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A novel multiplex PCR system for the detection of virulence associated genes of *E. coli* O157:H7 from food system

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Abstract

In view of the importance *E. coli* serotype 0157:H7 in human diseases, an easy and quick system is desirable to detect toxin producing strains. In this report, we describe standardization of a novel multiplex Polymerase Chain Reaction (mPCR) assay for simultaneous detection of four important genes associated with the organism *E. coli* 0157:H7 viz., *stx 1, stx 2, eae A, hly A* along with an internal amplification control (IAC). The mPCR method developed in the present study is sensitive enough to detect cells as low as 10^3 CFU ml^{-1} or g^{-1} of the food samples. The *E. coli* 0157:H7 strains having been identified to contain the gene in the mPCR were unequivocally detected positive for the serological and conventional culture method. As *E. coli* 0157:H7 is qualified as biowarfare agent; this mPCR system is of immense help in detecting them during emergencies of biological war and suspected outbreaks.

Keywords: Multiplex PCR, *E. coli* O157:H7, IAC, stx 1 and stx 2

INTRODUCTION

Escherichia coli O157:H7 is a food borne pathogen that has been associated with meat, meat-produce, unpasteurized milk, vegetables generally fertilized with contaminated cow manure, juice and water-related disease outbreaks [1]. This pathogen, which is known for its low infective dose and its ability to cause severe disease and death, emerged as a foodborne threat in the 1980s and early 1990s [2]. Food-borne outbreaks associated with *E. coli* 0157:H7 have been well documented worldwide. During 1996, a largest outbreak in the world caused by *E. coli* O157 in Japan, in which about 10,000 were, affected [3 and 4]. Infections due to *E. coli* 0157:H7 can result in severe bloody diarrhoea (haemorrhagic colitis, HC) and in life-threatening complications such as (HUS) haemolytic uraemic syndrome [5 and 6].

Rapid methods to detect *E. coli* O157:H7 are important to identify the source of outbreaks and to assure public safety. Both molecular and culture-based methods have been used for the detection of *E. coli* O157:H7. A disadvantage of this approach is that these methods are slow and labour intensive, they are not ideal for the analysis of the large number of samples during outbreaks [7].

Molecular approaches for bacterial detection avoid the need for culture and can be designed to be specific. PCR based techniques tends to be specific, more rapid and reliable and can eliminate the problems associated with the conventional culture methods. PCR formats available are either in the monoplex or duplex format and they lack IAC which is now become mandatory for all multiplex formats [8-15]. A muliplex PCR approach, can further improve the specificity of a PCR assay to the O157:H7 serotype, overcoming a problem observed using only single gene target PCR formats [16]. Information on important *E. coli* O157:H7 markers, such as the presence of O157, H7, intimin and Shiga-like toxin producing genes, can be obtained in one step. Thus multiplex PCR-based approaches offer the potential to be more rapid than other methods of *E. coli* O157:H7 detection. In the present study, we describe a newly developed robust and specific multiplex PCR assay for the simultaneous detection of four important genes associated with the *E. coli* 0157:H7, *stx1* and *stx 2*, *eae A* and *hly* genes along with an internal amplification control to check the false negative reactions in mPCR. The overall aim of this study, therefore, was to standardize and assess the suitability of newly developed multiplex PCR-based assay for its direct application onto food samples following one step enrichment in trypticase soy broth.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions.

E. coli O157:H7 strain no. US FDA-1 E. coli O157:H7 US FDA-1 was used as the reference strain, Obtained from (University of Fisheries, Mangalore). was used as the reference strains and the four isolates (EC-1, EC-2, EC-3, EC-4) isolated from different sources in our laboratory was also used in the present study.

The *E. coli* strains were cultured in modified trypticase soya broth with 20 mg of novobiocin per liter and further isolation was carried out using Sorbital Mc conkey agar supplemented with cefeximine and tellurite (CT-SMAC).

Collection of Samples

Samples were collected from different sources including slaughter houses, meat shops located in different parts of the Mysore city (Table-1). Each of the samples was inoculated into 95 ml trypticase soy enrichment broth supplemented with novobiocin and incubated at 37° C for 18 hours. Later inoculum was plated onto selective CT-SMAC agar. Sorbitol negative colourless/cream colonies exhibiting typical

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characteristic of *E. coli* O157:H7 were further subjected to biochemical, serological and molecular characterization.

Table 1. Isolation of *E. coli* O157:H7 from different samples

Samples	No of Samples Screened	Confirmed *E. coli* O157:H7 obtained
Chicken intestine	23	2
Chicken meat	23	0
Mutton	25	0
Beef	25	1
Pork	20	1
Fish	04	0

Primers and Internal Amplification Control

Four sets of primers were designed to detect genes *stx 1, stx 2, eaeA, hlyA* using the Gene Bank database sequences (Table 3). Conserved regions were selected and primers were designed with Gene runner software (USA). All primers were procured from Eurofins Biotech Pvt Ltd., (Germany) Bangalore. PCR products ranged from 166 bp to 779 bp in length. To check the presence of inhibitors within PCR mixture, an IAC was constructed. The primers used in this reaction had 5' overhanging ends, which were identical to the primer *rfb* gene, whereas 3' ends were complementary to a DNA sequence of *pUC* 18 (Table 3).

The PCR reaction mixture for generation of IAC DNA contained 1.0 µmol l^{-1} of each primer, 0.25 m mol l^{-1} each dNTP (MBI Fermentas, Canada), 0.5 units of *Taq* polymerase, 2.0 m mol l^{-1} MgCl2 in 1X PCR buffer (MBI Fermentas, Canada) with 250 pg of template DNA. The reaction procedure consisted of 30 cycles of denaturation at 94°C for 1 min, primer annealing at 58° C for 1min and extension at 72 ° C for 1min. The DNA was denatured for 4 min in the beginning and finally extended for 10 min at 72°C (Eppendorf master cycler gradient, Hamburg Germany). PCR product was purified using commercially available kit (Qiagen, Hilden, Germany). The concentration of IAC DNA was determined spectrophotometrically at 260 nm and was stored in DDW at -20°C. The following equation was used to calculate the copy number of the PCR product concentration: weight of PCR fragment (in g $\mu l^{-1)}$ X (6.023X10^{23}) / (660 g mol l^{-1} X number of base pairs of PCR fragment) = the number of genomic copy per microlitres [6].

DNA Extraction and Standardization of mPCR

DNA was extracted by boiling method from the overnight cultures [10]. Multiplex PCR was carried out in 50 µl reaction containing 0.4 µmol l^{-1} of *stx1*.F and *stx1*R, 0.4 µmol l^{-1} of *stx2* F *and stx2* R, and 0.4 µmol l^{-1} of *eae A* F and *eaeA* R primers, 0.2 mmol l^{-1} of each dNTPs, 10^3copies of IAC DNA, 1.2 unit of Taq polymerase, 2.0 mmol l^{-1} $MgCl_2$ in 1XPCR buffer (MBI Fermentas) with 1.0 µl of template DNA. Various concentrations of IAC DNA were tried before choosing 10^3 copies per reaction. Amplification consisted of initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, primer annealing at 58°C for 2 min and extension at 72°C for 2 min and a final extension a72°C for 10 min followed. The PCR products were analyzed on a 2% (wt/vol) Agarose gel.

Serotyping of *E. coli* Isolates

All the 87 presumptive *E. coli* isolates from 120 samples were examined by O and H antisera (MAST.CO.UK) to identify O157:H7 serotype by using plate agglutination test.

Spiking Studies

In order to validate the mPCR method for detection of *E. coli* O157:H7, milk and food samples were collected and artificially inoculated. Samples of rice based dishes (vegetable pulav) and milk (two each) were procured from the local market. Vegetable pulav (ten gram) and milk sample (10 ml) were inoculated with 100 µl of *E. coli* O157:H7 (US FDA-1) culture with cell concentration ranging from 10^{12} to 10^1CFU ml^{-1}. Each inoculated food sample were later tenfold diluted with peptone water, mixed well and incubated overnight (18 h) at 37°C. One milliliter from each sample was taken at the end of incubation period and processed for DNA extraction by boiling method. The DNA (1.5 µl) was used as template in PCR assay.

RESULTS

Isolation and Characterization

Eighty seven non-sorbitol fermenting colonies on SMAC agar containing cefeximine and tellurite were isolated from different meat and meat based products (120). Of these, 4 suspected isolates were confirmed as *E. coli* O157:H7 by biochemical (Table 2) and serological tests. These isolates along with the reference strains were further subjected to monoplex and multiplex PCR assay for the detection of virulent genes namely hemolysin, shiga like toxin and intimin genes.

Table 2. Biochemical and Molecular characterization of *E. coli* 0157:H7

	Biochemical tests								PCR results				
Isolates	**Sorbitol fermentation**	**Phenol red**	**Mannitol**	**Maltose**	**Cellobiose**	**Inositol**	**ODC**	**ADH**	***stx 1***	**stx 2**	***eae A***	***hly A***	**IAC**
E. coli O157:H7 (USFDA-1)	-ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
Isolate 1	-ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
Isolate 2	-ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
Isolate 3	-ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve
Isolate 4	-ve	+ve	+ve	+ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve

Table 3. Genes targeted and primer sequences

Gene	Primer sequence (5` to 3`)	Product size (bp)	Accession number
*stx*1F	ACACTGGATGATCTCAGTGG	614	EFO 79675.1
*stx*1R	CTGAATCCCCCTCCATTATG		
*stx*2 F	CCATGACAACGGACAGCAGTT	779	EU627768.1
*stx*2 R	CCTGTCAACTGAGCAGCACTTTG		
hlyA F	ACGATGTGGTTTATTCTGGA	166	EF116599
hlyA R	CTTCACGTCACCATACATAT		
eaeA F	GACCCGGCACAAGCATAAGC	290	FJ609835.1
eaeA R	CCACCTGCAGCAACAAGAGG		
IAC F	AAGATTGCGCTGAAGCCTTTG CCACACAACATACGAGCCG	521	S83460.1
IAC R	GTGCTTTTGATATTTTTCCGAGCGGACAGGTATCCGGTAAGC		U07164.1

Serotyping of *E. coli* Isolates

Among the 87 presumptive isolates, 4 were confirmed as the *E. coli* O157:H7 which showed granular agglutination with both O and H antisera within one minute.

Standardization of Multiplex PCR for the Detection of Selected Genes

The reaction conditions for the multiplex PCR assay were optimized to ensure that all of the target gene sequences were satisfactorily amplified. The primers were designed with care to avoid areas of homology with other organisms. The primers had almost equal annealing temperature, which reduced the possibility of non-specific amplification. The annealing temperature of 58^{o}C was finally selected based on nearly equal intensity of PCR products. Figure 1. shows the presence of amplified products after agarose gel electrophoresis. Reliable amplification of five bands of *stx-1, stx-2, eae A, hly* and IAC was obtained in standard *E. coli* 0157:H7 strain. As a negative control mPCR was tested with water and no amplicons were observed except IAC.

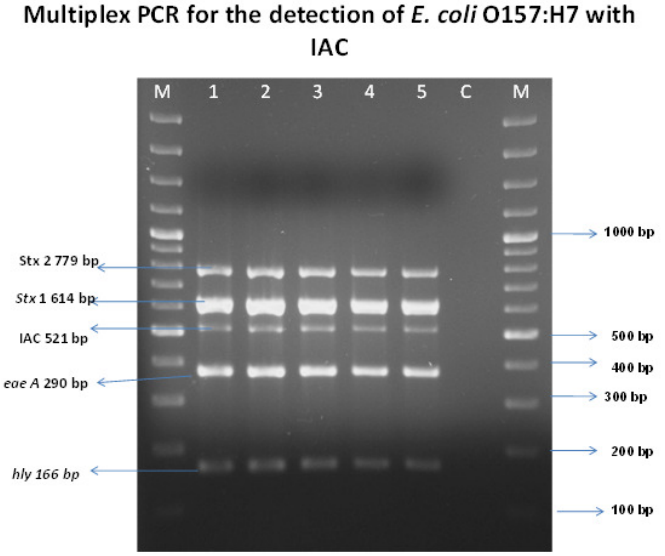

Fig 1. LM; USFDA1; L2-Isolate 1;L3-Isolate 2; L4-Isolate 3; L5-Isolate 4; L6-Control; LM

IAC and detection probability

The IAC co-amplified with target DNA and had amplicon size of 521 bps. Inclusion of varying concentrations of IAC DNA in mPCR mix did not change the detection limit of the assay and 10^3 copies were found to be optimum.

Robustness

There was no significant loss in the visibility of bands at less (10%, 20%) or more (10%, 20%) concentrations of PCR reagent and IAC DNA. The most prominent change of all concentrations was at 20% less concentrations. Temperature variation of ± 2^{o}C also did not make any appreciable change in the profile.

Analysis of artificially contaminated food samples

E. coli O157:H7could be detected in artificially inoculated vegetable pulav or milk samples after overnight enrichment in BHI. Detection limit was found to be as low as 10^3 CFU/g for both the samples tested.

DISCUSSION

E. coli O157:H7 is a worldwide threat to public health and the outbreaks historically have been associated with undercooked ground beef, meat and meat products, raw vegetables. *E. coli* O157:H7 strains possessing important virulence traits are required to be surveyed particularly when the strain is involved in disease outbreaks to assess the response strategies for containment.

In the present investigation, a multiplex PCR based assay was developed that detects all the important virulence associated genes viz., *stx1, stx2 eae A* and *hly* with an IAC for direct application onto food matrices.

For the mPCR reported here, all the primers were designed with the view to have a common annealing temperature to get preferable amplification at a single temperature and care was also taken to maintain at least 100 bps differences between product sizes for good resolution during agarose gel electrophoresis. In order to eliminate false negatives due to variation in performance of PCR thermal cyclers, incorrect PCR mixture, inefficiency in taq polymerase, personnel and the presence of PCR inhibitors in the sample matrices, an IAC is required to be integrated in the mPCR. Moreover, in mPCR system, presence of an IAC is now considered mandatory for diagnostic food microbiology [17]. In PCR with an IAC, a control signal should always be produced even when there is no target sequence present.

Another important criterion for a diagnostic PCR is robustness. The present assay worked in presence of ± 20% concentration of PCR reagents and IAC DNA. A temperature variation of ± 4^{o}C was

also well tolerated, which shows the robustness of the assay. Spiking studies revealed that the developed mPCR method is sensitive enough to detect cells as low as 10^3 CFU/ml or gram of the overnight-enriched food samples viz., milk and rice based vegetable pulav.

The adequacy of mPCR in identifying *E. coli* 0157:H7 strains in general and the toxin containing strains in particular following overnight enrichment of food and environmental samples in TS broth was well established. The *stx*1*, stx* 2 and *hlyA* genes being responsible for the expression of the potential toxin molecules can be easily be identified by the reported mPCR and this protocol would help in detecting them during biological emergencies. Use of this multiplex PCR assay can also help in providing the information required for appropriate action during suspected outbreaks of *E. coli* 0157:H7 food poisoning. Considering the low cost and the associated rapidity to detect the four genes simultaneously, it is believed that this may serve as a powerful tool for not only to obtain a reliable identification of *E. coli* 0157:H7 but also in assessing the toxin potential of the strain as well. The procedure is specially suited to fit into the daily work requirement of routine food quality control laboratories since detection and identification of the toxin genes of the pathogen from diverse food sources is becoming an important component of the diagnostic inventory of such laboratories. Given the long turnaround time associated with traditional culture methods, PCR in any case is a rapid and reliable screen for the detection of *E. coli* 0157:H7 harboring *stx*1 and *stx*2 toxin genes.

The recent outbreak of *E. coli* in Germany that has lead to scare all over the world with impacting not only the growing human loss (39 deaths) reported so far but also to an extent the economy of particular agriculture when immediate bans are imposed on the export of products like vegetables, grains and sprouts (CDC, EU, 2011)[18]. More intriguing is the nature of the *E. coli* strain involved. The sequencing data available from very first instance from number of strains recovered from this outbreak suggested the strain to be of EAEC group. The more detailed analysis of this strain further revealed it to be a hybrid of EAEC O104 and O157 of EHEC [18 and 19]. The important virulence associated components in this strain still stands to be the shiga like toxin, aggregating factors and a few hemolysins. The presently described mPCR that takes care of important virulent components of EHEC O157:H7 strains can definitely be helpful in providing early information onto the killing potential of such strains during the initial phase of outbreaks and can also help in molecular epidemiological investigations even to trace even the source of the outbreak.

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REFERENCES

[1] Campbell, G.R., J. Prosser., A. Glover and K. Killham. 2001. Detection of *E. coli* O157:H7 in soil and water using multiplex PCR. Journal of Applied Microbiology. 91: 1004-1010.

[2] Mead, P.S., Slutsker, L and Dietz, V. 1999. Food related illness and death in the United States. Emerging Infectious Diseases. 5: 607-625.

[3] Son Radu., Shahilah Abdul Mutalib., Gulam RusuL., Zainori Ahmad.,Tadaaki MorigakI., Norio Asai., Yung Bu Kim,Jun Okuda and Mitsuaki Nishibuchi. 1998. Detection of *Escherichia coli* O157:H7 in the Beef Marketed in Malaysia. Applied and Environmental Microbiology. 64(3): 1153–1156.

[4] BukharI. Z., J. Weihe., M. Lechevallier. 2006. Improved Detection Methods for *E. coli* 0157:H7. American Water, 1025 Laurel Oak Road, Voorhees, NJ 08043, USA Water Intelligence Online © IWA Publishing 2006 / UNIQUE ID: 200603AF91070F.

[5] Griffin, P. M. 1995. *Escherichia coli* O157:H7 and other enterohemorrhagic *Escherichia coli*, p. 739–761. *In* M. J. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg, and R. L. Guerrant (ed.), Infections of the gastrointestinal tract.Raven Press, Ltd., New York, N.Y.

[6] Kumar, H.S., Otta, S.K., Karunasagar, I., Karunasagar, I. 2001. Detection of Shiga-toxigenic *Escherichia coli* (STEC) in fresh seafood and meat marketed in Mangalore, India by PCR. Letters in Applied Microbiology. 33: 334-338.

[7] Karch, H and Bielaszewska, M. 2001. Sorbitol-fermenting Shiga toxin-producing Escherichia coli O157:H7 strains: epidemiology, phenotypic and molecular characteristics, and microbiological diagnosis. Journal of Clinical Microbiology 39: 2043–2049.

[8] Griffin, P. M., and R. V. Tauxe. 1991. The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. Epidemiological Reviews. 13:60–98.

[9] De Boer E, Heuvelink, A.E. 2000. Methods for the detection and isolation of shiga toxin producing *Escherichia coli*. Journal of Applied Microbiology. 88*:* S133-43.

[10] Theron, J., Morar, D., du preez, M., BrözeL., V. S. and Venter, S.N. 2001. A sensitive seminested PCR method for the detection of *Shigella* in spiked environmental water samples. Water Research. 35: 869–874.

[11] Paton, A.W., Paton, J.C. 2002. Direct detection and characterization of Shiga toxigenic Escherichia coli by multiplex PCR for stx1, stx2*, eae, ehxA* and *saa*. Journal of Clinical Microbiology. 40: 271–274.

[12] Wang, G., Clark, C.G. and Rodgers, F.G. 2002. Detection in *Escherichia coli* of the genes encoding the major virulence factors, the genes defining the O157:H7 serotype, and components of the type 2 shiga toxin family by multiplex PCR, Journal of Clinical Microbiology. 40: 3613-3619.

[13] Al-abri, S.S., Beeching, N.J., Nye, F.J. 2005. Traveller's diarrhoea. Lancet Infectious Diseases. 5:349-60.

[14] Dhanashree and P. Shrikar Mallya. 2008. Detection of shiga-toxigenic *Escherichia coli* (STEC) in diarrhoeagenic stool & meat samples in Mangalore, India. Indian Journal of Medical Research. 128: 271-277.

[15] Bonyadian, H., Momtaz, E., Rahimi, R., Habibian, A., Yazdani and M. Zamani. 2010. Identification & characterization of Shiga toxin-producing *Escherichia coli* isolates from patients with diarrhoea in Iran. Indian Journal of Medical Research 132. 328-331.

[16] Fratamico, P.M., S.K. Sackitey., M. Wiedmann and M.Y. Deng. 1995. Detection of *Escherichia coli* O157:H7 by multiplex PCR.

Journal of Clinical Microbiology. 33(8): 2188-2191.

[17] Hoofar, J., MalornY, B., Abdul mawjood, A., Cook, N., Wagner, M. and Fach, P. 2004. Practical considerations in design of internal amplification controls for diagnostic PCR assays. Journal of Clinical Microbiology. 42: 1863–1868.

[18] Center for Disease Control and Prevention. 2011. Investigation Update: Outbreak of Shiga toxin-producing *E. coli* O104 (STEC O104:H4) Infections Associated with Travel to Germany.

[19] James Andrews. 2011. May Change How We Deal With *E. coli*. Food Safety News, June. (www. foodsafetynews. Com /2011 /06 /E.coli-expert-weights-in-with-facts-about O104:H4).