Original article

Rapid and simple detection of *Escherichia coli* O157: H7 using multiplex PCR

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Background: *Escherichia coli* O157:H7 is a major foodborne pathogen and can be found globally. Traditional microbiological methods to detect *E.coli* O157:H7 are expensive and time consuming. However, detection assay using polymerase chain reaction (PCR) can be very specific and may be a suitable alternative.
Objective: The present study aimed to evaluate a simple and rapid diagnostic multiplex PCR assay for the selective identification of attaching and effacing genes of *E.coli* O157:H7.
Methods: One-step multiplex PCR using specific primers for eae (Intimin) and rfbE (O157 antigen) genes was carried out. PCR products were analyzed by agarose gel electrophoresis. The specificity of this method was determined using *E. coli* O157:H7, *Salmonella typhi*, *Pseudomonas aeruginosa*, and *Vibrio cholera*. Sensitivity was determined from serial dilutions of *E. coli* O157:H7 genomic DNA.
Results: Specific PCR products of 206 and 497 base pairs were obtained from the amplification of eae and rfbE, respectively. No cross-amplification of other bacterial genes was observed. Assay sensitivity was 380 femtogram.
Conclusion: This multiplex PCR assay can be used as a simple diagnostic test in clinical laboratories for the specific and rapid identification of *E. coli* O157:H7.

Keywords: *Escherichia coli* O157:H7, Detection, Specificity, Multiplex PCR, Sensitivity.

For about 50 years, *Escherichia coli* was not thought to be pathogenic at all, but with the identification of bacterial verotoxins called VTEC (Verotoxin-producing *E. coli*) and the discovery of toxins antigenically and functionally similar to shiga toxin produced by *Shigella dysenterica* (shiga-like toxins, SLTs), *E. coli* was classified into two groups: intestinal pathogenic (IPEC) and entraintestinal pathogenic (EXPEC).[1 - 3] *E. coli* are the two most common etiological agents of moderate-to-severe diarrhea in low-income countries. The pathogen is responsible for hemorrhagic colitis (HC); the disease is characterized by clinical manifestations such as abdominal cramps, watery to grossly bloody diarrhea.[4, 5] In 5.0 – 7.0 % of patients the disease can progress to the stages of hemolytic uremic syndrome (HUS) and disorder is characterized by microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure and thrombotic thrombocytopenic purpura (TTPP).[6, 7] According to a report of the USA Centers for Disease Control (CDC) and Prevention, the highest incidence of *E. coli* O157:H7 is due to food poisoning, which is one of the important cause of death of 525,000 children less than 5 years old each year.[8, 9] As a result rapid pathogen detection methods for raw food materials and processed foods are important for both consumers and producers in the food industry.[10, 11] In *E. coli* O157:H7 serotype, the major genes responsible for bacterial colonization in the gut are located in pathogenicity island area of 43 kbp called LEE (Locus of Enterocyto Effacement). Pathogenic factors include the type 3 secretion system and the proteins secreted in this pathway called Esps (*E. coli* secreted proteins), which are responsible for transmitting messages and events leading to the formation of attaching and effacing (A/E) wounds and formation of cup-like structures in the intestinal epithelial cells and disrupting water and electrolytes balance, and decreased absorption in the intestinal mucosa.[12 - 14] Locus of enterocyto effacement area also contains *eae* genes that encode intimin. Intimin is an important factor in the colonization of bacteria in the intestinal mucosa by binding to transmitter receptor Tir (Translocated Intimin receptor), which is secreted by the type 3 secretory system.[15, 16]

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Today, several methods are used for the identification of virulence factors from *E. coli* O157:H7 such as traditional culture on selective medium and characterization of suspicious colonies by biochemical tests.[17, 18] But multiplex PCR (m-PCR) is considered as one of the rapid, simple, reliable and cost effective tool for detect *E. coli* O157:H7 without enrichment culture.[19 - 21] In the presence work, molecular detection methods such as polymerase chain reaction (PCR) and m-PCR are appropriate alternative techniques for *in vitro* amplification of *eae* and *rfbE* genes. These techniques do not need any antigen and can even be detected in the presence of low levels of toxin genes.

Materials and methods

Bacterial strains and DNA preparation

The bacterial strain used in this study was *E. coli* O157:H7 (ATCC 43894) and were incubated on MacConkey agar (Merk, Germany). Then it was inoculated in 5 mL Luria-Bertani (LB; Merk, Germany) broth at 37 °C for overnight with shaking. On the following day, cells biomass of strain was harvested by centrifugation at 8,000 rpm for 4 min and DNA was extracted according to the manufacturer's handbook of Gene Transfer Pioneers (GTP) Kit (DM04050).

Specific primer design

The sequence of each targeted gene from *E. coli*-specific *rfbE* and *eae* gene, two of the major virulent genes in *E. coli* O157:H7, was obtained from NCBI GenBank, and a homologous search was performed using BLASTN on NCBI's Nucleotide collection (nr/nt) database. Complete coding sequence (Accession No. CP038423), specific primers (Table 1) for *E. coli* O157:H7 were performed with the Gene Runner (Molecular Biology Insights) software.

Gene amplification

After the primers were synthesized, in order to confirm any of the pairs of *rfbE* and *eae E. coli* O157:H7 gene primers, monoplex PCR reaction performed in a volume of 10 µl as follows: (1 µl) DNA template (38 ng/µl), 0.5 µl (10 pM) from each primer, 7 µl master mix of Taq DNA polymerase (amplicon PCR kit, Denmark) and (1 µl) double distilled water. The PCR conditions were set as denaturation at 94°C for 4 min, 30 cycles of 94°C for 1 min, 58°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min.

Multiplex PCR

The m-PCR mixture was adjusted to 10 µL and was carried out similarly to monoplex PCR with (0.5 µl) from *rfbE*-F and *rfbE*-R, F-eae and R-eae primers at one step tube. Finally, the amplified products were analyzed on 1.5% agarose gel electrophoresis.

Sensitivity and specificity of the m-PCR

Sensitivity of the m-PCR was determined with serial dilutions of *E. coli* O157: H7 DNA genomic in the range of 3.8 ng to 0.38 pg were prepared and utilized. On the other hand, the specificity of the m-PCR was determined according to a different bacteria containing *Salmonella typhi*, *Pseudomonas aeruginosa*, *Vibrio cholerae*. The results were analyzed by 1.5% agarose gel electrophoresis.

Table 1. Particulars of the primers used for monoplex and multiplex PCR screening reaction.

Primer name gene	Target gene	Primer sequence (52 – 32)	Amplicon size (bp)
Escherichia	***eae***		
eae-F		ATGGAACGGCAGAGGTTA	206
eae-R		AATGAAGACGTTATAGCCCA	
Escherichia	***rfbE***		
rfbE-F		AAG ATT GCG CTG AAG CCT TTG	497
rfbE-R		CAT TGG CAT CGT GTG GAC AG	

Results

After the DNA extraction (Figure 1A), we detected the *rfbE* and *eae* gene of *E. coli* O157:H7, individually according to monoplex PCR (Figure 1B). NCBI-BLAST analysis of this sequence shows that it has 100.0% homology with all the serovars of *E. coli* O157:H7 genus and 0% with all non- *E. coli* O157:H7 strains. The presence of 497 bp and 206 bp segments in agarose gel suggests *rfbE* and *eae* genes in the sample, respectively (Figure 1C). In order to determine the sensitivity of this m-PCR method, the serial dilution of *E. coli* O157:H7 genome (3.8 ng - 0.38 pg) used. PCR product analysis showed that amplification of two segments performed until 380 fg (Figure 2). Also, when different strains of bacteria such as *Salmonella typhi*, *Pseudomonas aeruginosa* and *Vibrio cholerae* used in this method, the results showed that the 206 bp and 497 bp segments was only detected *E. coli* O157:H7 containing *rfbE* and *eae* genes. But in the case of other samples, there were no band, which reveals the specificity of the designed primers (Figure 3). To assess this assay for native materials, *E.coli* O157-infected meat that artificially exposed with this bacteria were used for DNA extraction and PCR according to Figure 4, suitable results were achieved from this assay.

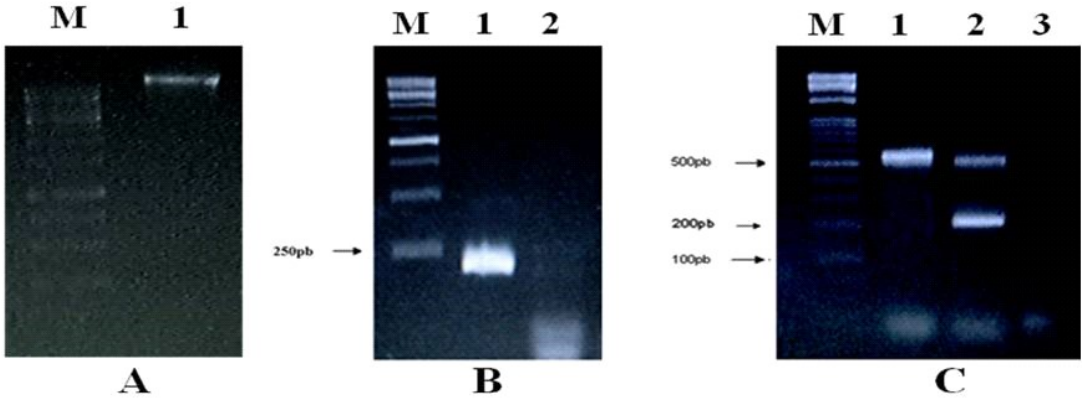

Figure 1. Visualization of 1.5% agarose gel electrophoresis. (A) DNA extracted from *E. coli* O157:H7. Lane 1, Bacterial genomic DNA extract from *E. coli* O157:H7. (B) Monoplex and multiplex PCR products analysis. Lane 1, *eae* gene amplicon (206 bp). Lane 2, negative control. (C) Monoplex PCR product analysis. Lane 1, *rfbE* gene (497 bp). Lane 2, *eae* gene and *rfbE* gene amplicons. Lane 3, negative control. M denotes 1 kb and 100 bp DNA ladder (Fermentas).

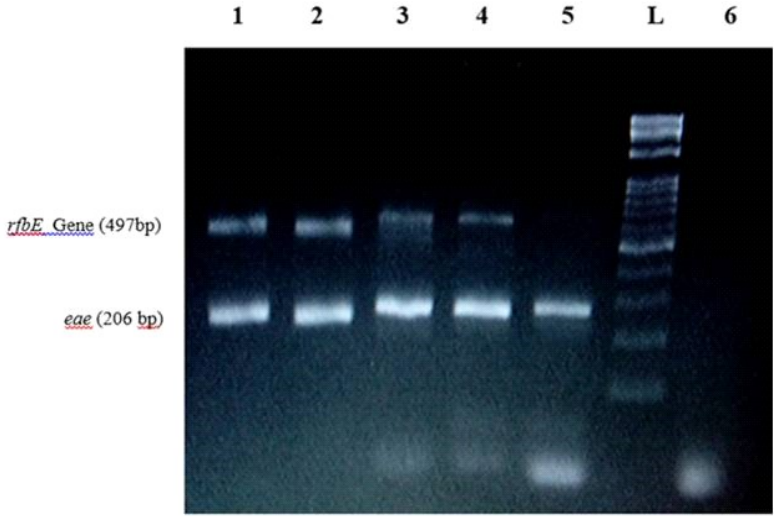

Figure 2. Visualization of 1.5% agarose gel electrophoresis in order to determination of the sensitivity of multiplex PCR using DNA serial dilutions. Lanes1: 3.8 ng, lane 2: 0.38 ng, lane 3: 38 pg, lane 4: 3.8 pg, lane 5: 0.38 pg, lane 6: negative control (without DNA), L denotes 100 bp DNA ladder (Fermentas).

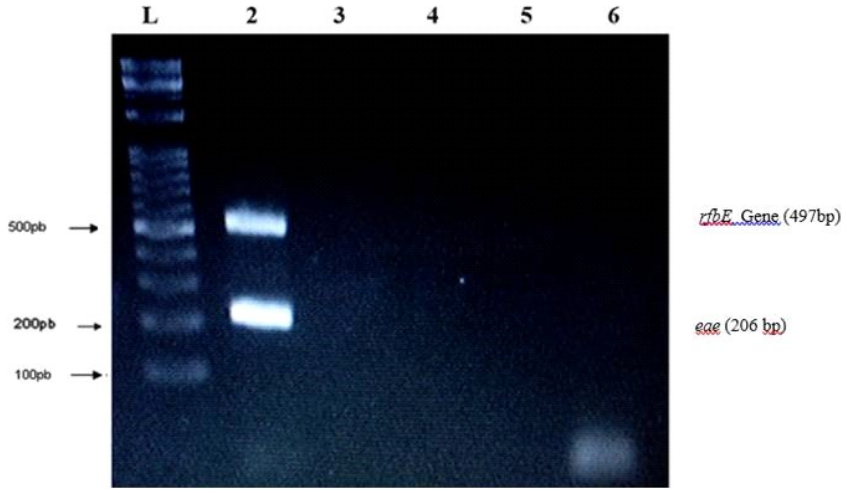

Figure 3. Visualization of 1.5% agarose gel electrophoresis for determination of the specificity of multiplex PCR using *eae* and *rfbE* gene-positive and negative strains. Lane 2: *E. coli* O157:H7; lane 3:*Salmonella typhi;* lane 4: *Pseudomonas aeruginosa;* lane 5:*Vibrio cholerae;* lane 6: negative control, L denotes 100 bp DNA ladder.

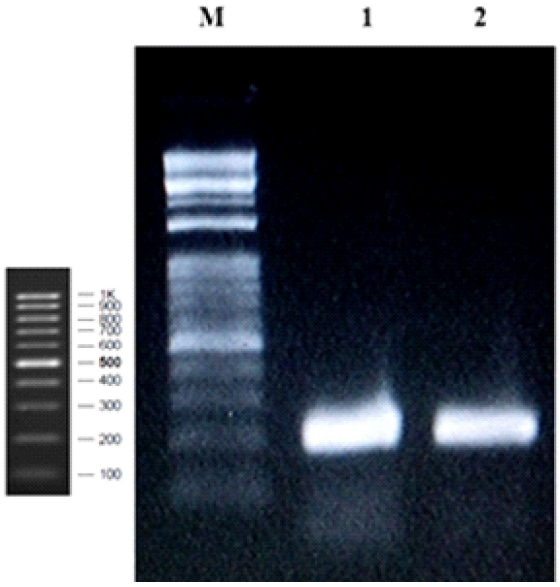

Figure 4. Visualization of 1.5% agarose gel electrophoresis for genome extraction on artificially-infected meat with *E. coli* O157:H7. Lane 1, 2: PCR products using eae-F and eae-R primers on genome extraction; M denotes 100 bp DNA ladder.

Discussion

E. coli O157:H7 is the most isolated strain among VTEC-related infections that an opportunistic and is the deadly and common prev-alent pathogen in foodborne-outbreaks in Europe, North America, India and Japan.[22,23] This pathogen has been isolated from a variety of food products such as beef, vegetables, chicken meat and other sources.[1-3] For this reasons, the World Health Organization (WHO) has been always emphasized the annual screening of such bacteria.[9] Traditionally, conventional microbiological culture methods such as selective enrichment plates media or biochemical tests as the gold standard though the most common methods for the detection of foodborne pathogens have been approved by the ISO.[24] But these methods are very time consuming, not sensitivity and there is a possibility to get false results therefore other specificity detection methods, such as multiplex PCR and loop-mediated isothermal amplification could potentially reduce the time of

analysis foodborne pathogens and these methods are the suitable alternatives compared to the conventional assay.[25 - 27] Multiplex PCR technology with high sensitivity and specificity has been widely applied to quickly diagnosis of multiple food-borne pathogens in a single reaction tube at the same time.[28] In the present study, we designed the two pair of primers for conserve regions of *E. coli* O157:H7 genomes that have successfully amplified *rfbE* and *eae* gene in order to detect of this pathogen. The *rfbE* gene encodes O157 antigen and *eae* gene is encoding the intimin protein, which is responsible for attachment and colonization of *E. coli* O157:H7.[15, 16] Two pairs of specific primers designed in this study were used for the multiplex PCR assay. The amplicons observed on agarose gel electrophoresis were of the size, 497 bp and 206 bp, respectively for *rfbE* and *eae* genes. There are previous investigations to develop multiplex PCR for the identification of *E. coli* O157:H7. Shinde DB, *et al.,* characterized the *E. coli* serotype O157:H7 in healthy Indian cattle by multiplex PCR using primers for four genes; *rfbE (O157), fliC (H7), VT1 (MK1), and VT2 (MK2)* genes.[23] Anglès d'Auriac MB, *et al.,* have investigated all tested 23 strains of *E. coli* and *S. dysenteriae* by using *wecA* primers, but did not report the numerical sensitivity.[20] Amézquita-Montes Z, *et al*., identified non-O157 STEC and ETEC in food products, especially ground beef by multiplex PCR targeting 2 virulence genes, namely *eae and bfpA* genes.[29] In 2019, Manage and co-workers reported that named cassette PCR was used for detection of pathogenic *E.coli* on potentially contaminated beef and it was compared with conventional liquid PCR. The data indicated that cassette PCR had 98.8% similarity with parallel common PCR for detection of STEC genes such as O157, eae, stx1 and stx2.[30] Also, Pinaka O, *et al*. used multiplex PCR and ELISA for screening detection of shiga toxin-producing colonies from *E.coli* O157:H7and non-O157 in animal feces, vegetables and humans.[31] However, in this research work, we verified that the specificity and sensitivity of these primers are suitable for the setup the multiplex PCR system. The sensitivity of m-PCR system is 380 fg/μL. Additionally, each pair of primers *rfbE* and *eae* demonstrated positive amplification only when the gene target was present. Also, artificially infection of meat with *E.coli* O157 was indicated that this simple PCR can be used for screening detection of real samples.

Conclusion

This rapid and reliable molecular detection method could be used for the design and development of a molecular detection kits in order to identify *E. coli* O157:H7.

Conflict of interest

The authors, hereby, declare no conflict of interest.

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