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Culture-Free Detection of Enterotoxigenic Escherichia Coli in Food by Polymerase Chain Reaction

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Abstract- Microbial quality of food has immense importance for protection of human health in rural and urban settings. Rapid and specific detection of enterotoxigenic Escherichia coli (ETEC) is critical for the management of the food-borne diarrheal diseases threatening human lives. Conventional methods for detection of ETEC involve enrichment and biochemical identification which are laborious, time consuming and less sensitive. Polymerase Chain Reaction, using specific primers is a powerful molecular technique for rapid and specific detection of ETEC. The present study aims to detect ETEC bearing virulent signature gene LT1 in food samples. Street fruit juices like Citrus (Citrus limetta), Pineapple (Ananas comosus), Sugarcane (Saccharum spp.) were procured from local market. Apart from these, vegetables used for garnishing the dishes such as Fenugreek (Trigonella foenum-graecum) Mint (Mentha spp.) and Coriander (Coriandrum sativum) leaves were purchased from local market. Multigenomic DNA templates were prepared by boiling prep and purified using Sodium acetate-ethanol method. Genomic DNA from pure cultures was also prepared and purified. This was followed by PCR targeting the LT1 gene to detect ETEC in food samples. Food samples collected from local market were analysed and found contaminated with ETEC. The amplified products from samples' DNA were same as compared to positive control. The amplification based assay developed here is rapid with high specificity for detection of ETEC in food samples.

Keywords: Enterotoxigenic Escherichia coli, PCR, Food samples

I. INTRODUCTION

Enterotoxigenic *Escherichia coli* (ETEC) is regarded as a major cause of *E. coli* mediated diarrhea in humans, affecting mainly children and travelers [1]. Apart from humans, ETEC has also important implications for the farming industry where it is a major pathogen of cattle. In 2010 alone, 28.7 million episodes have been reported due to ETEC and 45,713 deaths in WHO regions of South Asia [2]. Contaminated water and food have been implicated as vehicles for transmission of ETEC infection in humans [3]. A number of food matrices are the potential carriers of ETEC-related diarrhea. These include fresh fruits and vegetables (especially lettuce), shrimp, crab meat, salads and soft cheeses. ETEC infections, reported are always associated with poor hygiene and sanitation [4]. Apart from these, contaminated seafood and salads are frequently are the vehicles for of ETEC [5]. In the recent years, the frequency of diarrheal outbreaks of illness associated with consumption of raw fruits, vegetables has increased [6]. In general, food-borne illnesses have been traced to irrigation from contaminated water or unhygienic post-harvest management. Potential pre-harvest contamination sources of vegetables include soil, manure, human, farm animal feces and irrigation water [7]. These factors can influence the survival and growth of human pathogens on raw vegetables. ETEC secretes two types of enterotoxins (heat-labile, LT; and heat-stable, ST enterotoxins) encoded by *LT1* and *ST1* genes, respectively [8]. The heat-labile enterotoxins are classified into two major groups (LTI and LTII). *LTI* is expressed by *E. coli* strains that are pathogenic for both human and animals. The *LT1* gene commonly present in strains associated with human.

The conventional methods for detection and quantitative enumeration of ETEC in food items are cumbersome. These include cell culture techniques, enzyme linked immunosorbent assays, and membrane-based DNA hybridization assays [9]. All these methods are labour intensive and time consuming and they fail to detect viable but non culturable state of pathogens present in low concentrations [10, 11]. Polymerase Chain Reaction is a current powerful highly specific technology which allows amplification and detection of target. Several studies have been reported for the presence of ETEC in water and aquatic macrophytes [12, 13]. At present, the information on the presence and sensitive detection of ETEC in food samples is meager. Therefore, in the present study, a variety of food samples collected from local markets were analysed for culture-free enumeration of ETEC using PCR assay

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targeting *LT1* gene.

II. MATERIALS AND METHODS

A. Primers

For specific detection of ETEC harboring *LT1* gene in surface food samples, primers (F: 5′-GGCAGGCAAAAGAGAAATGG-3′ R: 5′-TTGGTCTCGGTCAGATATGTG-3′, position: 996- 1145, product size 150 bp) were adopted from Ram et al. [12] (Table 1).

Table 1. Nucleotide sequences of candidate oligomers of *LT1* gene of enterotoxigenic *Escherichia coli*.

Gene	Primer(5'-3')	**Product length (bp)**/position of primers	*Tm* (^{0}C)
LT1	GGCAGGCAAAAGAGAAATGG	**150**	54.5
	TTGGTCTCGGTCAGATATGTG	111-260	54.4

B. Specificity Of The Assay

The inclusivity and exclusivity of the assay was checked using reference strains of *E. coli* and other genera. The reference strain of *E. coli*: *E. coli* MTCC723 and *Salmonella* (*S. typhi* MTCC733), were procured from Microbial Type Culture Collection (MTCC) at Institute of Microbial Technology (IMTECH), Chandigarh, India. The strains were used for evaluating the specificity PCR primers used in this study (Table 1). All the bacterial strains were grown in LB broth (Hi Media, India) for 12 h at 37±1 °C (optical density 0.8 at 600 nm). DNA template was prepared from bacterial cultures (1×10^5 CFU/mL).

C. Food Samples

Street food items like Panipuri (water), Egg, Noodles and fruit juices like Citrus (*Citrus limetta*), Pineapple (*Ananas comosus*) and Sugarcane (*Saccharum* spp.) were procured from local market. Apart from these vegetables used for garnishing the dishes such as fenugreek (*Trigonella foenum-graecum*), mint (*Mentha* spp.) and coriander (*Coriandrum sativum*) leaves were purchased from local market. Fruit juices (2 l each) were filtered through sterilized muslin clothes to remove the coarse fibres. The leafy vegetables (50 g each) were properly rinsed in saline (200 ml) with gentle shaking followed by sonication (cycle 0.5 for 20 s) to transfer almost all the microflora in saline. After this the samples were concentrated to 500 µl by centrifugation at 14000 x g.

D. Isolation Of Multigenomic DNA

DNA template was prepared by boiling the 500 µl concentrated sample and removing the debris by centrifugation at 16,000 x g for 5 min at 4^{o}C. DNA was precipitated from supernatant using 0.3 M sodium acetate (pH 5.2) and ice cold ethanol (Ram *et al.*, 2007). The precipitated DNA was pelleted by centrifugation at 12000 x g for 5 min. DNA pellet was washed thrice with 70% ethanol and finally dissolved in 250 µl TE (pH 8.0). The purity and yield of isolated DNA samples were determined by Spectrophotometer.

E. Detection Of ETEC Using PCR

PCR targeting the virulent *LT1* gene was run to detect the ETEC in real life food samples. Genomic DNA from pure cultures and environmental isolates were prepared and purified. The reaction mixture in a final volume of 50 µl comprised of dNTPs (0.2 mM), Taq DNA polymerase (1.5 units), 10x reaction buffer (5 µl), $MgCl_2$ (1.5 mM), primers (0.4 µM, each) for *LT1* gene and DNA template (5 µl). The PCR program was as follows: initial denaturation at 95^{0}C for 3 min and then 45 cycles at 95 ^{0}C for 20 s, 55.8 ^{0}C for 30s, and 72^{0}C for 30s. Similar assay was performed with the environmental strains of *E. coli* and other genera. Purified multigenomic DNA (5 ul) from environmental samples were diagnosed for under identical PCR conditions.

III. RESULTS

A. Specificity of The Assay

The PCR reaction generated the product of 150 bp as evident on agarose gel electrophoresis (Figure 1). The PCR assay described here is highly specific to the ETEC. All strains of *E. coli* exhibiting *LT1* gene were positive in PCR assay (Table 2). However, no amplification of the target gene was observed in strains lacking the target gene such as *Vibrio cholerae* and other bacterial strains

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used in the study.

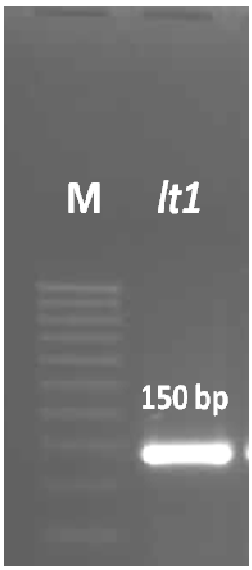


Fig. 1 Agarose gel electrophoretic analysis of amplicon of *LT1* gene

Table 2. Specificity of Molecular Beacon based real-time assays targeting *LT1* gene to detect enterotoxigenic *Escherichia coli*

Isolate/strain identity and source	Detection of [a]ETEC
[a]ETEC	
E. coli MTCC 723 ([b]MTCC, Chandigarh)	+
Other bacteria	
Salmonella typhi [b]MTCC733	-

[a]ETEC: Enterotoxigenic *Escherichia coli* ; [b]MTCC: Microbial Type Culture Collection at Institute of Microbial Technology (IMTECH), Chandigarh, India

B. Culture-Independent Detection Of Etec In Food Samples

Food samples collected from local market were analysed for the presence of ETEC. All the samples were found positive for the presence of ETEC. Vegetables (used for garnishing the dishes) like Fenugreek, Mint and Coriander were contaminated with ETEC (Table 3 & 4).

Table 3. Contamination of Vegetables used for garnishing the dishes by Enterotoxigenic *Escherichia coli*

S. No.	Food Samples	ETEC
Vegetables used for garnishing the dishes		
1.	Fenugreek (Methi)	+
2.	Mint	+
3.	Coriander	+

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Table 4. Contamination of street fruit juices by Enterotoxigenic *Escherichia coli*

S. No.	Juices	ETEC
1.	Citrus (Mausambi)	+
2.	Pineapple	+
3.	Sugarcane	+

IV. DISCUSSION

The present study has opened a new avenue in the culture independent detection of ETEC in food samples. *LT1* gene was targeted for the PCR assay. The primer pair was adopted from Ram *et al*, in which the limit of detection was 2 CFU [12]. Specificity of the assay was checked with strain of *E. coli* and other genus. The assay was negative for other bacterial strain lacking target gene. No amplification was observed by *S. typhi*. These observations validate the high specificity of the assay. ETEC can survive for longer periods up to 3 months in fresh and sea water [14]. This leads to the constant exposure and accumulation of ETEC. The contaminated vegetables are transported to the local markets. In the present study, all the leafy vegetables which are used to garnish the dishes were found contaminated with ETEC. The presence of ETEC was evident in egg sample. Eggs shells bearing fecal matter of chickens are contaminated with ETEC are stored at room temperature, which helps these organisms to multiply fast during transportation and handling [15]. The present assay overcomes the limitations of conventional detection methods. The culture-based assays are lengthy (18-96 h), and are unable to detect viable but non-culturable state of ETEC [11]. Therefore, the culture independent PCR developed in this study is rapid procedure with high specificity detection of ETEC in food samples.

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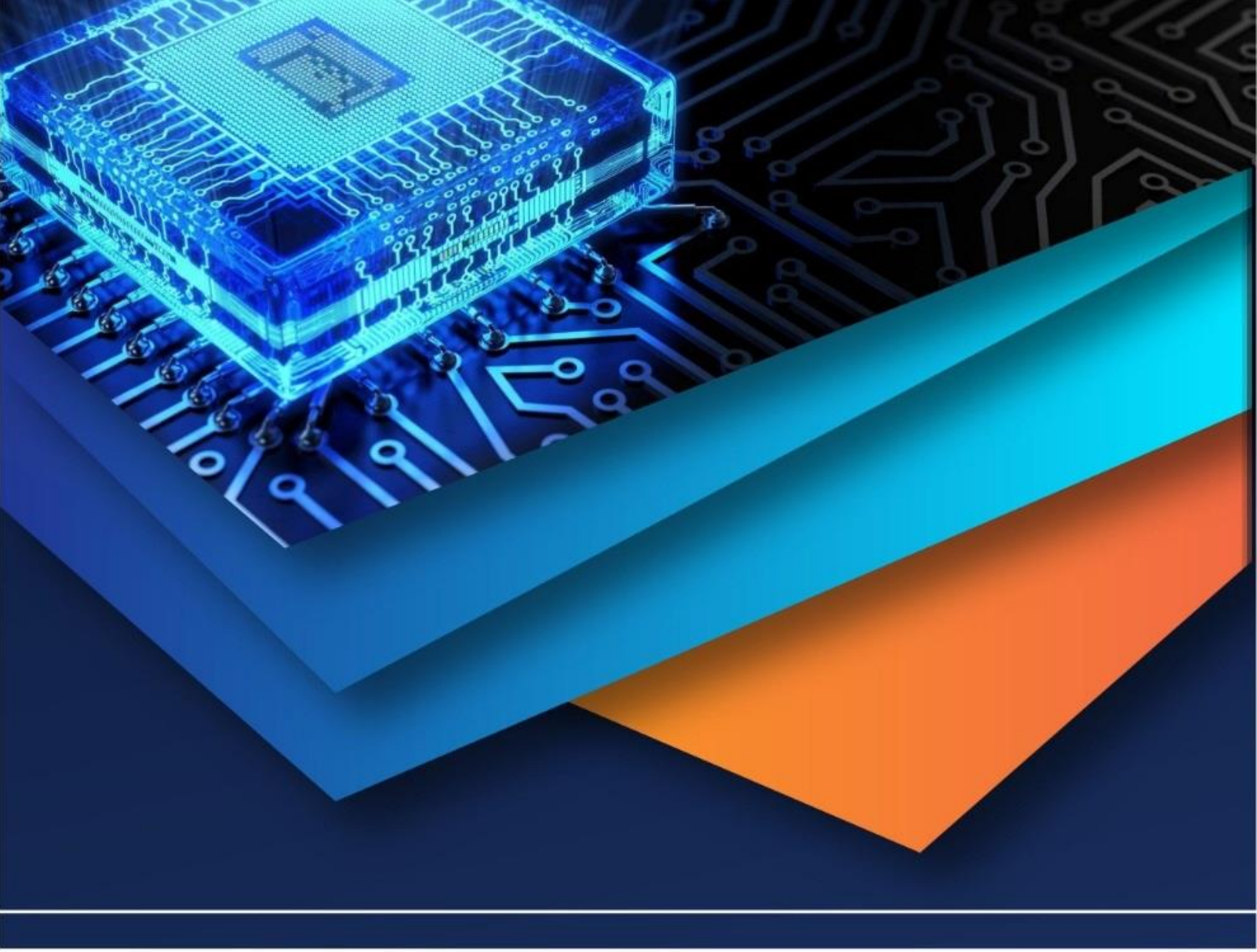
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