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Detection of Shiga-toxigenic *Escherichia coli* (STEC) in fresh seafood and meat marketed in Mangalore, India by PCR

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Aims: To study the incidence of Shiga-toxigenic *Escherichia coli* (STEC) in seafoods from India.

Methods and Results: *Escherichia coli* isolated from various seafoods such as fresh fish, clams and water were screened for the presence of *stx*, *hly*A and rfb_{O157} genes by PCR; 5% of clams and 3% of fresh fish samples were positive for non-O157 STEC.

Conclusions: STEC is prevalent in seafoods in India, and non-O157 serotype is more common.

Significance and Impact of the Study: Seafood could be a vehicle for transmission of STEC even in tropical countries.

INTRODUCTION

Shiga-toxigenic *Escherichia coli* (STEC) are increasingly being recognized as the causative agents of serious gastrointestinal diseases in humans, and have been responsible for several foodborne outbreaks (Karmali 1989; Ostroff *et al*. 1990). Infections due to STEC can result in severe bloody diarrhoea (haemorrhagic colitis, HC) and in life-threatening complications such as haemolytic–uraemic syndrome (HUS) (Tarr 1995). Food-borne outbreaks associated with STEC have been well documented worldwide. Although *E. coli* O157:H7 is currently the most common serotype in many parts of the world (Armstrong *et al*. 1996), serotypes O5, O26, O91, O111 and O113 are considered equally important in causing haemorrhagic colitis (Bettelheim 1996). Occurrence of STEC has been reported in a number of food products such as beef, pork, lamb, poultry and fish (Doyle and Schoeni 1987; Samadpour *et al*. 1994). However, most reported outbreaks of foodborne illness have been from developed countries (Griffin and Tauxe 1991). It is not known whether this situation is due to under-reporting in developing countries or to the relatively infrequent incidence of such organisms in foods in these countries. Radu *et al*. (1998) detected *E. coli* O157:H7 in beef marketed in Malaysia. Isolation of *E. coli* belonging to O157 and other O serogroups that exhibit cytotoxic activity in vero cells have been reported from patients with diarrhoea in India (Gupta *et al*. 1992) but the source of the strains is not known. In addition to the presence of one or more types of Shiga-toxin genes, STEC *E. coli* serotypes may also possess additional putative virulence factors such as a 60 MDa plasmid encoding haemolysin (Schmidt *et al*. 1994), and intimin, a putative adherence factor encoded by *eae*A (Louie *et al*. 1993). Detection of STEC *E. coli* in foods by PCR amplification of virulence-associated genes has been reported by a number of workers (Paton and Paton 1998). Further, the *rfb* genes encoding the O antigen have been used for the detection of O157 serotype (Desmarchelier *et al*. 1998).

In this study, the occurrence of Shiga toxin-producing *E. coli* was investigated in fresh fish, shellfish and meat sold in open markets in Mangalore, India, as the information regarding the occurrence of such *E. coli* in seafood and meat is totally lacking from this part of the world and the threat these organisms pose to public health is largely unknown.

MATERIALS AND METHODS

Sample collection and isolation of *E. coli*

Samples of fish, shrimp, clams and water were collected from various fish markets in Mangalore, India. Samples were placed on ice and transported immediately to the laboratory. Briefly, 25 g of the food sample were homogenized in 225 ml modified EC broth containing

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novobiocin (20 mg l^{-1}, Hi-Media) and then incubated statically for 6 h at 37°C. Enrichment of the first few samples was carried out for 6, 12 and 24 h. However, as more typical colonies were obtained at 6 h enrichment, this duration was used for subsequent samples. The culture was diluted in sterile tryptone water (1% tryptone, 0·5% sodium chloride) and spread onto sorbitol MacConkey agar (Hi-media). About 50–75 colonies were selected from plates yielding well separated colonies and subjected to standard biochemical tests for the identification of *E. coli*. Both sorbitol-fermenting and non-sorbitol-fermenting colonies were selected. An aliquot of the enrichment broth was centrifuged; the DNA was extracted as previously described (Sambrook *et al*. 1989) and used as the template for direct PCR. *Escherichia coli* EDL 933 was used as positive control.

PCR primers and reaction conditions

For PCR, primers were selected based on previously published information for *stx*1 and *stx*2, *hly*A and rfb_{O157} genes (Table 1). For RAPD, the following primers were used: 5′ GCG ATC CCA A 3′, 5′ CCG CAG CCA A 3′, 5′ AAC GCG CAA C 3′ and 5′ GTG GAT GCG A 3′ (Neilan 1995). All *E. coli* isolates tested in this study were grown in 3 ml Luria Bertani broth for 18 h and centrifuged at 10 000 *g* for 10 min. The resultant pellet was resuspended in 500 μl sterile distilled water and lysed by boiling for 10 min in a dry bath. Each lysate (3 μl) was amplified in a 50 μl reaction mixture containing 1 × PCR buffer, 200 μmol l^{-1} deoxynucleotide triphosphate mix, 0·5 μmol l^{-1} of each primer and 2·5 U taq polymerase (Bangalore Genei). PCR was performed individually using each primer pair according to previously described protocols (Karch and Meyer 1989; Desmarchelier *et al*. 1998; Fratamico *et al*. 1995). The products were separated on a 1·6% agarose gel, stained with ethidium bromide (0·5 μg ml^{-1}) and photographed using a gel documentation system (Herolab, Weisloch, Germany). For RAPD, the reaction conditions were as described by Neilan (1995). The isolates were tested for agglutination with O157 antiserum (Difco).

RESULTS AND DISCUSSION

As shown in Table 2, two of the 60 fish samples and two of the 48 clam samples were positive for *stx* and *hly*A genes by PCR. Interestingly, none of the samples was positive by PCR for rfb_{O157}. These results demonstrate the presence of STEC in seafoods of serotypes other than that of O157. All but one of the *stx*-positive isolates were sorbitol fermenters. In using the conventional method for the study of STEC, sorbitol-containing medium is used and generally, only colonies of sorbitol non-fermenters are picked up for further testing. The results of this study suggest that sorbitol-fermenting STEC may be prevalent in a significant number of samples in India, and this may be missed by conventional methods. Interestingly, both *stx*-positive strains from fish were positive for the *hly*A gene. However, one isolate each from the water and clam samples was positive for the presence of the EHEC-*hly*A gene but did not harbour the *stx* gene (Fig. 1a,b). It is generally assumed that the *hly*A gene encoded by a 60 MDa plasmid is an important virulence factor associated with Stx-producing *E. coli* (Boerlin *et al*. 1998). Although Fratamico *et al*. (1995) reported that PCR primers targeting the *hly*A gene are suitable for detection of STEC belonging to serogroup O157, later studies indicated that these primers recognize the EHEC *hly*A gene, which is not confined to the O157 serotype, and is therefore suitable for detection of all STEC (Paton and Paton 1998). Schmidt *et al*. (1995) surveyed diarrhoeagenic *E. coli* for the presence of EHEC *hly*A by PCR and noted the presence of this gene in all STEC O157:H7 strains and 50% of STEC of other

Table 2 Incidence of *Escherichia coli* virulence genes in different food samples detected by PCR

		Virulence genes detected by PCR		
Sample type	No. positive/tested	*Stx*	*hlyA*	rfb_{O157}
Fish	2/60	+	+	–
Clams	3/48	+ (2/3)	+	–
Water	1/32	–	+	–
Beef	2/2	+	+	+
Mutton	2/2	+	+	+

Table 1 Primers used for PCR in the present study

Gene	Primer sequence (5′ to 3′)	Product size (bp)	Reference
stx1 and *stx2*	TTTACGATAGACTTCTCGAC CACATATAAATTATTCGCTC	224	Karch and Meyer 1989
EHEC *hlyA*	ACGATGTGGTTTATTCTGGA CTTCACGTGACCATACATAT	166	Fratamico *et al*. 1995
rfb_{O157}	AAGATTGCGCTGAAGCCTTTG CATTGGCATCGTGTGGAC	479	Desmarhelier *et al*. 1998

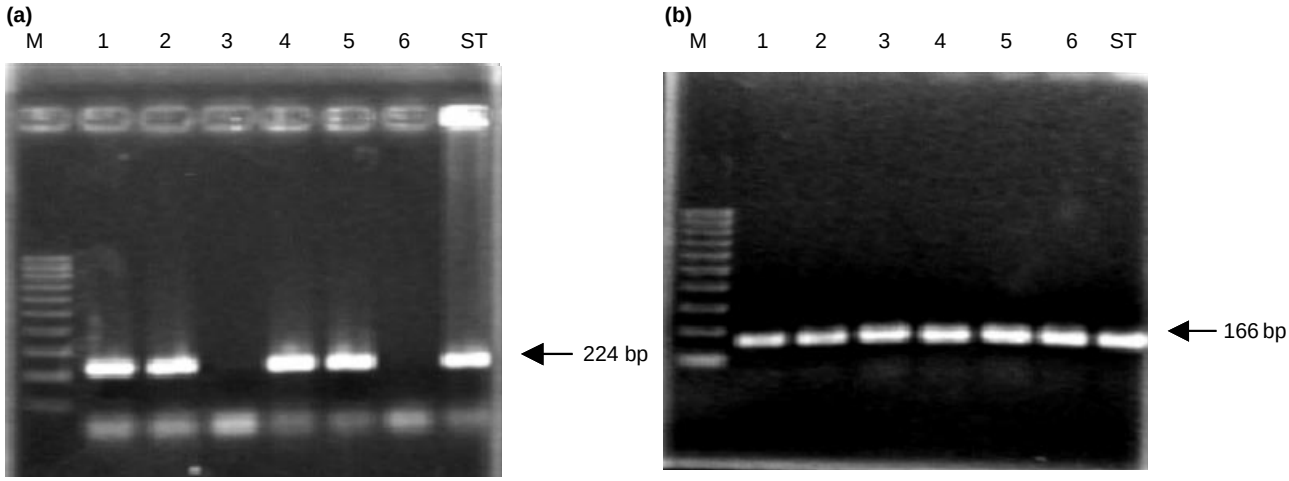

Fig. 1 Agarose gel electrophoresis of (a) *stx* and (b) *hylA* PCR products. Lane M: 100 bp molecular size marker; lanes 1, 2 and 3: isolates from clam; lanes 4 and 5: isolates from fish; lane 6: isolate from water; lane ST: EDL 933 (reference strain)

serotypes. However, all non-STEC diarrhoeagenic strains were negative for EHEC *hly*A. Thus, although some STEC strains may lack the *hly*A gene, its presence would be a good marker for STEC. In this study, the strains positive for *hly*A were negative for PCR with rfb_{O157} primers, which suggests that they belonged to serotypes other than O157. Also, they did not agglutinate with O157 anti-serum. The absence of the *stx* gene in these strains is interesting and could be explained by the loss of the bacteriophage coding this gene. It is accepted that bacteriophages are involved in carrying genes encoding Shiga toxins (Scotland *et al*. 1983; O'Brien *et al*. 1984). Therefore, the presence of *E. coli* harbouring the EHEC-*hly*A gene is significant, since there is increasing evidence that this haemolysin gene may be the marker for Shiga toxin-producing *E. coli* (Gyles *et al*. 1998), and this gene is absent in non-STEC diarrhoeagenic *E. coli* (Schmidt *et al*. 1995).

The results in Table 2 show that STEC are present in beef and mutton in India. Although the culture supernatant fluids were positive for the presence of the rfb_{O157} gene (Fig. 2), no isolations of *E. coli* containing this gene could be made. Considering the high sensitivity of PCR, it is suggested that a significant proportion of PCR-positive samples may not yield isolates (Paton and Paton 1998). This may happen when a small proportion of STEC are present with a large population of non-STEC. The results of this study suggest that PCR will be helpful in detecting samples harbouring small numbers of STEC which may be missed by traditional culture-based methods. Since cattle are considered to be reservoirs of STEC (Borczyk *et al*. 1987; Chapman *et al*. 1993; Fagan *et al*. 1999), these results assume significance in that, even in India, cattle and other animals could be reservoirs of STEC. Interestingly, although beef and mutton samples were positive for the presence of the gene rfb_{O157} of *E. coli* O157, in the case of fish and shellfish, the rfb_{O157} gene could not be detected, and the strains isolated from fish and shellfish belonged to serotypes other than O157. This further suggests that the source of contamination of sea foods might be different and the reservoirs of such *stx*-producing *E. coli* need to be identified. Further, the RAPD patterns of different strains were different (Fig. 3), suggesting that contamination of fish and shellfish by STEC is derived from diverse sources which are being further investigated. Although the RAPD pattern was studied using four primers, the results using one primer which gave consistently reproducible patterns with all strains is presented in Fig. 3. This primer may be useful for typing tropical isolates of STEC.

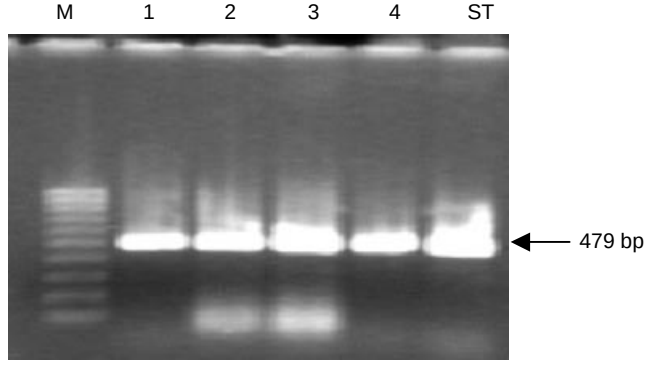

Fig. 2 Agarose gel electrophoresis of rfb_{0157} PCR products from enrichment broths. Lane M: 100 bp molecular size marker; lanes 1 and 2: beef samples; lanes 3 and 4: mutton samples; lane ST: EDL 933 (reference strain)

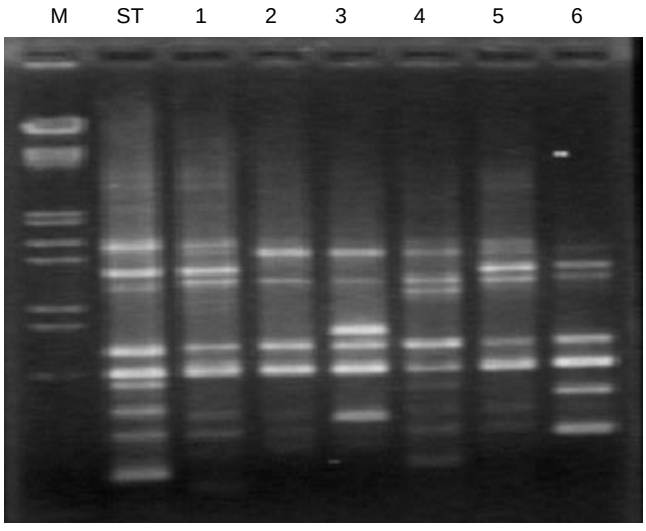

Fig. 3 RAPD profiles of different isolates used in this study. Lane M: λ DNA/*Eco* RI *Hind* III double digest molecular size marker; lane ST: EDL 933 (reference strain); lanes 1, 2 and 3: isolates from clam; lanes 4 and 5: isolates from fish; lane 6: isolate from water

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