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Optimization of multiplex PCR conditions for rapid detection of *Escherichia coli* O157:H7 virulence genes

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Abstract: The main source of *E. coli* 0157:H7 is cattle, but recent studies showed high percentage of outbreaks contributed by contaminated water. The occurrence of *E. coli* O157:H7 in environmental water samples poses a potential threat to human health. The aim of this study was to establish a protocol for the detection of the pathogen *E. coli* O157:H7 and *E. coli* virulence genes (*eaeA, rfbE, hly,* stx_1, and stx_2) in a multiplex PCR protocol using six specific primer pairs. The target genes produced species-specific amplicons at 625 bp, 397 bp, 296 bp, 166 bp, 210 bp and 484 bp for *E. coli* O157:H7 ($fliC_{h7}$ gene) and virulence genes (*eaeA, rfbE, hly,* stx_1, and stx_2) respectively. The results obtained show that the established PCR protocol is suitable for a rapid and specific analysis of the pathogenic *E. coli* O157:H7 in environmental water samples for the assessment of microbiological risks.

Keywords: ***Escherichia coli*** **O157:H7, multiplex PCR, optimization**

Introduction

E. coli O157:H7 is a part of the enterohemorrhagic group of *E. coli* (EHEC). This pathogen produces verotoxins that can cause thrombotic thrombocytopenic purpura (TTP), hemorrhagic colitis and hemolytic ureamic syndrome (HUS) (Law, 2000). Hemolytic ureamic syndrome (HUS), a life threatening complication that causes kidney failure, is developed by about 10% of patients, mostly in elderly people and children (Blackall and Marques, 2004).

In the year 1982 in US, a hemorrhagic colitis outbreak caused by hamburger consumption resulted in *E. coli* O157:H7 to be first recognized as an important human pathogen. Since then, numerous foodborne cases throughout the world for example in countries like Scotland, Japan, Canada and UK have been linked with this pathogen. In addition, *E. coli* O157:H7 is recognized as one of the most significant foodborne pathogen relating public health especially in South Africa, Europe, Japan and US (Hodges and Kimball, 2005).

Six genes of *E. coli* O157:H7 are generally targeted for PCR confirmation, namely *rfbE* (O157 antigen), *eae* (intimin), stx_1 (Shiga toxin 1), stx_2 (Shiga toxin 2), *hlyA* (hemolysin) and $fliC_{h7}$ (flagellar antigen) (Chapman, 2000). *E. coli* O157:H7 is able to form vero toxins and this virulence factor is encoded by stx_1 and stx_2 genes respectively. The gene *eaeA* encodes intimin, responsible for adherence of this pathogen to the intestinal lining and causing human illnesses. Meanwhile, hemolysin is encoded by *hlyA* gene (Boerling *et al.,* 1999), 0157 antigen by *rfbE* gene and flagellar antigen by $fliC_{h7}$ gene (Felds *et al.,* 1997).

Although the primary reservoirs of this pathogen are cattle and meat products, contaminated water has also been responsible for infection. Outbreaks of this pathogen in Japan, US and Europe, have been reportedly caused by contaminated drinking water (Bertrand and Roig, 2007). For humans, a minimal cell count of about 10–100 are sufficient to cause serious complications (Keene *et al.,* 1994).

E. coli 0157:H7 outbreaks are on the rise, hence it is important to develope a sensitive, rapid, and species-specific method to identify this pathogen in water and food. Commercial kits are available in the market for detection but is still deemed time consuming as they require long enrichments prior to detect microorganisms. Thus, a sensitive and rapid technique for detection of this pathogen is required. Recently, traditional microbiological culturing techniques are being replaced by polymerase chain reaction (PCR) based techniques for the identification and detection of *E. coli* 0157:H7 as it is less laborious and saves significant amount of time (Johnson *et al.,* 1995). PCR requires a small amount of DNA unlike the large numbers required for genetic-based molecular diagnostic methods (Feng, 1993). According to Shah *et al.,* (2009), PCR assays are proven specific and sensitive in detecting microbial pathogens such as *E. coli* 0157:H7. Several multiplex

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PCR protocols have been developed in the past to detect the major virulence genes of *E.coli* O157:H7 namely *rfbE, fliC*$_{h7}$, *eaeA, hlyA, stx*$_1$, and *stx*$_2$ in different combinations (Shah *et al.,* 2009; Fagan *et al*., 1999; Bai *et al.,* 2010). However, a procedure of multiplex PCR to detect all six *E. coli* 0157:H7 genes from water samples is lacking.

Therefore, the purpose of this study was to develop a rapid, sensitive, species-specific and reliable multiplex PCR procedure for the detection of pathogenic *E. coli* 0157:H7 in water samples by targeting the *fliC*$_{h7}$, *rfbE, eaeA, hlyA, stx*$_1$, and *stx*$_2$ genes. To achieve this purpose, the concentration of the magnesium chloride and primer annealing temperature of the samples were optimized. The optimized multiplex protocol was then tested for specificity and reproducibility.

Table 1. Primer pairs used for the optimization of multiplex PCR.

Primers	Sequences (5'- 3')	Target gene	Amplicon size (bp)	Reference
FLICH7-F	GCGCTGTCGAGTTCTATCGAGC	*fliC*$_{h7}$	625	Sarimehmetoglu *et al.*, 2009
FLICH7-R	CAACGGTGACTTTATCGCCATTCC			
rfbE-F	CAGGTGAAGGTGGAATGGTTGTC	*rfbE*	296	Bertrand *et al.*, 2007
rfbE-R	TTAGAATTGAGACCATCCAATAAG			
SLT1-F	TGTAACTGGAAAGGTGGAGTATACA	*stx*$_1$	210	Sarimehmetoglu *et al.*, 2009
SLT1-R	GCTATTCTGAGTCAACGAAAAATAAC			
SLT11-F	GTTTTTCTTCGGTATCCTATTCC	*stx*$_2$	484	Sarimehmetoglu *et al.*, 2009
SLT11-R	GATGCATCTCTGGTCATTGTATTAC			
AE22	ATTACCATCCACACAGACGGT	*eaeA*	397	Sarimehmetoglu *et al.*, 2009
AE20-2	ACAGCGTGGTTGGATCAACCT			
MFS1-F	ACGATGTGGTTTATTCTGGA	*hly*	166	Sarimehmetoglu *et al.*, 2009
MFS1-R	CTTCACGTCACCATACATAT			

Materials and Methods

Bacterial strains and culture conditions

The *E. coli* 0157:H7 strains that were used to conduct the optimization of multiplex PCR conditions were acquired from the American Type Culture Collection (ATCC; Rockville, MD). The strains were stored at -20˚C in modified Tryptic Soy Broth (mTSB (TSB + novobiocin); Merck, Darmstadt, Germany) containing 25% glycerol. For experiment purposes, the strains were then incubated in modified Tryptic Soy Broth at 37˚C overnight.

DNA template preparation

A modified boiled cell method (Tunung *et al.,* 2007; Chai *et al.,* 2007) was used to extract the genomic DNA from the grown strains. One millilitre of the culture broth was centrifuged at 13,200 x g for 2 min. The supernatant was thrown away and the cell pellet was resuspended in 500 µl of sterile distilled water followed by vigorous vortexing. Next, the homogenized cell suspension was boiled for 10 min; cooled at -20˚C for 10 min; and centrifuged again at 13,200 x g for 2 min. The supernatant, comprising DNA, was used to optimize of the multiplex PCR conditions.

PCR amplification

A 96-Well Veriti™ Thermal Cycler (Applied Biosystems, Foster City, CA) was used to perform the multiplex PCR protocol in a volume of 25 µl of reaction mixture containing 0.5 µl of *Taq* DNA Polymerase, 2.0 µl of DNA template solution, 5.0 µl of 5 x reaction buffer, 0.5 µM of deoxynucleoside triphosphates (dNTPs), 0.2 µM each of the 12 primers (6 primer pairs) and magnesium chloride ($MgCl_2$) (concentrations were optimized). Sterile distilled water was added accordingly to the 25 µl reaction mixture. Thermal cycling consisted of a 2 min initial denaturation at 94˚C and followed by 35 cycles of denaturation at 94˚C for 20 s, 1 min of annealing at 60˚C, and extension for 1 min at 72˚C, with a 10 min final extension at 72˚C followed by maintenance at 4˚C.

Agarose gel electrophoresis

From each PCR product an aliquot of 4 µl was subjected to 1.0% agarose gel electrophoresis containing 0.5 x TBE buffer (pH 8.0) and ethidium bromide was used to stain the gel. Electrophoresis was carried out at 80 Volt, 400 mA for 40 min with 0.8 µl of 100 bp DNA marker. The DNA bands were observed under ultraviolet (UV) light using gel documentation system (Syngene).

Optimization of multiplex PCR

The multiplex PCR parameters that were optimized included annealing temperature and magnesium chloride ($MgCl_2$) concentration while

other parameters were kept at constant. The annealing temperature was evaluated between 50°C to 60°C while the magnesium chloride ($MgCl_2$) concentration was varied between 1.5 mM to 3.5 mM.

Results

The parameters, annealing temperature and $MgCl_2$ concentration were optimized for *E. coli* 0157:H7 and *E. coli* 0157:H7 virulence genes detection. Figure 1, 2 and 3 showed the amplicons obtained using gel electrophoresis by optimizing the $MgCl_2$ concentration and annealing temperature of the ATCC samples. Bands observed are of genes $fliC_{h7}$ (625bp), *hly (*166bp), stx_1 (210bp), stx_2 (484bp), *rfbE* (296bp) and *eaeA* (397bp) and amplified by primer pairs FLICH-F/FLICH-R, MFSI-F/MFSI-R, SLTI-F/SLTI-R, SLTII-F/SLTII-R, rfbE-F/rfbe-R and AE22/AE20-2 respectively.

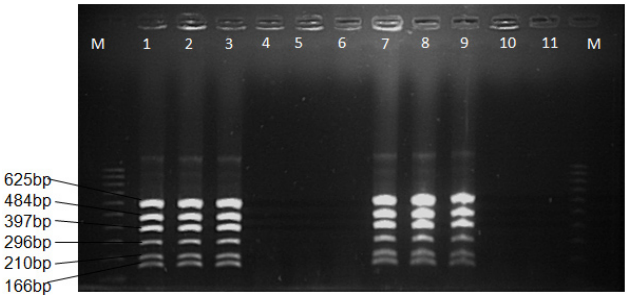

Figure 1. Gel electrophoresis image of amplicons showing the optimization of annealing temperature and $MgCl_2$ concentration for *E. coli* 0157:H7 and *E. coli* 0157:H7 virulence genes detection. Lane M: DNA ladder (100 bp), lanes 1-6: amplicons obtained using 1.5 mM $MgCl_2$ and lanes 7-11: amplicons obtained using 2.0 mM $MgCl_2$. The annealing temperatures for lane 1 and 7 was 50°C; 52°C for lane 2 and 8; 54°C for lane 3 and 9; 56°C for lane 4 and 10; 58°C for lane 5 and 11; and 60°C for lane 6

It was observed that the optimum $MgCl_2$ concentration for the multiplex PCR detection of *E. coli* O157:H7 and *E. coli* virulence genes ranged from 2.5 to 3.5 mM. However the bands observed at 3.0 mM of $MgCl_2$ (Figure 2 and Figure 3) showed the best results while there were no amplifications observed at some lanes with 1.5, 2.0 (Figure 1) and 2.5 mM $MgCl_2$ (Figure 2) Annealing temperature in the thermal cycling process were tested in temperatures ranging from 50°C to 60°C. Amplifications were most consistent at annealing temperatures of 50°C to 54°C for all ranges of $MgCl_2$ concentration tested (1.5-3.5 mM) however annealing temperature of 60°C was deemed the most suitable for the multiplex PCR as this temperature showed the best amplicons at 2.5 to 3.5 mM of $MgCl_2$.

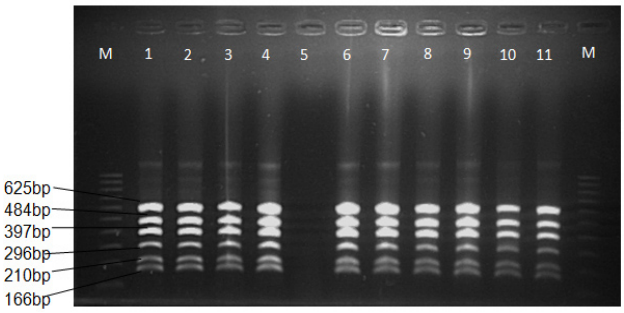

Figure 2. Gel electrophoresis image of amplicons showing the optimization of annealing temperature and $MgCl_2$ concentration for *E. coli* 0157:H7 and *E. coli* 0157:H7 virulence genes detection. Lane M: DNA ladder (100 bp), lane 1: amplicons obtained using 2.0 mM $MgCl_2$, lanes 2-7: with 2.5 mM $MgCl_2$ and lanes 8-11: with 3.0 mM $MgCl_2$. The annealing temperatures for lane 1 and 7 was 60°C; 50°C for lane 2 and 8; 52°C for lane 3 and 9; 54°C for lane 4 and 10; 56°C for lane 5 and 11; and 58°C for lane 6

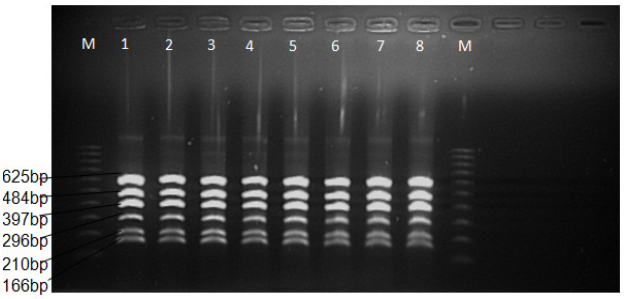

Figure 3. Gel electrophoresis image of amplicons showing the optimization of annealing temperature and $MgCl_2$ concentration for *E. coli* 0157:H7 and *E. coli* 0157:H7 virulence genes detection. Lane M: DNA ladder (100 bp), lanes 1 and 2: amplicons obtained using 3.0 mM $MgCl_2$ and lanes 3-8: with 3.5 mM $MgCl_2$. The annealing temperatures for lane 1 and 7 was 58°C; 50°C for lane 3; 52°C for lane 4; 54°C for lane 5; 56°C for lane 6; and 60°C for lane 2 and 8

The optimized multiplex PCR reaction protocol for *E. coli* 0157:H7 and *E. coli* 0157:H7 virulence genes detection contained the following: 0.5 µl of *Taq* DNA Polymerase, 2.0 µl of DNA template solution, 5.0 µl of 5 x reaction buffer, 0.5 µM of deoxynucleoside triphosphates mix (dNTPs), 11.6 µl of sterile distilled water, 0.2 µM each of the 12 primers (6 primer pairs), namely FLICH-F/FLICH-R, SLTI-F/SLTI-R, SLTII-F/SLTII-R, rfbE-F/rfbe-R, AE22/AE20-2 and MFSI-F/MFSI-R (total volume 2.4 µM) and 3.0 mM of magnesium chloride ($MgCl_2$) in a 25 µl total reaction mixture. Thermal cycling conditions were: 2 min of initial denaturation at 94˚C; followed by 35 cycles: 20s of denaturation at 94˚C, 1 min of primer annealing at 60˚C, and 1 min of extension at 72˚C; followed by final extension for 10 min at 72˚C; and maintenance at 4˚C.

Discussion

The detection of the pathogen *E. coli* O157:H7 is vital as incidence rate involving it in food and water samples is on the rise, causing a number of illnesses. In this study, we developed a rapid, reliable and specific method to successfully detect this microorganism in

water samples by analysing the six major virulence genes.

The genomic DNA extraction method applied should be rapid, simple, not hazardous and does not influence the success of the PCR. In addition, to minimise the possibility of contamination, few steps as possible should be involved (Sepp *et al.,* 1994). We applied the boiling cell method for DNA extraction, as it is effective in obtaining the genomic DNA of pathogenic bacteria (Park *et al.,* 2009). The boiling cell method has been used previously to extract genomic DNA from *E. coli* O157:H7 (Bai *et al.,* 2010), *Vibrio parahaemolyticus* (Lesley *et al.,* 2005) and *Campylobacter* spp. (Chai *et al.,* 2007). Amplicons observed (Figures 1-3) using the DNA template proves boiling cell method is reliable, rapid and simple for the extraction of genomic DNA from *E. coli* O157:H7.

The type of food matrix being sampled and presence of pathogenic microorganisms in low number often make it harder to detect, identify and quantify foodborne pathogens. However, the applications of polymerase chain reaction have made the tasks of detecting these pathogens simpler and faster (Toze, 1999). Meanwhile, the process of multiplex PCR involves designing each primer set in a single PCR mixture to amplify amplicons that are specific to the target DNA sequences. The alteration of multiplex PCR parameters such as *Taq* DNA polymerase, primers concentration, Mg^{2+} and deoxyribonucleoside triphosphates (dNTPs) permits the formation of the desired genes. Furthermore, the annealing temperature of the multiplex PCR reaction is optimized to achieve distinct bands for each primer sets (Elizaquivel and Aznar, 2008).

It is essential to modify the $MgCl_2$ concentrations in order to achieve a reliable PCR procedure for the identification of pathogenic foodborne microorganisms. Free magnesium ion will react with free dNTPs to form soluble complexes to synthesis the PCR products. Too little free magnesium ion present when using *Taq* DNA polymerase, will yield in low or no PCR product, while a variety of unwanted products, primer-dimer artifacts and misincorporation will be promoted if too much free magnesium ion are present. Besides that, a high annealing temperature and a balanced ratio of dNTP concentrations and free magnesium ion will result final products with higher fidelity (Roux, 1995). After testing the $MgCl_2$ at different concentrations, we concluded that 3.0 mM was the most suitable as it produced amplicons with the most even intensities (Figure 3).

Annealing temperature has been identified as a crucial parameter during the optimization of PCR protocol, as it is easily measured and modified. Moreover, nonspecific amplification can be reduced by optimizing the annealing temperature of a PCR procedure. Annealing temperature for PCR amplification is related to the melting temperature of the primers utilized as it is usually more or less than 5°C of the melting temperature of the primers. The annealing temperature is normally increased in increments of 2°C to 5°C in subsequent runs if unwanted products are observed. To a greater extent, high annealing temperature will result in greater specificity. Our study portrayed the best amplicons at high annealing temperature of 60°C (Figure 3). This contradicts with the results of Sipos *et al.* (2007) that the better results were observed at lower annealing temperature than higher annealing temperature. This difference in outcome may have been caused by the method of sample loading used. In this study, samples were loaded in the wells of agarose gel, in contrast with the capillary electrophoresis and automated sample loading applied in Sipos *et al.* (2007).

According to Scheu *et al.* (1998), primer (oligonucleotides) sequences which are unique for the target species determines specificity in detecting a microorganism. The *rfbE* gene is a fifth gene in a 12-rfb gene cluster and is responsible for the O157 antigen biosynthesis. Moreover, this gene separates 0157 serotypes of *E. coli* from non-0157 *E. coli* serotypes. The *rfbE* gene has been used in previous studies for the identification of the 0157 serotypes but results in cross-reactions with some non-0157 *E. coli* strains (Paton and Paton, 1998; Chapman *et al.,* 2001). In this study, we utilized primers similar to those from Bertrand and Roig (2007), which resulted in specific amplicons of the 0157 *E. coli* serotype. The ability to form verotoxins, which are toxic to Vero cells, are the main virulence factors of *E. coli* O157:H7, encoded by the stx_1 and stx_2 genes respectively. Hence, the primer pairs SLTI-F/ SLTI-R and SLTII-F/SLTII-R, encoding stx_1 and stx_2 genes were observed to be specific for *E. coli* O157:H7. Furthermore, the primer pair FLICH7-F / FLICH7-R was specific for *E. coli* O157:H7 and the flagellar H7 gene is encoded by $fliC_{h7}$ (Felds *et al.,* 1997) In addition, the primer pairs MFS1-F/MFS1-R (*hlyA* gene) and AE22/AE20-2 (*eaeA* gene), are responsible for enterohemolysin (Boerling *et al.,* 1999) and intestinal related illnesses (Law, 2000) respectively were also specific to *E. coli* O157:H7.

In previous studies, notably by Hu *et al.* (1999) and Fratamico *et al.* (2000), a five-gene multiplex PCR protocol to detect $fliC_{h7}$, stx_1, stx_2, *eae, hlyA and rfbE* in different combinations was developed. Apart from that, Bai *et al.* (2010) has successfully managed

to identify all six genes as in our study. The results of Bai *et al.* (2010) correlates with this study as all six genes namely *fliC*$_{h7}$, *stx*$_1$, *stx*$_2$, *eae, hlyA and rfbE* were detected in a multiplex PCR procedure. Nevertheless, through this study we developed a protocol to detect the presence of all these six *E. coli* O157:H7 genes in water samples using multiplex PCR by combining different sequences of oligonucleotides from Sarimehmetoglu *et al.* (2009) and Bertrand and Roig (2007).

In conclusion, *E. coli* O157:H7 is a pathogenic microorganism that can cause several human foodborne illnesses with severe complications and thus cannot be neglected. Furthermore, cases of this pathogen being reported in water samples are increasing steadily. Through this study we were successful in developing a multiplex PCR method that detects all of the 6 major virulence genes belonging to *E. coli* O157:H7 from water samples. This particular typing method is rapid, specific and reliable to detect the presence of *E. coli* O157:H7 for future surveillance studies and sensitive screening in monitoring cases of outbreaks.

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