Int J Entric Pathog. 2014 February; 2(1): e16431. DOI: 10.17795/ijep16431

Published online 2014 February 08. **Research Article**

Multiplex Real-Time PCR Assay for the Detection of *LT, STIa and STIb* Genes in Enterotoxigenic *Escherichia coli*

Pejman Abbasi [1]; Mohammad Kargar [2,*]; Abbas Doosti [3]; Jalal Mardaneh [1]; Mohammad Ali Dehyadegari [1]; Sadegh Ghorbani-Dalini [4]

[1]Alborzi Clinical Microbiology Research Center, Namazee Hospital, Shiraz University of Medical Sciences, Shiraz, I.R. Iran
[2]Department of Microbiology, Islamic Azad University, Jahrom, I.R. Iran
[3]Biotechnology Research Center, Islamic Azad University, Shahrekord Branch, Shahrekord, I.R. Iran
[4]Department of Biology, University of Sistan and Baluchistan, Zahedan, I.R. Iran

*Corresponding author: Mohammad Kargar, Department of Microbiology, Islamic Azad University, Jahrom, I.R. Iran. Tel: +98-9177164249, E-mail: mkaragarmicro418@yahoo.com

Received: November 27, 2013; **Revised:** December 15, 2013; **Accepted:** December 24, 2013

Background: Diarrhea is an important cause of illness and death among all age groups on a global scale. Enterotoxigenic *Escherichia coli (ETEC)* protozoa were established as a causative agent of diarrhea in developing and developed countries. The identification of diarrheagenic *E. coli* (DEC) strains needs to detect the factors that determine the virulence of these organisms.

Objectives: In this study, we aimed to use the multiplex real-time (MRT) and multiplex PCR assays for identification of ETEC in patients with diarrhea in Shiraz, Iran.

Patients and Methods: A total of 430 stool samples were collected from patients with diarrhea in Shiraz, in 2012. Diarrheagenic *E. coli* (DEC) strains were isolated by standard biochemical analysis. We used MRT-PCR and multiplex PCR (MPCR) assays to detect the presence of *LT, STIa* and *STIb* genes in ETEC.

Results: In this study, 430 stool samples were tested and 52 (12.1%) were identified as contaminated with *E. coli* using standard biochemical tests. The ETEC were detected in eight patients (15.4%) with diarrhea by the MRT-PCR and MPCR methods. The results of this study showed that four out of eight strains (50%) were STI a producer, and three out of eight strains were ST producers (37.5%). Also, one strain (12.5%) contained both *STIa* and *LT* genes, simultaneously.

Conclusions: This is the first study performed in Shiraz to identify ETEC intestinal pathogens in patients with diarrhea. The results of this study showed that MRT-PCR can be used as a replacement for the conventional MPCR assay to detect the ETEC strains.

Keywords: Enterotoxigenic *Escherichia coli*; Shiraz; Real-Time Polymerase Chain Reaction

1. Background

Diarrhea remains a leading cause of mortality and morbidity worldwide, particularly in developing countries (1). *Escherichia coli (E. coli)* is a heterogeneous group of typically non-pathogenic gram-negative bacteria, which are a natural part of the intestinal flora of animals and humans (2). However, the pathogenic strains are associated with several diseases, including diarrhea, urinary tract infections and meningitis (3). Diarrheagenic *E. coli* (DEC) strains are important causes of diarrhea in the developing world and, to a lesser extent, in the developed world (4). The *E. coli* were isolated and identified using the biochemical tests (5). *Escherichia coli* strains associated with diarrhea have been classified into six groups, based on clinical, epidemiological and molecular criteria (6, 7), namely enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC), and diffusely adherent *E. coli* (DAEC) (8-10). Among these six recognized diarrheagenic categories of *E. coli*, the ETEC is the most common group, particularly in the developing countries (11). The ETEC produce one or both of the two enterotoxins, heat-labile enterotoxin (LT) and heat-stable enterotoxin (ST), that have been fully characterized, cloned, and sequenced, and their genetic control in transmissible plasmids was identified (12-14). The heat-stable enterotoxin produced by ETEC is a low-molecular weight protein consisting of 18 or 19 amino acids. The ST is divided into two groups, including *STa* (or *STI*) and *STb* (or *STII*). The *STa* produced by ETEC strains can cause infection in both animals and humans, whereas *STb* is primarily associated with infection in animals (15). The *STa* is further subdivided into STh (*STaI*) and *STp* (*STaII*) named after the first discovery in humans and pigs, respectively. The diarrhea caused by ETEC is characterized by a rapid onset of watery, non-bloody diarrhea with remarkable volume, accompanied by little or no fever. Other common manifestations

Implication for health policy/practice/research/medical education:
The prevalence and epidemiological features of enterotoxigenic *Escherichia coli* as a causative agent of diarrhea has a regional variation. A similar pattern has also been observed between and within countries from the same geographical area.

of this diarrheal infection are malaise, abdominal cramping pain, vomiting and nausea (16).

Because of the time demanding diagnosis and lack of controlled studies to provide guidelines, antimicrobials are not commonly used for the treatment of diarrhea caused by ETEC strains (17, 18). The ETEC can be identified by different molecular methods, based on the presence of different plasmid containing virulence genes, which are absent in other *E. coli*. The ETEC virulence genes have been investigated and characterized extensively (6). Non-culture-based diagnostic tests are more attractive to clinical laboratories, medical centers, and the biomedical industries because they hold the potential for results that can be obtained in-house, in much shorter time, and at a lower cost than that of standard culture-based methods.

2. Objectives

In this study, we aimed to use the multiplex real-time (MRT) and multiplex PCR (MPCR) assays to identify the enterotoxigenic *E. coli* (ETEC) infection in patients with diarrhea in Shiraz, I.R. Iran.

3. Patients and Methods

3.1. Definition

Diarrhea was defined as at least three loose bowel movements in a 24-hour period accompanied by at least one of the following symptoms: nausea, vomiting, abdominal cramps, or fever > 38˚C.

3.2. Clinical Sampling and Culture Process

A total of 430 stool samples were collected from patients with diarrhea in Shiraz, in 2012. During transportation to the laboratory, the samples were stored in PBS transport medium on ice packs. A loop full of diarrheal sample was streaked on MacConkey agar and incubated for 24 hours, at 37˚C. The pink colonies were then sub cultured on eosin methylene blue (EMB), on which the colonies exhibit green metallic sheen color. For a further conformation, biochemical tests were performed.

3.3. DNA Extraction

A sweep of three colonies were inoculated in Luria-Bartani (LB) broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) and incubated overnight at 37˚C, with shaking. All isolated *E. coli* strains were grown on Luria-Bertani agar (Sigma Aldrich, St. Louis, Missouri, USA) over night at 37˚C. The *E .coli* genomic DNA was extracted using a DNA extraction kit (Qiagen UK Ltd., Crawley, UK) according to manufacturer's instructors.

3.4. Primer Selection

The primers were selected to detect three different virulence genes (*LT*, *STIa*, *STIb*) simultaneously, in a single reaction (Table 1).

3.5. Multiplex PCR Assays

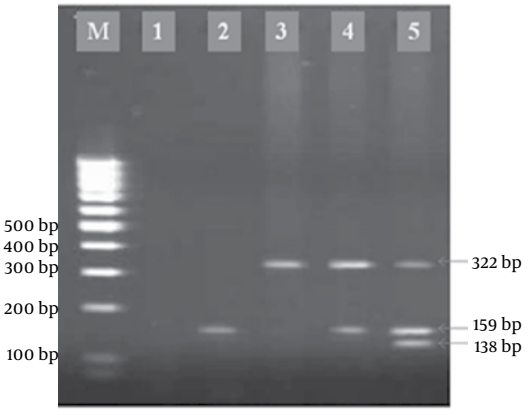
Each MPCR assay was performed at a final reaction volume of 25 μL, containing 2 μL of the template DNA, 200 mM deoxynucleoside triphosphates, 4 mM $MgCl_2$, 1.5 U Taq DNA polymerase (Sinagen Company, Teheran, Iran), and 0.2 mM of each primer. Cycling program was as follow: 95ºC for 5 minutes to initially denature the DNA, then 35 cycles consisting of 1 minute at 94˚C, 1 minute at 58˚C, 1 minute at 72˚C, and finally, a single prolonged extension at 72˚C for 5 minutes. A negative control lacking DNA template was included in each experiment to exclude the possibility of the reagent contamination. The *E. coli* strain used as control in the PCR test included *E. coli* ATCC 35401 (LT+, ST+). The amplified product was visualized by gel electrophoresis in 1.5% agarose gel containing ethidium bromide for 45 minutes at 100 V and then visualized under UV light (Figure 1).

Table 1. Primers Used for Detection of Enterotoxigenic *E. coli* Genes [a]

ETEC	Amplicon Size	Reference
STIa gene	159	(7)
F: 5′TTTCCCCTCTTTTAGTCAGTCAA3′		
R: 5′GCAGGATTACAACACAATTCACAGCAG3′		
STIb gene	138	(7)
F: 5′TGCTAAACCAGTAGAGTCTTCAAAA3′		
R: 5′GCAGGATTACAACACAATTCACAGC3′		
LT gene	322	(7)
F: 5′TCTCTATGTGCATACGGAGC3′		
R: 5′CCATACTGATTGCCGCAAT3′		

[a] Abbreviations: ETEC, Enterotoxigenic *E. coli;* F, forward; R, Reverse.

Figure 1. Agarose Gel of Amplicons From the Multiplex PCR



The molecular ladder is shown in lane M (100 bp); nonpathogenic *E. coli* in lane 1; strain identification and amplicons are shown in lanes 2 (*STIa* positive), 3 (LT positive) and 4 (*STIa* and LT positive); *E. coli* ATCC 35401 is shown in lane 5.

3.6. Multiplex Real Time PCR Assays

Real-time PCR assay was conducted in a final volume of 25 μL, same as MPCR, plus 1 μL of CYBR Green I (Invitrogen, Carlsbad, CA, The USA) which was added to the reaction. Reactions were performed on Rotor-Gene 6000 (Corbett Research Pty Ltd., Sydney, Australia) by cycling conditioning at 95˚C for 5 minutes, followed by 45 cycles of 95˚C for 30 seconds and 58˚C for 40 seconds. Finally, melting curve analysis was performed from 70 - 99˚C, with a ramping rate of 2.5˚C/s and analysis of fluorescence at each 2˚C for 5 seconds. All reactions were repeated in triplicates, and positive and negative controls were used in each run. All data were analyzed by Rotor-Gene 6000 software version 1.7 (Corbett Research Pty Ltd., Sydney, Australia).

4. Results

In this study, a total of 430 patients (older than 3 years of age) with diarrhea were evaluated in Shiraz, I.R. Iran. Out of the 430 stool samples analyzed, 52 (12.1%) were identified as contaminated with *E. coli* using standard biochemical tests. There were 29 (56%) males and 23 (44%) females (3 to 63 years old). In this study, we used MRT-PCR and MPCR to detect the presence of *LT*, *STIa* and *STIb* genes in ETEC. The ETEC were detected in eight (15.4%) patients with diarrhea. Out of the eight ETEC strains, *LT* and *STIa* genes were detected in three and four strains, respectively, and one strain containing both *STIa* and *LT* genes was found. None of the strains, which were identified as ETEC by molecular methods, contained the *StIb* gene. The patients were categorized into three different groups, according to their age: [3 - 23], [24 - 60] and over 61 years old (Table 2).

Table 2. Distribution of the Eight Enterotoxigenic *E. coli* Strains in Patients in Accordance With Season, Age, Gender and Clinical Symptoms

Clinical and Other Characterization	ETEC[a]. No.
Season	
Spring	3
Summer	2
Fall	3
Winter	not seen
Age, y	
(3-23)	5
(24-60)	3
(61 >)	not seen
Gender	
Male	5
Female	3
Clinical symptoms	
Diarrhea Bloody	not seen
Diarrhea Watery	8
Fever	5
Vomiting	3

[a] Abbreviations: ETEC, enterotoxigenic *E. coli*

5. Discussion

Diarrhea is an important cause of mortality and morbidity in different areas of the world (developed and developing countries) and among all age groups. The outbreak surveillance programs provide important information about the changing profiles of foodborne pathogenic microorganisms and carriers, as well as information about the achievements and failures of public health agencies to maintain the safety of the food supplies (16). The ETEC was established as a causative pathogen of diarrhea in developing and developed countries (19). Several countries in the world, including Bangladesh, Egypt and Mexico, have reported ETEC as the most common cause of diarrhea among all *E. coli* intestinal pathotypes (9, 20-23). In the present study, ETEC were detected in eight (15.4%) patients with diarrhea. This is the first study performed in Shiraz to identify the ETEC intestinal pathogens in patients with diarrhea. Our finding is approximately similar to that reported by Jafari et al. (24), in which they found that the rate of ETEC was 12.8%, and also similar to the results of the studies by Presterl et al. and Jafari et al. (20, 22). The results of this study showed that, four out of eight strains (50%) were *STI* a producers, and three out of eight strains were LT producers (37.5%). Also, one strain (12.5%) contained both *STIa* and *LT* genes, simultaneously. Our finding is similar to the results of the study by Jafari et al. (22), in Tehran. Other studies also showed the predominance of ST producing ETEC (15, 25). In the present study, *StIb* gene was not detected in any of the samples, whereas a study showed the predominance of *STIb* producing ETEC (16). The prevalence and other epidemiological features of this pathogen, as causative agent of diarrhea, vary in different regions. This variation is also seen between and within countries in the same geographical area (6). The ETEC is recognized as a leading cause of diarrhea in countries with inadequate sanitary conditions and among travelers to those countries. However, it has been infrequently identified as a cause of food-related outbreaks of gastroenteritis in several developed countries, such as USA. The ETEC might be underrecognized as a cause of foodborne outbreaks because laboratory tests that are used to identify this organism are not widely available (16). This study provides information on clinical manifestations, including watery diarrhea, bloody diarrhea, fever, and vomiting. Our findings are approximately similar to those reported by Jafari et al, in Tehran (24). In many developing countries, antibiotics are readily accessible without any medical prescription. Self-prescribing of antimicrobial agents are common, primarily for nonspecific illnesses and for complaints such as headache, fever, diarrhea, minor respiratory and gastrointestinal infections and abdominal pain (26). The rapid detection of DEC has important treatment implications. Treatment of diarrhea does not usually depend on the etiological diagnosis, yet such diagnosis bears an implication on overall management of patients. Some DEC strains (e.g. ETEC)

respond to antimicrobial agents, while others (e.g., STEC) do not. Therefore, antibiotic consumptions should be avoided. Optimal treatment depends on rapid detection of the specific pathogens (6). The identification of ETEC strains requires these organisms be differentiated from other DEC pathogroups. It is not sufficient to identify a strain as diarrheagenic, based on O serotyping, because it does not correlate, in most cases, with the presence of virulence factors. Thus, for identification of diarrheagenic *E. coli* strains, factors that determine the virulence of these organisms should be identified. The limitations associated with traditional diagnostic techniques can be overcome by PCR, which is a sensitive, specific and rapid diagnostic method.

Real-time PCR is a faster, more robust assay, because it does not require post-PCR procedures to detect amplification products (7, 27). Molecular-based techniques, such as the MPCR method, showed specificity and sensitivity for the most common classes of DEC strains of various clinical sources, genotypes, and stereotypes. The results of this investigation prove that it is possible to perform synchronous amplification of virulence genes from different *E. coli* strains such as EHEC, ETEC, and EPEC, and apply this method to diagnose the patients with hemolytic-uremic syndrome and sporadic enterocolitis, and also individuals involved in food-borne outbreaks.

Acknowledgements

The authors thank to the Biotechnology Research Center of Islamic Azad University, Shahrekord Branch, for their sincere supports.

Authors' Contribution

All authors had participated equally.

Financial Disclosure

There was no conflict of interest.

Funding/Support

The study was a self-funded research.

References

1. Barletta F, Ochoa TJ, Ecker L, Gil AI, Lanata CF, Cleary TG. Validation of five-colony pool analysis using multiplex real-time PCR for detection of diarrheagenic Escherichia coli. *J Clin Microbiol.* 2009;**47**(6):1915–7.
2. Kagkli DM, Folloni S, Barbau-Piednoir E, Van den Eede G, Van den Bulcke M. Towards a pathogenic Escherichia coli detection platform using multiplex SYBR(R)Green Real-time PCR methods and high resolution melting analysis. *PLoS One.* 2012;**7**(6).
3. Karimi Darehabi H, Naseri MH, Menbari S, Mobaleghi J, Kalantar E. Antibiotic Resistance Pattern of Escherichia coli Groups A, B1, B2 and D Isolated from Frozen Foods and Children with Diarrhea in Sanandaj, Iran. *Int J Entric Pathog.* 2013;**1**(1):2.
4. Nayani S. Study on the Prevalence of Virulent genes in Escherichia coli isolated from Fish using multiplex PCR. *Helix.* 2013;**2**:316–9.
5. Kalantar E, Alikhani MY, Naseri MH, Torabi V. Antibiotic resistance patterns of STEC and ETEC strains: A study on frozen foods of animal origin and children with acute diarrhea. *JMID.* 2013;**3**(1):31–5.
6. Hegde A, Ballal M, Shenoy S. Detection of diarrheagenic Escherichia coli by multiplex PCR. *Indian J Med Microbiol.* 2012;**30**(3):279–84.
7. Guion CE, Ochoa TJ, Walker CM, Barletta F, Cleary TG. Detection of diarrheagenic Escherichia coli by use of melting-curve analysis and real-time multiplex PCR. *J Clin Microbiol.* 2008;**46**(5):1752–7.
8. Kuwayama M, Shigemoto N, Oohara S, Tanizawa Y, Yamada H, Takeda Y, et al. Simultaneous detection of virulence factors from a colony in diarrheagenic Escherichia coli by a multiplex PCR assay with Alexa Fluor-labeled primers. *J Microbiol Methods.* 2011;**86**(1):119–20.
9. Gomez-Duarte OG, Romero-Herazo YC, Paez-Canro CZ, Eslava-Schmalbach JH, Arzuza O. Enterotoxigenic Escherichia coli associated with childhood diarrhoea in Colombia, South America. *J Inf Dev Ctries.* 2013;**7**(5):372–81.
10. Jin Y, Seung H, Oh Y, Jung J, Jeon S, Lee J, et al. Epidemiological Relationship of Enterotoxigenic Escherichia coli and Enteroaggregative E. coli Isolated from Patients with Diarrhea in Seoul. *J Bacteriol Virol.* 2013;**43**(1):37–44.
11. Nataro JP, Kaper JB. Diarrheagenic Escherichia coli. *Clin Microbiol Rev.* 1998;**11**(1):142–201.
12. So M, Dallas WS, Falkow S. Characterization of an Escherichia coli plasmid encoding for synthesis of heat-labile toxin: molecular cloning of the toxin determinant. *Infect Immun.* 1978;**21**(2):405–11.
13. Qadri F, Svennerholm AM, Faruque AS, Sack RB. Enterotoxigenic Escherichia coli in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clin Microbiol Rev.* 2005;**18**(3):465–83.
14. Schultsz C, van den Ende J, Cobelens F, Vervoort T, van Gompel A, Wetsteyn JC, et al. Diarrheagenic Escherichia coli and acute and persistent diarrhea in returned travelers. *J Clin Microbiol.* 2000;**38**(10):3550–4.
15. Nocker A, Camper AK. Selective removal of DNA from dead cells of mixed bacterial communities by use of ethidium monoazide. *Appl Environ Microbiol.* 2006;**72**(3):1997–2004.
16. Yoder JS, Cesario S, Plotkin V, Ma X, Kelly-Shannon K, Dworkin MS. Outbreak of enterotoxigenic Escherichia coli infection with an unusually long duration of illness. *Clin Infect Dis.* 2006;**42**(11):1513–7.
17. Johnson JR, Delavari P, Kuskowski M, Stell AL. Phylogenetic distribution of extraintestinal virulence-associated traits in Escherichia coli. *J Infect Dis.* 2001;**183**(1):78–88.
18. Fischer Walker CL, Sack D, Black RE. Etiology of diarrhea in older children, adolescents and adults: a systematic review. *PLoS Negl Trop Dis.* 2010;**4**(8).
19. Al-Gallas N, Bahri O, Bouratbeen A, Ben Haasen A, Ben Aissa R. Etiology of acute diarrhea in children and adults in Tunis, Tunisia, with emphasis on diarrheagenic Escherichia coli: prevalence, phenotyping, and molecular epidemiology. *Am J Trop Med Hyg.* 2007;**77**(3):571–82.
20. Presterl E, Zwick RH, Reichmann S, Aichelburg A, Winkler S, Kremsner PG, et al. Frequency and virulence properties of diarrheagenic Escherichia coli in children with diarrhea in Gabon. *Am J Trop Med Hyg.* 2003;**69**(4):406–10.
21. Shaheen HI, Khalil SB, Rao MR, Abu Elyazeed R, Wierzba TF, Perusski LF, Jr., et al. Phenotypic profiles of enterotoxigenic Escherichia coli associated with early childhood diarrhea in rural Egypt. *J Clin Microbiol.* 2004;**42**(12):5588–95.
22. Jafari F, Shokrzadeh L, Hamidian M, Salmanzadeh-Ahrabi S, Zali MR. Acute diarrhea due to enteropathogenic bacteria in patients at hospitals in Tehran. *Jpn J Infect Dis.* 2008;**61**(4):269–73.
23. Moriel DG, Rosini R, Seib KL, Serino L, Pizza M, Rappuoli R. Escherichia coli: great diversity around a common core. *MBio.* 2012;**3**(3).
24. Jafari F, Garcia-Gil LJ, Salmanzadeh-Ahrabi S, Shokrzadeh L, Aslani MM, Pourhoseingholi MA, et al. Diagnosis and prevalence of enteropathogenic bacteria in children less than 5 years of age with acute diarrhea in Tehran children's hospitals. *J Infect.* 2009;**58**(1):21–7.
25. Kagambega A, Martikainen O, Siitonen A, Traore AS, Barro N,

Haukka K. Prevalence of diarrheagenic Escherichia coli virulence genes in the feces of slaughtered cattle, chickens, and pigs in Burkina Faso. *Microbiol Open.* 2012;**1**(3):276–84.
26. Mardaneh J, Poor SA, Afrugh P. Prevalence of Shigella species and Antimicrobial Resistance Patterns of Isolated Strains from Infected Pediatrics in Tehran. *Int J Entric Pathog.* 2013;**1**(1):28–31.
27. Souza TB, Lozer DM, Kitagawa SM, Spano LC, Silva NP, Scaletsky IC. Real-time multiplex PCR assay and melting curve analysis for identifying diarrheagenic Escherichia coli. *J Clin Microbiol.* 2013;**51**(3):1031–3.