Gastroenterology and Hepatology From Bed to Bench

BRIEF REPORT

Design of an improved multiplex PCR method for diagnosis of enterohaemoraghic *E.coli* and enteropathogic *E.coli* pathotypes

Mohammad Soleimani, Abbas Morovvati, Seyede Zohreh Hosseini, Mohammad Reza Zolfaghari
Islamic Azad University, Qom Branch, Qom, Iran

ABSTRACT

Aim: We aimed to develop a multiplex PCR assay for specific detection of EPEC and EHEC pathotypes based on specific marker genes.

Background: About 2.5 million infant's morbidity in developing countries occurs by *E.coli* pathotypes because of diarrhea and intestinal diseases. The traditional phenotypic methods are time consuming and sometimes detection and differentiation of the pathotypes are not done easily. Multiplex PCR technology is used as a sensitive, specific and rapid molecular method for detection of various pathogens.

Patients and methods: PCR reactions were performed with primers which targeted the virulence genes selected for each category (stx_1, stx_2 genes for EHEC and *bfpA* for EPEC).For preparation of a positive control, the PCR products were cloned in pTZ57R/T plasmid. The same PCR reactions were done but in presence of genomes of various negative control bacteria for evaluation of test specificity.

Results: As expected, gel agarose electrophoresis of PCR products of the stx_1, stx_2 and *bfpA*, showed 329bp, 586bp and459bp bands respectively. Result of amplification using negative control genomes as template was negative.

Conclusion: The multiplex PCR assay followed by capillary electrophoresis presented in the present paper provides a simple, reliable, and rapid procedure that in a single reaction identifies the four main pathotypes of E. coli. This assay will replace the previous molecular genetics methods used in our laboratory and work as an important supplement to the more time consuming phenotypic assays.

Keywords: Multiplex PCR assay, Diarrheagenic, *Escherichia coli*.

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Introduction

Escherichia coli strains are considered the most abundant commensal bacteria in human gut (1).These bacteria are members of a large group of bacterial germs that inhabit the intestinal tract of humans and other warm-blooded animals (2). Prevalence of diarrheal diseases caused by Diarrheagenic *E.coli* (DAEC) continues to be a health problem worldwide, especially in developing countries (3). *E.coli* isolates are classified on the basis of phenotypic, genotypic characteristics and virulence properties. Enteropathogenic *E.coli* (EPEC), Enterotoxigenic *E.coli* (ETEC), Enteroinvasive *E.coli* (EIEC), Enteroaggregative *E.coli* (EAEC), Diffuselyadherent *E.coli* (DAEC), and Enterohemorrhagic *E.coli* (EHEC) have been validated as the main Diarrheagenic *E.coli* pathogroups (4, 5). Enterohaemoraghic *E.coli* (EHEC) could be found in humans, cattle, and goats (5,6). EHEC are also called Verocytotoxigenic

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Reprint or Correspondence: Mohammad Soleimani, PhD. Islamic Azad University, Qom Branch, Qom, Iran
E-mail: soleimani@yahoo.com

E. coli (VTEC) because of their cytotoxic effect on Vero cells (7).They cause diarrhea, colitis and the hemolytic uremic syndrome (HUS). EHEC causes bloody colitis in approximately 10% of patients (8). Also EHEC cause epidemic, sporadic, and so often fatal infections worldwide. The pathotype contain the *eae* gene for attachment and effacing and*stx1*, *stx2* Shiga toxin genes (9,10). EPEC is causative agent of diarrhea in humans, rabbits, dogs, cats and horses (5,6). Also EPEC is the causative agent of summer diarrhea in infants (11). EPEC Adherence Factor (EAF) and Bundle Forming Pilli (*bfpA*) are major virulence factors (11,12). It is estimated that2.5 million infant deaths occur annually as a result of infection with Diarrheagenic *E.coli* (13).

Given *E.coli* pathotypes are a public health concern, so it is required to develop a rapid, sensitive, and specific diagnostic assay for their identification. Determination of *E.coli* pathotypes by traditional methods is time-consuming and demands technique al expertise (14,15). In addition, cross reaction with other gram negative pathogens may occur (16,17).

In the present study we aimed to develop a multiplex PCR assay for specific identification of EPEC and EHEC pathotypes based on specific marker genes. We selected marker genes exhibiting the highest degree of homology among the accepted sequences found in the databases.

Methods

The bacterial genomes of EHEC and EPEC pathotypes as positive control and genome of some other bacteria as negative control were purchased from the Pasture Institute of Iran.

The target genes were *bfpA* in EPEC and stx_1, stx_2 in EHEC. The stx_1, stx_2 and *bfpA* primers were designed by the Gene runner software (version 3.05) and were synthesized by Sina Clon Bioscience Co. The primer sequences and their loci on the genome and the length of the amplified fragments are presented in table 1.

PCR reactions were done for detection of the three genes in volume of 25 µl. This reaction volume consisted of 2mM Mg^{2+}, 2 mM dNTPs, 100 ng bacterial genome, 0.5 µM forward and reverses primers and 1 unit Taq DNA polymerase enzyme. Amplification using a certain program was done for 35 cycles in conditions of the initial denaturation at 94˚C for 4minutes, denaturation at 94˚C for 1 minute, and annealing temperature of 60˚C for 40 seconds and extension time of 72˚C for 45 seconds. It's notable that the reactions were done for all the three genes in two modes of uniplex and multiplex.

To assess the primers specificity, the PCR reaction was done with the above conditions on the negative control bacterial genomes. Moreover, to make sure about the quality of the extracted DNA used in the PCR reaction and lack of the inhibitor accompanied with the DNA, amplification of *16SrRNA* chromosomal gene which is present in all bacteria was done on the basis of Chiang study (18).

Table1. The primers sequence, the length of the amplified fragments and other their specifications

Pathotype	Target gene	Primer name	Primer sequence	Tm (˚C)	Product length (bp)
EPEC	*bfpA*	F-bfpA	5´-TGCTGCCACCGTTACCGCAG	59.97	459
		R-bfpA	5´-GCAGTTGCCGCTTCAGCAGG	59.16	
EHEC	stx_1	F-stx1	5´-CGCATAGTGGAACCTCACTGACGC	59.89	329
		R-stx1	5´-TGCCATTCTGGCAACTCGCGA	59.39	
	stx_2	F-stx2	5´-TAACCACACCCCACCGGGCA	60.04	586
		R-stx2	5´-GGCCACAGTCCCCAGTATCGC	59.25	

For creating stable positive control, the PCR products were cloned. For this after purification of the products using Accuprep PCR purification kit (Bioneer), ligation reaction between pTZ57R/T plasmid and purified the three genes was done separately according to work instructions of the InsTA clone PCR cloning kit (Fermentas). After preparing competent *E.coli* JM107, the cells were transformed. For final confirmation of the insert receiving clones, enzymatic digestion was done after plasmid extraction by means of Accuprep plasmid mini Extraction kit (Bioneer). The confirmed recombinant plasmids were named pTZ57R/T-*bfpA*, pTZ57R/T-stx_1 and pTZ57R/T-stx_2, representing plasmids contained *bfpA*, stx_1 and stx_2 genes respectively.

To determine the reaction sensitivity; the minimum copy number of the target genes to be able to show a visible bond in the PCR, was calculated. For this reason 10- fold serial dilutions (10^{-1} -10^{-11}) of the pTZ57R/T-*bfpA*, pTZ57R/T-stx_1 and pTZ57R/T-stx_2 plasmids with certain concentration was prepared. After PCR on the serial dilutions of plasmids, the last dilution that showed a visible band was determined as the limit of detection (LOD) of the method. Finally, the DNA concentrations of the last dilutions were calculated and converted as the copy number of the respective gene.

Results

Amplified fragments of the stx_1, stx_2 and *bfpA* with lengths of 329bp, 586bp and 459bp respectively were determined on the agarose gel (Figure 1).

The PCR reaction related to the stx_1, stx_2 and *bfpA* genes, didn't show any band using genomes of control negative bacteria as template (data not shown). It denotes the PCR specificity in this study. Also, the PCR positive result related to *16SrRNA* gene with the length of 475 bp, demonstrated the presence of the PCR inhibitor free DNA in negative control samples (data not shown).

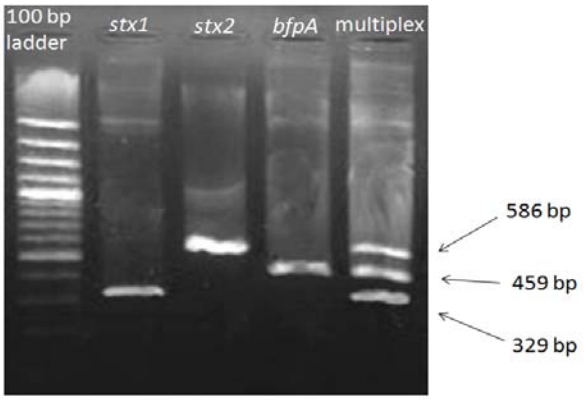

Figure1. Both uniplex and multiplex PCR amplification of stx_1 (329bp), stx_2 (586bp) and *bfpA* (459bp) genes

Results of the enzymatic digestions confirmed right cloning of the target genes and creation the pTZ57R/T-*bfpA*, pTZ57R/T-stx_1 and pTZ57R/T-stx_2recombinant plasmids (data not shown). In sensitivity determination assays, the last dilutions of the pTZ57R/T-*bfpA*, pTZ57R/T-stx_1 and pTZ57R/T-stx_2recombinant plasmids that showed clear band on agarose gel were10^{-5}, 10^{-6} and10^{-9}respectively. Regarding the results, the least detectable copy number for the stx_1, stx_2 and *bfpA* genes in a 25 µl PCR reaction equals 45 and 31 and 545copies respectively.

Discussion

About 2.5 million infant's morbidity in developing countries occurs by *E.coli* pathotypes because of diarrhea and intestinal diseases. Routine tests based on traditional phenotypic methods are so time consuming and cumbersome and sometimes detection and differentiation of the pathotypes are not done easily. On the other hand PCR as a reliable, sensitive and precise method can be considered as an appropriate approach for diagnosis and control of these pathotypes (19). In this study the uniplex and multiplex PCR for rapid detection of *E.coli* pathotypes was designed. Stx_1 and stx_2 plasmid genes for EHEC and *bfpA*

plasmid gene were used for EPEC pathotype. These genes in mentioned pathotypes are unique (20). Cocolin et al. in 2000 used *eaeA* gene for detection of EPEC. Also Yazdi et al. in 2011 used *eaeA* gene in their study for detection of this pathotype. For molecular detection of EPEC in most cases and also in this study the plasmid gene *bfpA* was beenused (20-22). Regarding EHEC, Vidal et al. in 2004 and Maricell et al. in 2005 used stx_1 and stx_2 genes(19, 23).

One of the most important points in Multiplex PCR technique is designing primers that in this study for stx_1gene 12 sequences, stx_2 gene 20 sequences and for the *bfpA* 6 sequences were downloaded from the NCBI sequence database, and then through alignment, the conserved regions were used for designing primers. It's notable that in most other studies just one sequence has been used for designing primers. Another important issue for designing primers is their Tm values which must be the same and the PCR products which must be distinctive on agarose gel. In this study compared with other similar studies all the mentioned points are put into action. In studies by Vidal et al. in 2004 and Kimata et al. in 2005 the PCR products were not differentiable and the band distances were too short (1, 19)but in the current study the design was done in a way that the band distances are at least 100 bp so that they are detectable.

In our study, the high specificity of the designed primers was proved and the negative control bacterial genomes which mostly were from the Enterobacteriaceae family and bacteria that cause the same diseases did not show any amplification. In addition other researchers have used such bacteria for evaluation of the PCR specificity in *E.coli* pathotypes molecular diagnosis.

In this study, the PCR products were cloned in pTZ57R/T plasmid and in addition to usage in consequent steps of the study the confirmed plasmids were used as positive controls. In most studies, the genomic DNA has been used as the positive control, Paton's study in 1998is an example in this regard (10). It's evident that gene cloning in a plasmid vector is a simple way to infinite access to desired amount of the positive control.

One of the most important criteria of the molecular detection kits is the limit of detection. So researches have used various methods for the corresponding calculation. One of these methods is serial dilution from live bacteria and then counting and determination of the colony forming unit (CFU). In this method after genome extraction from different bacterial dilutions the LOD is computed via PCR. Contrary to having high precision, need for using live bacteria, is relatively expensive and may lead to problems such as bacterial growth failure and LOD determination complications. Another LOD approximation method is serial dilution preparation from the genomic DNA and then PCR assay which has low precision being its disadvantage. But gene containing plasmid dilution based methods are less expensive, simpler and faster in addition to being able to directly perform the steps on the template DNA that is one of the best LOD determination methods. Therefore this method was used in the current study. Knowing that the target genes had been cloned in plasmid vectors and preparing serial dilutions from them the LOD was determined for each gene. Accordingly, in a 25 µl PCR reaction the LOD was 45 copies for stx_1 gene, 31 copies for stx_2gene and 545 copies for *bfpA* gene. Cocolin et al. in 2000 determined their ultimate PCR LOD for EPEC and EHEC pathotypes as 10^3 CFU(21). Kong et al. in 1999 determined their ultimate PCR LOD for these pathotype as 10^2CFU(24). Nguyan et al. in 2005 were determined their ultimate PCR LOD for this pathotype as 10^3 CFU in one milliliter (25).

Our diagnostic method is the first report of an optimized M-PCR for the pathotypes in Iran that

while designing primers, with analytical sensitivity and specificity determination, all the criteria necessary for a diagnostic molecular kit are met. Domestication and design of these methods in Iran is an important stride towards flourishing and development of medical diagnostic laboratories for exact diagnosis of these bacteria.

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