAGG	384	Paton and Paton, 1998

Primer sequence used for detection of enteroinvasive *E. coli*

Gene	Primer sequence (5' – 3')	Size of the product (bp)	References
ipaH	CTC GGC ACG TTT TAA TAG TCTGG GTG GAG AGC TGA AGT TTC TCTGC	933	Vidal *et al.,* 2004

Primers sequence used for detection of enteroadherent- aggregative *E. coli*

| Gene	Primer sequence (5' – 3')	Size of the product (bp)	References
aafII	CAC AGG CAA CTG AAA TAA GTC TGG ATT CCC ATG ATG TCA AGC ACT TC	378	Vidal *et al.,* 2004

Primers sequence used for detection of diffusely adherent *E. coli*

Gene	Primer sequence (5' – 3')	Size of the product (bp)	References
daaE	GAA CGT TGG TTA ATG TGG GGT AA TAT TCA CCG GTC GGT TAT CAG T	542	Vidal *et al.,* 2004

Table.1 Cycling conditions for amplification of different genes of *E. coli*

Pathotype	Gene	Denaturation	Annealing	Extension	No. of cycles
EHEC	*stx1* and *stx2*	94 ℃ -1 min	58 ℃ -90 sec	72 ℃ -90 sec	35
	stx2	95 ℃-30 sec	58 ℃ -40 sec	72 ℃ -90 sec	35
	Stx common	94 ℃ -1 min	58 ℃ -90 sec	72 ℃ -90 sec	36
EPEC& EHEC	*eae A* common	95 ℃ -30 sec	58 ℃ -40 sec	72 ℃ -1 min	35
ETEC	*lt*	95 ℃ -1 min	60 ℃ -1 min	72 ℃ -2 min	30
	st	95 ℃ -1 min	60 ℃ -1 min	72 ℃ -1 min	30
EIEC	*ipaH*	94 ℃ -1.5 min	60 ℃ -90 sec	72 ℃ -90 sec	35
DAEC	*daaE*	94 ℃ -1.5 min	60 ℃ -90 sec	72 ℃ -90 sec	35
EAEC	*aafII*	94 ℃ -1.5 min	60 ℃ -90 sec	72 ℃ -90 sec	35

Fig.1 Detection of *eaeA* gene of enteropathogenic and enterohaemorrhagic
E. coli in seafood samples
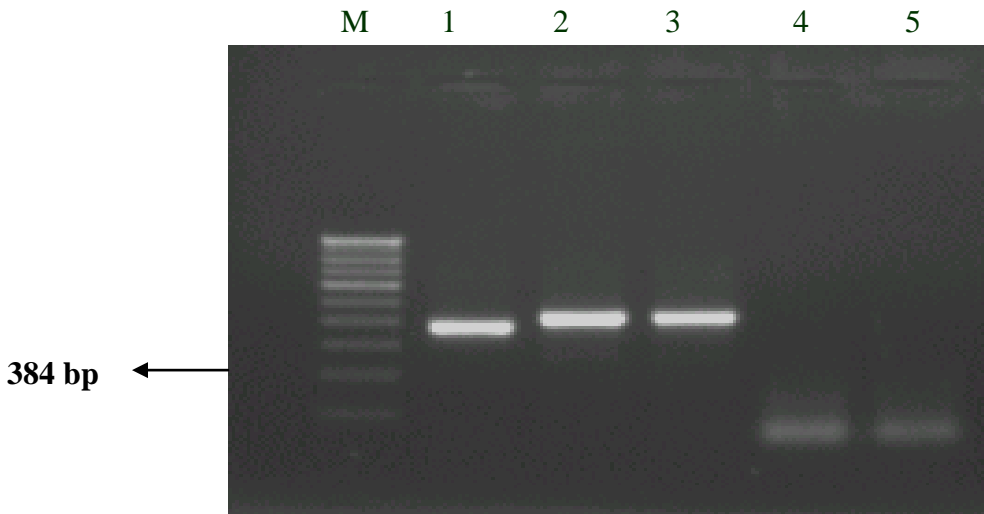
Lane M: 100 bp DNA ladder
Lane 1: Reference strain EDL 933 (*E. coli* O157:H7)
Lane 2: Squid sample positive for *eaeA* gene
Lane 3: Fish sample positive for *eaeA* gene
Lane 4 -5: Negative control

Shellfish are known to harbour faecal coliforms since they filter large quantities of water. Being coastal and intertidal, it is to be expected that if there is faecal pollution in the coastal environments, the sanitary quality of water is bound to be affected and consequently the oysters. In an attempt to recover ETEC and EHEC from fish samples in retail markets of Cochin, Thampuran *et al.,* (2005) observed that atypical *E. coli* with blood haemolysing property were present in fish and shellfish. The present study showed the absence of ETEC pathotype from aquatic environment and seafood samples. Pathogenic *E. coli* in environment samples off Velar estuary revealed that the organism was detected in fish, crustaceans and bivalves with percentage positivity being 30.1%, 29.7%,

and 51%, respectively (Ramesh *et al.,* 1991). Hatha *et al.,* (1993) isolated 107 strains of pathogenic *E. coli* from water, sediment and fish off Bhavani River. The fish and shellfish from various cities of north India were screened for pathogenic *E. coli* by Singh and Kulashrestha (1994). Thampuran *et al.,* (2005) reported that typical *E. coli* O157 or labile toxin- producing *E. coli* was absent in the fish and fishery environments of Cochin (India). Enterotoxigenic *E. coli* (ETEC) were identified as the etiologic agent in a large number of foodborne outbreaks at a sushi restaurant in Nevada. Poor food-handling practices and infected food handlers likely contributed to this outbreak (Jain *et al.,* 2008). Dupray *et al.,* (1999) detected *stx* genes in shellfish enrichments but no attempt was made to isolate STEC strains from positive samples. The results of this study suggest that PCR will be helpful in detecting samples possessing small numbers of pathogenic *E. coli*, which may be missed by conventional methods (Kumar *et al.,* 2001). Dhanashree and Mallya (2008) showed a low incidence of STEC and high prevalence of *eaeA* positive *E. coli* other than STEC in stool and meat samples. This supports the findings of the present study with low prevalence of *eaeA* positive *E. coli* in seafood. Sehgal *et al.,* (2008) observed enterohaemorrhagic *E. coli* isolates to be distributed among domestic and wild animals and the maximum number of isolates of *E. coli* O157 was detected in samples received from coastal belt areas.

In this study, 3 of 66 samples showed the presence of pathogenic *E. coli*. These 3 samples (two squid and one flat fish) were collected from a landing centre in Mangalore. These 3 seafood samples were positive for *eaeA* virulence gene of EHEC/EPEC and negative for other virulence genes of pathogenic *E. coli*. The prevalence of *eaeA*-positive *E. coli* while being negative for other virulence genes of enterohaemorrhagic *E. coli*

like *stx* and *hlyA* as determined in this study, was 4.54% in seafood samples. This result suggests that the isolates obtained in this study may possibly be atypical EPEC. The presence of non- EPEC serotypes that carry *eaeA* and lack *bfpA* and Shiga toxin (*stx*) gene sequences have been found in acute diarrhea (Vieira *et al.,* 2001). Brown and Hartland (2002) reported on the genotypic characteristics of *E. coli* strains of non-enteropathogenic *E. coli* (EPEC) serogroups that carry *eaeA* and lack the EPEC adherence factor and Shiga toxin genes. Reports from developed countries have shown that atypical EPEC strains (*eaeA*+, *bfpA*- and *stx*-) are more prevalent than typical EPEC strains (Paciorek, 2002; Trabulsi *et al.,* 1996). However, *eaeA* positive *E. coli* requires further study with regard to their virulence and epidemiologic significance. Sewage contamination of fish harvesting areas may be the major reason for the presence of *E. coli*, but contamination can occur through the use of non- potable water or ice in the landing centers or fish markets (Kumar *et al.,* 2005). The use of animal manures can result in the contamination by certain pathogenic types such as Shiga toxin-producing *E. coli* for which bovine reservoirs have been identified (Johannessen *et al.,* 2005). Therefore, it is important that all stages of fish production, handling and processing are monitored for *E. coli* contamination.

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