Multiplex Polymerase Chain Reaction (PCR) efficiency in detection of pathogenic *Escherichia coli* O157:H7

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Abstract

Shiga toxin-producing *E. coli* (STEC) is an important foodborne pathogen causing diarrhea, hemorrhagic colitis and hemolytic-uremic syndrome in humans. STEC is an implicated in the vast majority of outbreaks, widely via consumption of STEC contaminated beef, as important vehicle of transmission of this organism to human. The *E. coli* O157:H7 serotype is traditionally identified by serological identification of the somatic antigen (O157) and structural flagella (H7). In this study, the bacteria were identified as STEC serotype O157:H7 with three primer pairs that amplified fragments of *secD*, *rfbE* and *fliC* genes in PCR assays. These primer pairs specifically amplified different sizes of target genes: a 244bp region of the *E. coli* diagnostic marker gene (*secD*); a 317bp region of the O157 lipopolysacharide (LPS) gene (*rfbE*); and a 381bp region of the H7 flagellin gene (*fliC*). The singleplex, duplex and triplex PCR assay developed in this study have a sensitivity limit at 2.8×10^3, 2.8×10^5 and 2.8×10^7 CFU/ml of *E. coli* O157:H7, respectively. Sensitivity to detect trace amount of *E. coli* O157:H7 DNA was reduced as the number of primer used was increased for competing to the same DNA template.

Introduction

E. coli O157:H7 serotype is a gram negative bacteria, rod shaped and pathogenic to human. The pathogenecity of shiga toxin-producing *E. coli* (STEC) O157:H7 serotype is associated with virulence genes of the shiga toxin 1 (*stx1*) and shiga toxin 2 (*stx2*) or combinations of both. The *E. coli* O157:H7 infection can cause food poisoning with symptoms like diarrhea, vomiting and fever. In serious infection, it can lead to hemorrhagic colitis and hemolytic uremic syndrome (HUS) (Griffin *et al.*, 1998; Fach *et al.*, 2003). *E. coli* O157:H7 infection into human occurs usually through food contamination of these bacteria in food chain during food preparation and packaging (Son *et al.*, 2001).

In June 2011, the world was shocked by Shiga toxin-producing *E. coli* (STEC) outbreak in Europe. The highest reported number of cases was in Germany with 45 deaths, and more than 3000 cases had been reported. The outbreak in Germany was linked to a novel strain *E. coli* O104:H4 serotype that had acquired the genes to produce shiga toxins (Bielaszewska *et al.*, 2011).

It is important to identify the bacteria contamination at the early stage in the food chain rather than identification after ingestion to prevent infection to human (Shriver-Lake *et al.*, 2007). It is

recommended that when preparing food at home, basic hygiene practices and thorough cooking are essential since STEC is heat sensitive. The Ministry of Health, in its Annual Report in 2007 (MOH, 2007a), reported that the main contributing factor accounting for more than 50% of poisoning episodes in the country is linked to unsanitary food handling procedures. A total of 997 cases were reported throughout the country from the 1st of January to the 2nd of February, 2008 (Soon *et al.*, 2011).

Conventional method for detection of *E. coli* O157:H7 typically require up to 72 hours, involve enrichment and plating on selective media followed by biochemical and serological characterization. These methods are time-consuming and not suitable for routine analysis where speed, sensitivity and quantification are critical. Thus, a rapid and early detection method is essentially required to control the outbreak effectively. The PCR diagnostic technique is a DNA-based detection and is one of the most specific and sensitive method for routine confirmatory assay for the bacterium.

The objective of this study is to compare the efficiency of singleplex, duplex and triplex PCR in detection of *E. coli* O157:H7. The primers used in PCR assay were specific for targeting either *fliC* or *rfbE* and *secD* gene. Genes such as *rfbE* encoding O antigen for O157 serotype and *fliC* encoding H7-

specific flagellar antigen have been frequently used in the detection of *E. coli* O157:H7 by PCR (Bai *et al.*, 2010). Duplex and triplex PCR is also known as multiplex PCR. Multiplex PCR is a diagnostic technique that used two or more primers for simultaneous amplification of bacteria specific genes in one reaction tube.

Several multiplex PCR have been developed with combination of major virulence genes (Tobias and Vutukuru, 2012; Chandra *et al.*, 2013). In this study, major virulence genes such as *stx1* and *stx2* were not chosen as targets in multiplex PCR. This is due to other common STEC serotypes such as O26, O45, O103, O111, O121 and O145 strains have been reported that also produce shiga toxin 1 and shiga toxin 2 (Pradel *et al.*, 2008). Therefore, the significance of targeting for amplifies of virulence genes remains controversial (Gordillo *et al.*, 2011). There is no published procedure on multiplex PCR to detect the combination of diagnostic marker *E. coli* with serotype-specific marker *E. coli* O157:H7. In this study, the bacteria were identified as STEC serotype O157:H7 with combination of both marker genes in multiplex PCR assays to differentiate between pathogenic and non-pathogenic *E. coli* and ensure specificity in the detection of the bacterium.

Materials and Methods

Bacterial strains and culture media

E. coli O157:H7 bacterial isolates was obtained from the Department of Food Science, Faculty of Food Science and Technology, UPM. Non-O157:H7 strains of *E. coli* K64(B9) and K67(B12):H derived from the culture collection of the Department of Microbiology and Parasitology, Faculty of Medicine and Health Sciences, UPM were used as negative controls. Sorbitol MacConkey agar (SMAC) was used for the isolation of *E. coli* O157:H7 following enrichment in Tryptic Soy Broth (TSB) and were used for the DNA isolation.

Inoculation

For a final concentration of 2.8 x 10^2 *E. coli* O157:H7 CFU/25 g ground beef, one ml of 1 x 10^6 *E. coli* O157:H7 serial dilution was added to 225 ml buffered peptone water (BPW). The *E. coli* O157:H7 serial dilutions were then plated (100 µl) in triplicate onto Tryptic Soy Agar (TSA) for enumeration of inoculum. Inoculated BPW (225 ml) was then added to the filter stomacher bag, which were thoroughly hand massaged and then stomached at 190 rpm for 30 s and incubated overnight at 37°C. On the next day, one ml of overnight culture was added to 9 ml

Table 1. The primer sequences used in the multiplex

Primer Pairs	Primer Sequence
secD (forward)	5'-ATCGTGGTGATTGTCATCG-3',
secD (reverse)	5'-ACGCCCATTAATGCTTCAC-3',
rfbE (forward)	5'-TCTTTCCTCTGCGGTCCTA-3';
rfbE (reverse)	5'-CAGGTGAAGGTGGAATGGT-3'
fliC (forward)	5'- ATAATCTACGCCGCCAACT-3',
fliC (reverse)	5'-GACTCCATCCAGGACGAAA-3'.

of EC broth and from there one ml was transferred onto a microcentrifuge tube for DNA extraction. The extracted DNA was labeled as 1 x 10^0 DNA solutions.

Colony Forming Unit (CFU)

To get single colony of *E. coli* O157:H7, 100 µl of the DNA serial dilution was plated in triplicate on Tryptic Soy Agar (TSA) and incubated overnight at 37°C. The CFU/ml of culture was calculated using a formula as follow:

Number of CFU per milliliter (C) = Colony number (N_{CFU}) x Dilution factor (DF) x Volume of sample plated (10^1)

DNA isolation

Total DNA was isolated from one ml of TSB broth culture grown overnight for all the bacterial strains used in the study. DNA was extracted with a DNA isolation kit (Qiagen, Germany) according to the manufacturer's instructions. The genomic DNA was checked for the concentration and purity using spectrophotometer (Shimadzu 1601, Japan).

DNA serial dilution

The 10-fold DNA serial dilution was prepared by diluting the DNA at appropriate concentrations (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}). To perform a 1 : 10 dilution of DNA stock (1 x 10^0), 20 µl of DNA was added to a 180 µl sterile distilled water, followed by a 1 : 10 dilution of 1 x 10^{-1} DNA and so on.

Primer design

The oligonucleotides were derived from the published DNA sequences of *E. coli* using Primer Premier 5.0 software. In multiplex PCR, three primer pairs were designed to amplify the *secD*, *rfbE* and *fliC* gene. The primer sequences used in the multiplex are shown in Table 1.

Multiplex-PCR conditions

The multiplex mixtures were prepared with slight modification according to the manufacturer's instructions (Promega). In general, multiplex mixtures contained 400 µM deoxynucleoside triphosphates, 1X PCR buffer, 5 mM MgCl$_2$, 2.5U of *Taq* DNA polymerase and 2.5 µl of DNA template (serially diluted). Multiplex mixtures comprised primers at the following concentrations: 0.8 µM

secD and 1 µM each of rfbE and fliC. Nuclease free water (NFW) was used as negative control in place of DNA template. DNA amplification was carried out in a thermalcycler (Bioresearch) using an initial denaturation step at 95°C for 1 min, followed by 30 cycles of amplification with denaturation at 95°C for 50 s, annealing at 57°C for 30 s and extension at 72°C for 30 s, ending with final extension at 72°C for 3 min. The fragments were separated on 1.2% agarose gel, followed by ethidium bromide staining and photographed under UV light. DNA molecular size standards (100bp ladder, Vivantis, Malaysia) were included in each agarose gel electrophoresis run.

DNA sequencing

The multiplex PCR products were purified with a GF-1 PCR Clean-up kit, (Vivantis, Malaysia) according to the manufacturer's instruction. The purified multiplex PCR products were sequenced using Big Dye™ Terminator Cycle Sequencing Ready.

Results and Discussion

Multiplex PCR

Identification of *E. coli* O157:H7 isolates by multiplex PCR using primers pairs for *secD*, *rfbE* and *fliC* genes confirmed to amplify a 244-, 317- and 381-bp fragment, respectively. The specific presence of *rfbE* and *fliC* gene in *E. coli* O157:H7 strains differentiate them from other *E. coli* serotypes, *E. coli* K64(B9) and K67(B12):H that lacking this gene (Figure 1). Only *E. coli* carry *secD* gene, which was the internal gene for all *E. coli* species. Thus, *secD* gene can be used as diagnostic marker to distinguish the *E. coli* strains from other bacterial species. The reason *secD* gene was essential in this study was due to the *stx1* and *stx2* genes, which both encoded for Shiga toxin 1 and Shiga toxin 2, respectively, were relatively homogeneous family of toxins that show identity with the Shiga toxins of *Shigella dysenteriae* (Wang *et al.*, 2002) and other common STEC serotypes (Pradel *et al.*, 2008). In addition, other two set of primers was designed to specifically amplify the *fliC* and *rfbE* genes that only carried by *E. coli* O157:H7 strain. Thus, the pathogenic *E. coli* O15:H7 strain can be identified with all designed primer.

Sensitivity of multiplex PCR

In multiplex PCR primer pair was chosen based on similar amplification efficiencies for their respective target. This was achieved through the utilization of primers with nearly identical optimum annealing temperatures (Sanchez *et al.*, 2009).
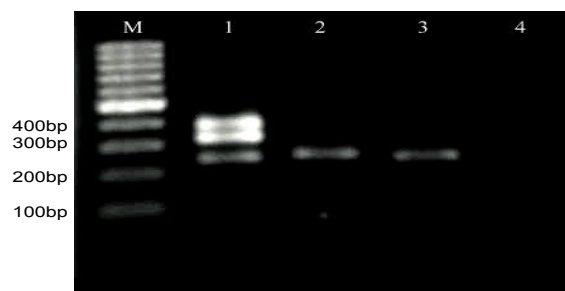

Figure 1. Multiplex PCR amplification of *E. coli* isolates of different strains. Lane 1 = *E. coli* O157:H7 (*fliC*, *rfbE*, and *secD*); Lane 2 = is *E. coli* K64(B9) (*secD*); Lane 3= *E. coli* K67(B12):H (*secD*); Lane 4 = nuclease free water (NFW); M = 100bp DNA marker.

Annealing temperature of all primer pairs were designed to be relatively uniform, which was about 57°C. Special attention to primer design parameters such as homology of primers with their target nucleic acid sequences, their length, the GC content and their concentrations were also considered. To minimize or reduced unspecific binding in multiplex PCR, gradient PCR have been made with each primer pair prior to multiplex PCR. Trial-and-errors have been made to make sure all target bands appears in a good intensity without primer dimers. The optimization included alteration of other PCR components such as PCR buffer constituents, dNTPs and enzyme concentrations in multiplex PCR to improve in the sensitivity or specificity of multiplex PCR (Bai *et al.*, 2010). Multiplex PCR is a chosen method for rapid identification of *E. coli* O157:H7 to species level as it could speed up and simplify the identification procedures to in one working day (Son *et al.*, 2001).

The objective of this study is to simplify identification procedure by simultaneous amplification of several target genes of *E. coli* O157:H7 in a single PCR to identify this pathogen compared to conventional procedure. In this study, multiplex PCR assay was developed for simultaneous amplification of *E. coli* O157:H7 in a single tube and the sensitivity of singleplex, duplex and triplex PCR assay for detection of *secD*, *rfbE* and *fliC* gene were compared. The sensitivity of multiplex PCR was determined using 10-fold serial dilution of beef spiked with 2.8 x 10^2 CFU/ml of *E. coli* O157:H7. The reciprocal of the highest dilution positive by PCR adjusted to concentration CFU per milliliter after DNA extraction is defined as CFU/ml. The specificity of the multiplex PCR for *secD*, *rfbE* and *fliC* is demonstrated by amplicons of the predicted sizes of 244-, 317- and 381-bp fragment, respectively. Following agarose gel electrophoresis, the minimum concentrations of *E. coli* O157:H7 detectable by PCR using one, two and three set of primers were 2.8 x 10^3, 2.8 x 10^5 and 2.8

Table 2. Distance tree of sequencing result. *Escherichia coli* O157:H7 sequenced nucleotides showed high similarity with *Escherichia coli* O157:H7 EDL933 complete genome

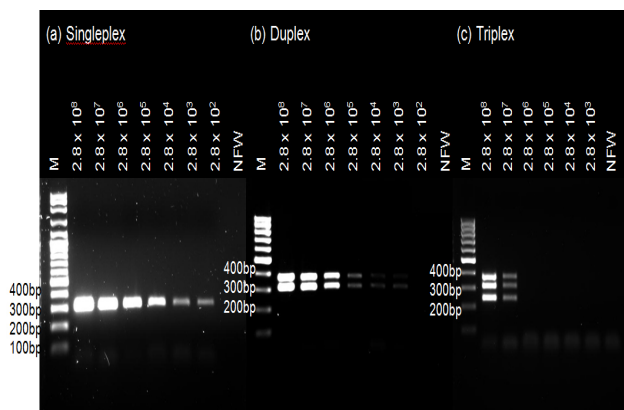|	Primer	Sequencing Size (bp)	Estimated Size (bp)	Homology (%)	Protein Coded
secD	Forward	234	244	98	Secretion
	Reverse	218			membrane
rfbE	Forward	293	317	99	Perosamine
	Reverse	299			synthetase
fliC	Forward	360	381	99	Flagellin
	Reverse	358			

Figure 2. Sensitivity comparison of singleplex (*rfbE*), duplex (*fliC* and *rfbE*) and triplex (*fliC*, *rfbE*, and *secD*) PCR. The concentration of total *E. coli* O157:H7 DNA in series of 10-fold dilution was defined as CFU/ml. Nuclease free water (NFW) was used as negative control in place of DNA template. M = 100bp DNA marker.

x 10^7 CFU/ml, respectively. The sensitivity of PCR was demonstrated by decreasing in amplification capability with increasing in 10-fold DNA serial dilution preparation and primer pairs (Figure 2).

These results showed that the sensitivity of multiplex PCR decreased as the total numbers of primers involved in PCR increased. Fainter band was observed with lesser amount of target DNA and with the increased number of primer pairs in the PCR which translated into reduced PCR sensitivity. The amount of DNA template and primer pairs become the limitation factors in PCR amplification since more diluted DNA required additional amplification time and more primer pairs involved, more competition for the same DNA template occurred. This is in agreement with Gordillo *et al.* (2011), who demonstrated that the detection sensitivities of PCR assays using two set of primer pairs decreased with regard to the serial dilutions of *E. coli* O157:H7 inoculated in a sample of meat product.

Sequencing

The distance tree of deduced sequenced oligonucleotides of *E. coli* O157:H7 is shown in Table 2. Comparison of the sequenced nucleotide sequences of the *E. coli* O157:H7 isolates with *E. coli* O157:H7 EDL933 complete genome exhibited 98% to 99% homology indicating that they are closely

related. There was no significant different between the deduced oligonucleotide sequence of *E. coli* O157:H7 isolates compared with the oligonucleotide sequence *E. coli* O157:H7 EDL933 deposited in gene bank.

Conclusion

There was a general correlation of the number of primer pairs and DNA template concentration used with efficiency in amplification. The efficiency decreased consistently when 10-fold serial dilutions of DNA and more number of primer pairs were used. Therefore, the smaller the amount of template and more number of primer pairs to begin with, the less efficient of the amplification. In conclusion, although the multiplex PCR assay is less efficient than singleplex PCR but it is rapid and amplified simultaneously target genes for specific determination of *E. coli* O157:H7 contamination in food as the amplification occurs in a single tube. The use of rapid and simultaneous detection methods such as multiplex PCR has a great potential for the detection of *E. coli* O157:H7 in food with respect to the monitoring hygiene.

Acknowledgements

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