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Simultaneous detection of six major virulent genes of *Escherichia coli* O157:H7 from seafood by multiplex PCR and its validation through spiked study

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Abstract

Enterohaemorrhagic *Escherichia coli* (EHEC), mainly the serotype O157:H7 is a global concern in foodborne outbreaks. The incidence of *E. coli* O157:H7 in seafood sold in India has been reported as 8.4%. The conventional method for the detection of *E. coli* O157:H7 from seafood is cumbersome and time consuming making this strain particularly challenging to detect. In this study a multiplex PCR was developed targeting six major virulence factors of the pathogen *viz.* shigatoxin 1 and 2, attachment and effacement factor, flagellar antigen, hemolysin gene and O157 antigen. The sensitivity of the multiplex PCR developed in the study was found to be 120 pg μl^{-1} of DNA and that of *E. coli* 0157:H7 spiked samples was 2 cfu ml^{-1}. The multiplex PCR developed in this study showed 100% specificity in identifying *E. coli* O157:H7, effectively distinguishing it from non-target bacteria. This highly sensitive and specific multiplex PCR is the first of its kind to detect *E. coli* O157:H7 in seafood by targeting six major virulence genes.

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Introduction

Enterohaemorrhagic *Escherichia coli* (EHEC), one of the pathotypes of Diarrheagenic *E. coli* (DEC), is a major food-borne pathogen with high mortality rate causing severe gastrointestinal diseases, haemorrhagic colitis and haemolytic uremic syndrome (HUS). *E. coli* O157:H7, O26, O91, O103, O104, 0111, O113, O117, O118, O121, O128, O145 and non-flagellated O157:H- are the serogroups reported till now in EHEC, among them *E. coli* O157:H7 is regarded as one of the deadliest strains due to its high epidemiological impact and mortality rate. A multistate outbreak of *E. coli* O157:H7 occurred in United States in 2019, linked to romaine lettuce with 210 reported cases (5 mortality) and a similar major outbreak occurred in 2018 with 209 case reports (CDCP, 2018). There are several reports on the occurrence of *E. coli* O157:H7 in diarrheic cattle faeces (Omisakin *et al*., 2003; Hussein and Bollinger, 2005; Atnafie *et al*., 2017), human stool (Bielaszewska *et al*., 1997; Mead and Griffin, 1998; Anu *et al*., 2023), vegetables (Lin *et al*., 1996; Delaquis *et al*., 2007), raw beef and beef products (Chapman *et al.*, 2000; Chapman *et al*., 2001; Cagney, 2004) and poultry birds (Heuvelink *et al*., 1999; Best *et al*., 2003). With hospital and domestic sewage being discharged into the major water sources, fish and shellfish harvested from these contaminated waters may harbour *E. coli* O157:H7, potentially acting as vehicles of transmission to humans. Sehgal *et al*. (2008) reported the prevalence of *E. coli* O157 in seafood as 8.4% from India during 1996 to 2005 and Vaiyapuri (2016) reported 0.8% incidence of shigatoxin (*stx1*) producing *E. coli* in seafood from retail markets in Cochin, Kerala. Prakashan *et al*. (2018) reported incidence of shigatoxigenic *E. coli* (STEC) in seafood marketed in Mumbai, India. A study by PCR based detection of shigatoxin gene in STEC isolates from seafood showed 16.6 and 33.33% in fish and shellfish sold in Mumbai, India, respectively (Prakasan *et al*., 2018).

Reports on *E. coli* O157:H7 mediated food borne disease outbreaks in developing countries like India, has been very limited due to the unidentified origin of diarrhea in the country or the lack of systematic study to find the etiologic agent behind the scene (Bender *et al*., 2004). Detection of *E. coli* O157:H7 is cumbersome as it involves enrichment, plating on selective media and serological confirmation. Even though there are several rapid serological techniques available for the detection of *E. coli* O157:H7, these methods may lead to false-positive results as antisera of O157:H7cross-reacts with *Citrobacter freundii*, *Escherichia hermanii* and other bacteria (Nataro and Kaper, 1998). Thus, DNA based detection methods are more reliable, specific and sensitive than serological test in detecting the presence of this pathogen from various food products. Multiplex PCR is the most sensitive method, as it enables the detection of multiple targets in a single reaction. Even though various multiplex PCR methods have been developed by previous researchers for the detection of *E. coli* O157:H7, from pork, chicken, vegetables (Nguyen *et al*., 2016; Tegegne *et al.,* 2024), egg, cheese, milk products (Kawasaki *et al*., 2009), there is no specific multiplex PCR technique developed with high specificity for the detection of *E. coli* O157:H7 from seafood. Hence this study made an attempt to develop a sensitive and specific multiplex PCR to detect the food borne pathogen, *E. coli* O157:H7 from seafood, targeting the six major virulence factors *viz.* shigatoxin (*stx1* and *stx2*), attachment and effacement factor (*eaeA*), flagellar antigen (*fliC*), haemolysin gene (*hlyA*) and O157 antigen (*rfbE* O157) which are specific for *E. coli* O157:H7.

Materials and methods

Bacterial strain and culture conditions

E. coli O157:H7 (ATCC 43895) standard strain, procured from ICAR-Central Institute Of Fisheries Education (ICAR-CIFE), Mumbai, India, was grown overnight at 37°C in tripticase soy broth (TSB) and purified by streaking on Sorbitol MacConkey agar supplemented with cefixime and tellurite (CT-SMAC). Various seafood pathogens *viz. Listeria monocytogenes* (ATCC 19111), *Vibrio parahaemolyticus* (MTCC-451) and *Edwardsiella tarda* (MTCC-2400) and fish spoilage bacteria *Pseudomonas aeruginosa* (MTCC-1688) were procured from American Type Culture Collection (ATCC), United States and Microbial Type Culture Collection (MTCC), Chandigarh, India.

DNA template preparation

Genetic material (DNA) of *E coli* O157:H7 was extracted by phenol-chlorofom-cetyl trimethyl ammonium bromide (CTAB) method according to Sambrook *et al*. (1989). After the extraction, the DNA was suspended in 1x TE buffer (pH 8.0) stored at -20°C till further use and the concentration of the extracted DNA was measured using Nanodrop (Thermoscientific, USA).

Primer sequences

Six sets of primers specific for virulent genes of *E. coli* O157:H7 *viz. stx1, stx2, eaeA, fliC, hlyA* and *rfbEO157* (Integrated DNA Technologies-IDT, USA) were used in this study and the details are given in Table 1. Primer sequences were evaluated for self-priming or self-complementary, non-specific targets and also analysed its specificity for each target gene sequences under study using multiple sequence alignment tool (Corpet, 1988).

Standardisation of uniplex PCR amplification

The uniplex PCR reaction for 6 virulence genes of *E. coli* O157:H7 was carried in a 30 µl reaction mixture containing 3 µl 10x *Taq* buffer (100 mM Tris-HCl, 500 mM KCl, 2 mM $MgCl_2$), 2.5 mM each dNTPs, 1 µl of each primer at 10 pmol μl^{-1} concentration, 1 U of *Taq* DNA polymerase (Himedia, India) and 1 µl of DNA template. The cycling protocol consisted of an initial denaturation at 96°C for 2 min followed by 35 cycles of denaturation at 96°C for 30 s, annealing at 55°C for 20 s, elongation at 72°C for 30 s, and a final extension at 72°C for 7 min (Thermal Cycler, Bio Rad, USA).

Standardisation of Multiplex PCR amplification

After a series of tests, the following optimal conditions for the six gene multiplex PCR procedure was established with a reaction volume of 30 µl consisting of 8 µl 10x *Taq* buffer (100 mM Tris-HCl, 500 mM KCl, 2 mM $MgCl_2$), 2.5 mM each dNTPs, 1 µl of each primer at 10 pmol μl^{-1} concentration, 2 U of *Taq* DNA polymerase and 2 µl of DNA template. Amplification protocol of initial denaturation at 96°C for 5 min followed by 35 cycles of denaturation at 96°C for 30 s, annealing at 55°C for 30 s, elongation at 72°C for 30 s, followed by a final extension at 72°C for 10 min (Thermal Cycler, Bio Rad, USA).

Table 1. Primer sequences for the detection of six virulent factors of *E. coli* O157:H7 with amplicon size.

Target gene	Primer sequence	Amplicon size (bp)	Reference
fliC	F: AGCTGCAACGGTAAGTGATTT R: GGCAGCAAGCGGGTTGGTC	949	Wang *et al*. (2000)
stx_1	F: TGTCGCATAGTGGAACCTCA R: TGCGCACTGAGAAGAAGAGA	655	Bai *et al*. (2010)
hlyA	F: GCATCATCAAGCGTACGTTCC R: AATGAGCCAAGCTGGTTAAGCT	534	Paton and Paton (1998)
stx_2	F: CCATGACAACGGACAGCGTT R: TGTCGCCAGTTATCTGACATTC	477	Fagan *et al.* (1999)
eaeA	F: CATTATGGAACGGCAGAGGT R: ACGGATATCGAAGCCATTTG	375	Bai *et al*. (2010)
$rfbE_{0157}$	F: CAGGTGAAGGTGGAATGGTTGTC R: TTAGAATTGAGACCATCCAATAAG	296	Bertrand and Roig (2007)

Evaluation of specificity and sensitivity of the multiplex PCR

The multiplex PCR protocol developed was verified for its specificity to detect the virulence genes *viz.* (*fliC*), *stx1* and *stx2*, *eae*A, *rfbE* O157 and *hlyA*, in *E. coli* O157:H7. To check the efficiency of the protocol, it was subjected to amplify non-target bacterial pathogens including, *L. monocytogenes*, *V. parahaemolyticus* and *E. iella tarda* and fish spoilage bacteria *viz*. *P. aeruginosa*, along with the targeted one. To archive the specificity of the assay, a mixture of DNA of both targeted and non-targeted pathogens were also used as template and subjected to multiplex PCR protocol developed in this study.

The sensitivity of the multiplex PCR protocol was evaluated as per the method of Xu *et al*. (2012). The bacterial DNA at a concentration of 120 ng μl^{-1} was serially diluted at 10 fold dilutions upto 10^{-5} and was used as template in multiplex PCR.

Detection of sensitivity of multiplex PCR using spiked sample

Ten seafood samples including sardine (2), mackerel (2), shrimp (4) and clams (2) procured from retail markets in Kochi, were placed in aseptic bags with ice and immediately transported to the laboratory for use within 2 h. To assure the absence of natural contamination of *E. coli* O157:H7 in seafood samples, 25 g of each sample were homogenised in a smasher and filtered through 5 µm micropore filter before DNA isolation (Kawasaki *et al.,* 2009). 2 ml of the filtrate was used for DNA extraction, using C-TAB method (Sambrook *et al*., 1989). The presence of *E. coli* O157:H7 was evaluated by multiplex PCR developed in this study (Hussain *et al*., 2014). Seafood samples without any natural contamination of *E. coli* O157:H7 was selected for spike study and it was pasteurised at 100°C for 180 s. After pasteurisation, the fish was divided to 4 groups (25 g each), and it was artificially inoculated with 10-fold dilution (10^{-4} to 10^{-7}) of overnight culture of *E. coli* O157:H7. The initial count of bacteria in the artificially inoculated fish was enumerated as 310, 36, 2 and 0 cfu ml^{-1}, with 10-fold dilution of overnight broth culture of *E. coli* O157:H7. For the artificial inoculation of *E. coli* O157:H7, 100 µl of each dilution were mixed with 25 g of seafood sample and 225 ml of LB broth was added. It was pummeled in a smasher and incubated at 37°C for 12 h in a shaking incubator. After the incubation period, the filtrate was used for the extraction of genomic DNA and then the sensitivity of the protocol developed was analysed by multiplex PCR.

Results and discussion

Standardisation of uniplex and multiplex PCR protocols

Initially, the primer pairs targeting major virulent factors specific for *E. coli* O157:H7 *viz.* shigatoxin (stx_1 and stx_2), flagellar antigen (*fliC*), attachment and effacement factor (*eaeA*), hemolysin gene (*hlyA*) and O157 antigen (*rfbEO157*) was adopted from previous researchers (Paton and Paton, 1998; Wang *et al*., 2000; Bertr and and Roig, 2007; Bai *et al*., (2010);) and it is represented in Table 1. After the selection of primer pairs for each targeted gene, its specificity towards the targeted genes was analysed by uniplex PCR. For this, master mix and uniplex PCR protocol were optimised by a series of trials (Fig. 1, Lanes 2-7). After the successful amplification of six virulence genes, all six primer pairs were simultaneously added to the reaction mixture and PCR was performed. Only some of the genes were amplified after the PCR. After a series of tests, master mix for multiplex PCR (concentration of Mg^{2+}, dNTP, primers and *Taq* polymerase) and PCR protocol (number of cycles, time and duration of initial denaturation, annealing and final extension) were optimised as mentioned under standardisation of multiplex PCR (Materials and methods). Thus all the six amplified products (multiplex PCR) were resolved successfully in 2% agarose gel electrophoresis in its specific product size (Fig. 1, Lane 1). Hence, the standardised mastermix and multiplex PCR protocol can be utilised for detecting contamination of *E. coli* O157:H7 in seafood.

Specificity of multiplex PCR

The multiplex PCR protocol developed in this study successfully ampliflied all the six targeted genes in *E. coli* O157:H7. The six primer pairs could specifically bind to the targeted sites without any cross reactions against non-target fish pathogens and fish spoilage bacteria used in this study (Fig. 2). The multiplex PCR protocol developed in this study followed the basic rules of inclusivity and exclusivity, thus the protocol had an excellent specificity to amplify the targeted virulent factors of *E. coli* O157:H7 and thereby successfully detected the pathogen among other bacterial pathogens and normal flora of fresh seafood sample. Specificity study of the multiplex PCR developed by Nguyen *et al*. (2016) for the detection of three food-borne pathogens supports this study as they have analysed specificity of their protocol with non-targeted food-borne pathogens. Previous study by Bai *et al*. (2010), failed to

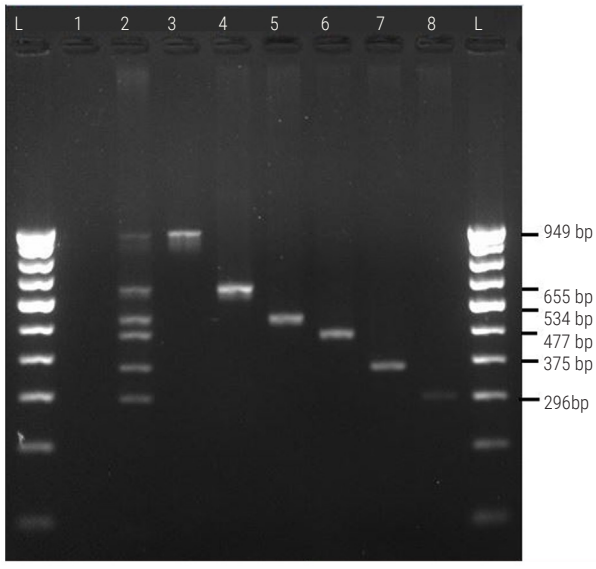

Fig. 1. Agarose gel showing uniplex and multiplex PCR amplified products. L- 100 bp DNA ladder, NC- Negative control, 1- Multiplex PCR amplified product, 2- Flagellar antigen (*fliC*), 3- Shigatoxin 1 (*stx1*), 4- Hemolysin (*hlyA*), shigatoxin 2 (*stx2*), attachment and effacement factor (*eaeA*) and O157 antigen (*rfbE*O157)

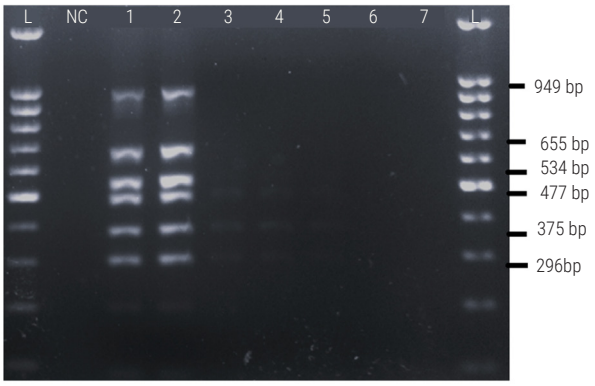

Fig. 2. Gel image showing specificity of multiplex PCR developed against targeted pathogen over non-targeted pathogen. L- 100 bp ladder, NC- Negative control, 1- Multiplex PCR using mixture of DNA of both targeted and non-targeted pathogen as template, 2-Multiplex PCR using DNA of *E. coli* O157:H7 as template, 3-6 showing negative result for multiplex PCR using DNA of non-targeted pathogen *Listeria monocytogenes*, *Vibrio parahemolyticus*, *Edwardsiella tarda* and *Pseudomonas aeruginosa*, respectively

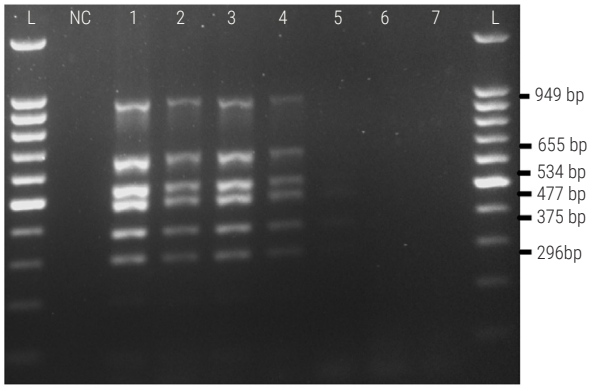

Fig. 3. Gel image showing sensitivity of multiplex PCR using various dilutions of DNA as template. L- 100 bp DNA ladder, NC- Negative control, 1- 6- Multiplex PCR using 120 ng μl^{-1}, 12 ng μl^{-1}, 1.2 ng μl^{-1}, 120 pg μl^{-1}, 12 pg μl^{-1} and 1.2 pg μl^{-1} of DNA as template

report the specificity of the multiplex PCR developed in their study to detect the presence of *E. coli* O157:H7 targeting six virulent factors in cattle faeces. Thus the specificity of standardised protocol and master mix developed in this study enhances its suitability for the routine monitoring of *E. coli* O157:H7 in seafood.

Sensitivity of multiplex PCR

For analysing the detection sensitivity of the multiplex PCR developed in this study, the extracted DNA with an initial concentration of 120 ng μl^{-1} was serially diluted upto 10^{-5} so as to get the concentrations as 12 ng μl^{-1}, 1.2 ng μl^{-1}, 120 pg μl^{-1}, 12 pg μl^{-1}, 1.2 pg μl^{-1}. After performing the PCR using various dilutions of DNA as template, it was found that the protocol successfully amplified 120 pg μl^{-1} concentration of DNA and also it resolved in gel electrophoresis (Fig. 3). There was a qualitative decrease in the resolution of amplicons in the agarose gel with the decrease in the DNA concentration, down till 120 pg μl^{-1} concentration of DNA, in which all the six targeted genes were detected simultaneously. Two independent duplicate assay of multiplex PCR showed highly reliable results. In a study by Giau *et al*. (2015) had developed multiplex PCR targeting 2 virulent genes of *E. coli* O157:H7 from different types of food, but failed to evaluate the sensitivity of the same. Thus, the multiplex PCR developed in this study can be effectively used for the detection of *E. coli* O157:H7 in seafood. High sensitivity of the PCR ensures accurate detection of the pathogen at a lower concentration of 120 pg μl^{-1}.

Detection of *E. coli* O157:H7 in spiked samples

Among the 10 seafood samples, there was no natural contamination of *E. coli* O157:H7 and the samples in which the target pathogen was spiked detected successfully (Fig. 3). Both finfish and shellfish were included in the study and the protocol was found to be most sensitive and specific for the detection of targeted pathogen. Till date, there are reports for spiked study in food for the detection of *E. coli* O157:H7 (Bai *et al*. 2010; Hu *et al*., 1990), but there are no reports for the same in seafood. To assess the ability of the protocol to detect the pathogen in seafood sample within short time period, the spiked samples were enriched only for 12 h and within that time, the protocol was able to detect even the minimum load of target successfully. Thus the multiplex PCR found to have 100% sensitivity and specificity in detecting *E. coli* O157:H7 in seafood.

Detection sensitivity of multiplex PCR in spiked seafood samples

The infectious dose of *E. coli* O157:H7 ranges from <100 or even <10 cfu g^{-1} to cause an acute diarrhea, thus a highly sensitive technique is required for the detection of the pathogen. On spiked sample study, the multiplex PCR developed detected *E. coli* O157:H7 at all different inoculation levels, down to 2 cfu ml^{-1} of seafood sample (Fig. 4), thus the protocol detected the pathogen even at very low numbers. This protocol is found to be more sensitive than already developed protocols for the detection of *E. coli* O157:H7 from various food samples, *viz.* sensitivity of 10 cfu of *E. coli* O157:H7 in a study targeting plasmid DNA, SLT-1, SLT-2 and *eaeA* gene simultaneously (Fratamico *et al*., 1995) and sensitivity of 6 cfu for the detection of *E. coli* O157:H7 (H7, SLT-II, intimin, O157 and SLT-I) from bovine faeces (Hu *et al*., 1999). Thus, the multiplex PCR developed in this study is satisfactorily sensitive to detect *E. coli* O157:H7 directly from seafood.

Multiplex PCR is a powerful tool for detecting *E. coli* O157 in seafood. This technique allows for the simultaneous amplification of multiple target genes, enhancing the efficiency and speed of pathogen identification. In this study, a new multiplex PCR protocol was developed and standardised, enabling the detection of *E. coli* O157:H7 in seafood by amplifying six virulence genes of the targeted bacteria. The optimised mastermix and multiplex PCR protocol developed in this study offer a high degree of sensitivity with 100% inclusivity and exclusivity ensuring accurate detection with low detection limit of 2 cfu ml^{-1}. This makes it a valuable method for the routine monitoring of seafood samples, ensuring seafood safety and quality.

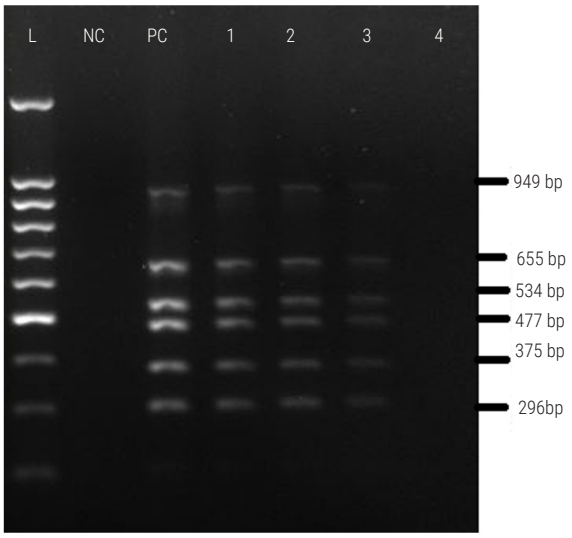

Fig. 4. Agarose gel showing sensitivity of multiplex PCR at different cfu of *E. coli* O157:H7 spiked in seafood. L-100 bp DNA ladder, NC- Negative control, PC- Positive control; 1-4 multiplex PCR product of *E coli* O157:H7 spiked at a concentration of 310 cfu ml^{-1}, 36 cfu ml^{-1}, 2 cfu ml^{-1}, 0 cfu ml^{-1}, respectively

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