



## Study on the Prevalence of Virulent genes in *Escherichia coli* isolated from Fish using multiplex PCR

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Received date: 29<sup>th</sup> October 2012, Accepted date: 1<sup>ST</sup> November 2012, Published date: 1<sup>st</sup> March 2013

### Abstract:

A one shot multiplex Polymerase Chain Reaction PCR was developed for detecting 6 virulent genes of diarrheagenic *E.coli* from fish and shrimps. In order to differentiate between 5 categories of diarrheagenic *E.coli*, I selected the target genes *eae* for Enteropathogenic *E.coli* (EPEC), *ipaH* for Enteroinvasive *E.coli* (EIEC), *stx* for Enterohaemorrhagic *E.coli* (EHEC), *aggR* for Enteroaggregative *E.coli* (EAggEC), *elt* for Labile toxin producing Enterotoxigenic *E.coli*, *est* for Stable toxin producing Enterotoxigenic *E.coli* (ETEC). Using our multiplex PCR about 18 clinical isolated strains of *E.coli* in our Laboratory were successfully categorized and easily analyzed for the presence of virulence plasmids. Stable toxin producing ETEC was found to be prevalent among fish and shrimps as 12 cultures gave positive result for *est* gene, that is for stable toxin producing *E.coli*. Primer pairs used in the assay do not interfere with each other and generated amplification product of 147 bp. Then the targeted gene is also amplified in a single PCR reaction in one tube and detected by electrophoresis.

**Keywords:** Primers, PCR, EPEC, EIEC, EHEC, EAggEC and ETEC

### Introduction:

*Escherichia coli* is the most important etiologic agent of diarrhea and represents a major public health problem in developing countries. Identification of diarrheagenic *E.coli* strains requires that these organisms be differentiated from nonpathogenic members of the normal flora. Sero grouping of O-antigen is not sufficient to identify a strain as diarrheagenic, because it does not correlate, in most cases with the presence of virulence factors [1]. Thus identification of diarrheagenic *E.coli* strains needs to detect factors that determine the virulence of these organisms. With the advent of PCR, it has become possible to detect pathogenic genes in bacterial isolates, allowing the rapid diagnosis of diarrheagenic *E.coli* [2]. This study is attempted to develop a multiplex PCR for five pathogenic strains of *E.Coli*.

### Pathogenic strains of *E.Coli*:

**Enteropathogenic *E.coli*:** EPEC do not produce any enterotoxins or cytotoxins in the cells to invade, attach and efface. These bacteria induce a characteristic attaching and effacing lesion in epithelial cells in which microvilli are lost and the underlying cell membrane is raised to form a pedestal which can extend outward for up to 10  $\mu$ m. The genes encoding for this virulence are encoded on the locus of erythrocyte effacement (LEE), a secretion of a number of proteins including EspA and EspB which subsequently trigger a series of responses in the host cell [3]. EPEC presents with watery diarrhoea which can vary in its severity and duration.

**Enteroinvasive *E.coli*:** Enteroinvasive *Escherichia coli* (EIEC) was first shown in 1971 to cause diarrheal disease in otherwise healthy volunteers. It is known to cause shigellosis-like symptoms in both adults and children. EIEC strains cause a disease that is indistinguishable at the symptomatic level from the dysentery [4]. They actively invade colonic cells and spread laterally to adjacent cells.

**Enterohemorrhagic *E.coli*:** EHEC strains bind tightly to cultured mammalian cells and produce the same type of attachment effacement phenomenon as EPEC strains. Although EHEC strains cause dysentery similar to that caused by shigella species but they do not invade mucosal cells by the production of a toxin, Vero cytotoxic shiga like toxin. The clinical features of EHEC infection are most striking. Initial symptoms are of colicky abdominal pain followed by diarrhoea and, in about half of cases, vomiting.

### Enteroaggregative *E.coli*:

EAggEC that bind to small intestinal cells are not invasive and cause no obvious histological changes in the intestinal cells to which they adhere. They differ from ETEC strains primarily in that they do not adhere uniformly over the surface of the intestinal mucosa but tend to clump in small aggregates. EAggEC strains produce an ST like toxin (EAST) and a haemolysin like toxin (120 kDa in size). The

infection presents with watery mucoid diarrhoea with no fever or marked blood.

**Enterotoxigenic *E.coli*:** ETEC strains adhere to the small intestinal mucosa through colonization factor (CF) antigen and produce symptoms not by invading the mucosa but by producing toxins that act on mucosal cells to cause diarrhea [5]. These strains produce two types of enterotoxins .A cholera like toxin called heat-labile toxin (LT). A diarrheal toxin called heat-stable toxin (ST) (retain 100°C for 30 min) ST is a family of small toxins which can be divided into two groups .methanol soluble (STa) and methanol insoluble (STb). STa causes increase in cGMP levels in the host cell cytoplasm which leads to fluid loss. cGMP like cAMP is an important signaling molecule in eukaryotic cells and changes in cGMP affect a number of cellular processes including activities if ion pumps.LT-I and STa are encoded on plasmids.

**Survival of *Escherichia coli* in the intestine of fish**

An outbreak of diarrheal illness caused by ingestion of food contaminated with enterotoxigenic

*E. coli* was described in Japan [6]. The illness was strongly associated with eating tuna paste. Brazilian authors isolated 18 enterotoxigenic strains of *E. coli* (ETEC) from 3 of 24 samples of fresh fish originating from Brazilian markets; 13 of them produced a thermolabile enterotoxin [7].The authors explained the presence of toxic strains of *E. coli* in samples collected from fish (not from water) from one fish market by a longer survival of bacteria on an adequate substrate, i.e. inside the living organism. The isolation of 317 *E. coli* isolates tested for thermostable (ST) and thermolabile (LT) toxins has been described in another Brazilian study [8] only one produced ST and none produced LT toxin. Furthermore, although transfer of antibiotic resistance from a fish pathogen did take place in the environment of fish intestinal content, the likelihood of this being a high-risk route for acquisition of resistance by bacteria that can affect humans is low.

**Table 1: Virulent genes of pathogenic *E.coli* targeting in the *E.coli* multiplex PCR**

Primer	Sequence 5' to 3'	Target Gene	Amplicon size(bp)
SK1 SK2	CCCGAATTCGGCACAAGCATAAGC CCCGGATCCGTCTCGCCAGTATTCG	Eae	881 b.p
ipaIII ipaIV	GTTCCCTTGACCTTTCCGATACCGTC GCCGGTCAGCCACCCTCTGAGAGTAC	ipaH	619 b.p
VTcomu VTcomd	GAGCGAAATAATTTATATGTG TGATGATGGCAATTCAGTAT	stx	518 b.p
LT L LT R	TCTCTATGTGCATACGGAGC CCATACTGATTGCCGCAAT	Elt	322 b.p
AL65 AL125	TTAATAGCACCCGGTACAAGCAGG CCTGACTCTTCAAAAAGAGAAAATTAC	est	147 b.p
aggRKS1 aggRKS2	GTATACACAAAAGAAGGAAGC GTTCCCTTGACCTTTCCGATACCGTC	aggR	254 b.p

**Materials and Methods:**

**Isolation of DNA from *E.coli* cells:**

Reagent: 1 x TE ( Tris , EDTA) buffer Culture : 18 *E.coli* cultures ( 17 laboratory cultures from the samples freshwater fish [Labeo rohita (rohu) ,Colossoma macropomum ( pacu) ] and marine water [Rastrelliger kanagurta (kaanagartha) *Penaeus indicus* (white Shrimp) ] and one type culture that is *E.Coli* 2622 .18 cultures were inoculated in LB broth and incubated at 37°C for 24 hours. 1ml of broth was taken in eppendoff tubes and Centrifuged at 10000 rpm for 5min. then supernatant was discarded the pellets containing cultures were stored. Then the

pellets were resuspended in 200µl of TE buffer and Incubated at 95°C in water bath for 5min.

**Preparation of Master Mix for PCR:**

The components of Master mix are: DNA template that contains the region of the DNA fragment to be amplified,Primers , PCR mix(100ul Taqpolymerase ,200UmDeoxynucleotide triphosphates (dNTPs),1XBuffersolution, Divalent cation 1.5mM Mg or Mn ions andMonovalent cation - 500 mM potassium ions. Master mix reagents can be obtained from the companies.

**PCR Procedure:**

About 0.125 μM concentration of SK1, SK2, ipaIII and ipaIV primers, 0.25 μM concentration of VTcom-u, VTcom-d, LT-L, LT-R, agg 1 and agg 2 primers and 0.5 μM concentration of AL 65 and AL 125 were added into each PCR vial. Then 30 μl of PCR mix that contains 6mM Tris HCL (pH 8.3); 30mM KCL; 0.06% Triton X-100; 0.9mM Mgcl2; 1.5 U of Taq DNA polymerase; 0.12 deoxynucleoside triphosphate into each vial. Finally add 5 μl of template DNA of different cultures separately into the vials. The PCR program was 95 °C for 1 min, 52 °C for 1 min and 72 °C for 1 min for 30 cycles and 72 °C for 10 min. The primers would lead to give respective size of amplicons shown in Table 1

Some of the cultures, after running the multiplex PCR were followed by performing Single PCR for conformation. For single PCR 2.5 μl of template and 18 μl of PCR mix was added in to each vial. A single primer of AL65 (1.25 μl) and AL125 (1.25 μl), LTL (0.6 μl) and LTR (1.25 μl), RKS1 (0.6 μl) and RKS2 (0.6 μl) were added separately into each vial and then subjected to single PCR.

**Agarose Gel Electrophoresis**

**Reagents:** 1. 2.5% of Agarose in 1x TAE Buffer  
Composition of 10X Tris Acetate EDTA (TAE)- 48.4g of Trisbase, 11.4 mL of glacial acetic acid (17.4 M), 3.7 g of EDTA, disodium salt in 1 Litre deionized water.

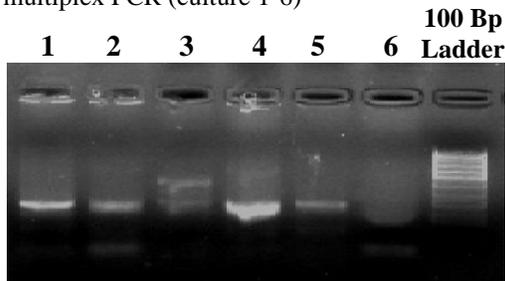
2. Ethidium Bromide
3. Amplified DNA
4. Loading Dye:

Composition of loading dye: 1litre of deionised water- 0.25g of bromophenol blue 0.25g of xylene cyanol, 50.00g of sucrose, 1ml of 1M Tris

**Preparation and Casting of 2.5% Agarose Gel**

About 2.5% Agarose in 20 ml of TAE buffer was prepared and boiled for dissolving. After boiling, the solution was slightly cooled and 2 μl of Ethidium bromide (1 μg/μl) was added carefully. PCR products along with loading gel were then electrophorised and visualized by Gel doc image system.

Figure 1: Agarose gel electrophoresis of products from multiplex PCR (culture 1-6)



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Figure 2: Agarose gel electrophoresis of products from multiplex PCR (culture 7-12)

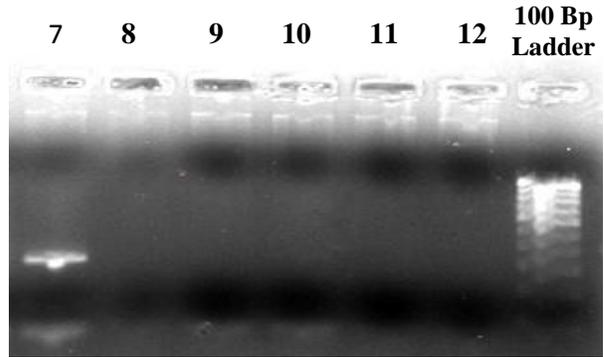
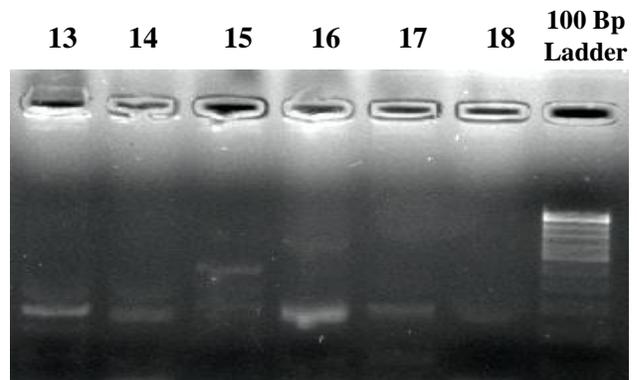


Figure 3: Agarose gel electrophoresis of products from multiplex PCR (culture 13-18)



**Single PCR**

1	3	3	16	16	16	3	100
RKS	AL	RK	AL	LTR,	RKS	LT	b.p
1,	65,	S1,	65,	LTL	1,	R,	ladder
RKS	AL	RK	AL		RKS	LT	
2	125	S2	125		2	L	

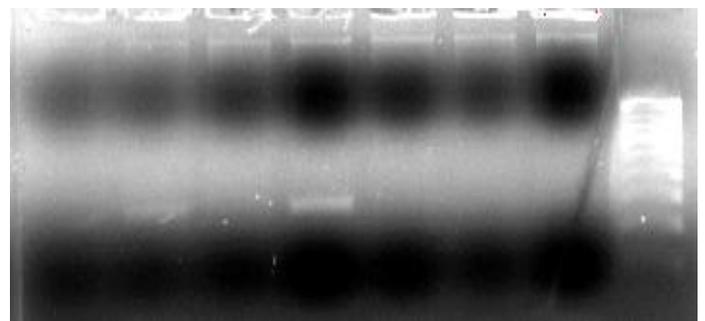


Figure 4: Agarose Gel Electrophoresis of selected cultures from single PCR

**Result and Discussion:**

From the Figures 1, 2, 3 and 4 it can be inferred that among 18 cultures ,12 cultures gave positive result for est gene ,that is for stable toxin producing E.coli and 6 cultures are negative for all the virulent genes.Among 8 cultures isolated from freshwater fishes , 3 had shown positive result for est gene and among 9 cultures from marine water fishes 7 had produced stable toxin .Suprisingly , Type culture has also shown positive result for est gene.Therefore it is found that stable toxin is more prevalent among fishes through Multiplex PCR.

**Conclusion:**

When the DNA isolated from those pure cultures are subjected to multiplex PCR by using different primers respected to different virulent genes of E.coli, it is found that 66 % of the cultures had produced stable toxin and there was a 100% positive result from kaanagarta and 60% positive result in the case of shrimps. From the results, it is opined that mere presence of *E.coli* in fish should not be considered as biological hazard but the presence of virulence genes in the *E.coli* should be considered as health hazard.

**Acknowledgment:**

I express my profound sense of gratitude to Dr. Madhusudana Rao - Chief Scientist, Dr.M.M .Prasad Director and Mr. Panda – Technical Assistant of Central Institute of Fisheries Technology for giving me the opportunity to work under their guidance.

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