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Multiplex PCR for Simultaneous Detection of Virulence Genes in *Escherichia coli*

Ke Xin Yu and **Kwai Lin Thong***

Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
* thongkl@um.edu.my (corresponding author)
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ABSTRACT Diarrhea caused by *Escherichia coli* infection is a major cause of public health problems in developing countries. In view of the deficits and limitations of conventional methods of detecting the virulence determinants, a multiplex Polymerase Chain Reaction (PCR) assay was optimized and developed to provide an effective, rapid and specific diagnostic tool to simultaneously detect virulence genes such as heat-stable toxin 1 (ST1), heat-labile toxin 1 (LT1), heat-labile toxin 2 (LT2), verotoxin1 (VT1), verotoxin 2 (VT2) and attachment and effacement (*eaeA*) in pathogenic *E. coli*. Five sets of primers targeting these six virulence genes were optimized by using postitive control *E. coli* strains. The optimized conditions consisted of 3.0 mM of $MgCl_2$, 0.2 mM of dNTPs, 1.5 U of *Taq* DNA polymerase (Promega), 0.70 μM of VT primer, 0.60 μM of LT2 primer and 0.07 μM each of LT1 primer, ST primer and AE primer. The mPCR assay was then applied to a panel of 87 *E. coli* isolates from different sources. One food isolate (EC 375) was positive for *eaeA* gene while another environmental isolate had ST, LT1, *eaeA* and VT genes. The study shows that the mPCR assay is a useful tool to differentiate the pathogenic potential (pathotypes) of *E. coli* by presence of known virulence genes.

ABSTRAK Diarrhea yang disebabkan oleh jangkitan *Escherichia coli* adalah penyebab utama masalah kesihatan masyarakat di negara yang sedang membangun. Oleh itu, memandangkan kekurangan dan limitasi kaedah tradisional, Esei multiplex Polymerase Chain Reaction (PCR) dalam penyelidikan ini memainkan peranan sebagai peralatan yang berguna untuk mengesan kehadiran gen virulen seperti 'Heat-stable toxin 1' (ST1), 'Heat-labile toxin 1' (LT1), 'Heat-labile toxin 2' (LT2), 'Verotoxin 1' (VT1), 'Verotoxin 2' (VT2) dan 'Attachment and Effacement' (*eaeA*) dalam patogenik *E. coli*. Lima pasang primer mengesan enam gen virulen telah dioptimasi dengan menggunakan kawalan positif *E. coli*. Rumusan yang optimal mengandungi $MgCl_2$ sebanyak 3.0 mM, dNTPs sebanyak 0.2 mM, *Taq* DNA polymerase sebanyak 1.5 U (Promega), VT primer sebanyak 0.70 μM, LT2 primer sebanyak 0.60 μM, LT1 primer, ST primer and AE primer sebanyak 0.07 μM masing-masing. Satu sampel makanan telah menunjukkan kehadiran gen *eaeA* dan gen ST, LT1, *eaeA* and VT terbukti hadir dalam satu sampel air. Penemuan dalam penyelidikan ini menunjukkan multiplex PCR ini adalah suatu kaedah yang berpotensi dalam mengesan *E. coli* virulen gen.

(*Escherichia coli*, multiplex Polymerase Chain Reaction, virulence genes)

INTRODUCTION

Escherichia coli is well recognized as the main commensal inhabitant of mammals' gastrointestinal tract. However, it is also associated with several intestinal infections in both humans and animals [1]. Pathogenic *E. coli* strains cause several types of human diarrhea. A few categories of diarrheagenic *E. coli* are now classified on the principle of virulence factors production. These well studied members of diarrheagenic *E. coli* include enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAggEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) [2,3]. Infections associated with these pathotypes are of public health concern.

Enterohemorrhagic *E. coli* (EHEC), also known as Shiga-producing *E. coli* (STEC), has been recognized as a cause of serious diarrhea syndromes associated with hemorrhagic colitis (HC). It is also responsible for hemolytic uremic syndrome (HUS), which includes microangiopathic hemolytic anemia, thrombocytopenia and acute renal failure [1]. *E. coli* O157:H7 serotype is the prototypical EHEC and most often implicated in illness worldwide [4]. EHEC is recognized by its production of verotoxin (VT) or Shiga-like toxin (SLT) [5]. The organism also possesses *eaeA* gene that codes for 'attaching and effacing' proteins and *ehxA* gene that codes the enterohemolysin [6]. EHEC human infections are mostly food or water borne and majority associated with ingestion of undercooked ground beef, raw milk, contaminated water and others [4].

Enteropathogenic *E. coli* (EPEC) causes a watery diarrhea with low degree of fever and vomiting [7]. The diarrheal illness occurs in young infants of less than two years of age in developing countries and is considered to be among the major causes of sporadic infant diarrhea in developing countries [8]. EPEC produces a non fimbrial adhesion designated intimin (coded by *eaeA* gene) and plasmid-encoded protein referred to as EPEC adherence factor (EAF) [7]. Besides that, EPEC also possesses a virulence factor called bundle-forming pili (*bfp* gene) [8]. Outbreaks of EPEC infections include contaminated drinking water and foods. On the other hand, bottle feeding and the physical environment (toys, carriages, bath area) of an EPEC infected infant have been a significant risk factor of EPEC infection [7].

Enterotoxigenic *E. coli* (ETEC) is an important cause of diarrhea in infants and travelers in underdeveloped countries or regions of poor sanitation. The term traveler's diarrhea is frequently used as a syndrome of travelers from high sanitary standard countries visiting low sanitary standard areas who suffer from watery diarrhea. ETEC is not recognized as a causative agent of diarrhea in developed countries [9]. Enterotoxins produced by ETEC include the LT (heat-labile) toxin and the ST (heat-stable) toxin. ETEC may produce either LT or ST alone or in combination [1].

Identification of the virulence factors that allow pathogenic strains to be distinguished from the non-pathogenic strains or normal gut flora becomes an essential element in diagnostic work. Detection of specific toxins or virulence genes of the pathogenic *E. coli* groups becomes more reliable and efficient compared to the laborious conventional procedures. Therefore, it is widely accepted that detection of virulence factors associated with *E. coli* is a reliable approach for the molecular-based diagnosis of infectious disease. In recent years, the applications of Polymerase Chain Reaction (PCR) for the detection of virulence genes of *E. coli* have been described [10, 11].

PCR is a powerful technique for detection of pathogens because of its rapidity, specificity and sensitivity. It is an effective procedure for generating large quantities of a specific DNA sequence in vitro [12]. It can detect the presence of a known DNA sequence in very small and crude samples. In addition, multiplex PCR assay permits simultaneous detection of more than one DNA sequence. It allows parallel screening of different targeted genes in the same reaction. This facilitates the use of the multiplex PCR assay in routine testing and reduces the time required to achieve results [13].

Studies associated with detection of pathogenic *E. coli* by multiplex PCR assay have been carried in Malaysia. These studies mainly focused on detection of one or two categories of pathotypes of *E. coli*. For example, Samuel *et al.* [14] detected ETEC, Norazah *et al.* [15] screened only for EPEC, Radu *et al.* [16] and Al Haj *et al.* [17] detected shiga toxin producing *E. coli* (STEC) and EPEC, respectively. On the contrary, studies related to simultaneous detection of different virulence genes of various pathotypes of ETEC, EPEC and EHEC *E. coli* strains is lacking. Kong *et al.* [18] developed a multiplex PCR to detect *E. coli* strains with virulence genes include VT, LT1, ST and *eaeA* genes. In this study, a multiplex PCR assay which detects for six virulence genes of ETEC, EPEC and EHEC was modified based on Kong's study and applied on indigenous *E. coli* isolates from environment water, food samples and humans in Malaysia.

Detection of pathogenic *E. coli* strains by identifying the presence of virulence characteristics is important in food safety and water quality monitoring. In view of emerging importance of ETEC, EPEC and EHEC *E. coli* strains, the prevalence of virulence genes in potential sources of water and food borne disease needs to be investigated.

MATERIALS AND METHODS

Bacterial strains

All 87 *Escherichia coli* isolates were from three main sources: environmental water obtained from Petaling Jaya (n = 12, from ponds and lakes), foods (n = 28, from vegetables, seafood Tofu, fish cakes, satay and rendang) and hospitalized patients (n = 47). Human isolates were provided Institute of Medical Research Malaysia (IMR). Other *E. coli* strains were previously isolated by researchers in the BioMedical Science Laboratory, Institute of Postgraduate Studies, UM, according to standard microbiological and biochemical tests. Prior to analysis, all isolates were re-checked and re-confirmed as *E. coli* by plating on Eosin Methylene Blue agar plate and the presence of alkaline phosphatase gene (a housekeeping gene of *E. coli*) by a monoplex PCR.

The positive control *E. coli* strains used in the PCR assay were kindly provided by Dr. Richard C. Y. Kong (City University of Hong Kong). The control strains included known LT2-positive and VT-positive isolate *E. coli* SA 53, known LT1-positive and ST-positive isolate *E. coli* ATCC 35401 and known VT-positive and *eaeA*-positive isolate *E. coli* O157 [18].

Culture of organisms and extraction of total DNA

All *E. coli* isolates were incubated at 37 °C overnight on Luria-Bertani agar plates. Thereafter, one colony from the plate was picked and boiled in 50µl of sterile distilled water for 5 minutes, chilled on ice for 10 minutes and then centrifuged at 13 000 x *g*) for 90 seconds. A 5µl of the supernatant (≈100ng DNA) was used as DNA template in the PCR.

Oligonucleotide primers

All oligonucleotide primers were synthesized by Operon Biotechnologies GmbH, Germany. The respective nucleotide sequences and expected size of the PCR products of the primers sets are shown in Table 1.

Table 1. Oligonucleotide sequences of primers used in the detection of housekeeping gene and virulence genes of *E. coli*

TARGET GENE	PRIMER	SEQUENCE (5' – 3')	EXPECTED PRODUCT SIZE (bp)	REFERENCE
Alkaline phosphatase (*phoA*)	Pho-F Pho-R	GTG ACA AAA GCC CGG ACA CCA TAA ATG CCT TAC ACT GTC ATT ACG TTG CGG ATT TGG CGT	903	Kong *et al.* [18]
Heat-stable toxin 1 (ST1)	ST1-F ST1-R	CTT TCC CCT CTT TTA GTC AG TAA CAT GGA GCA CAG GCA GG	175	Kong *et al.* [18]
Heat-labile toxin 1 (LT1)	LT1-F LT1-R	TTA CGG CGT TAC TAT CCT CTC TA GGT CTC GGT CAG ATA TGT GAT TC	275	Kong *et al.* [18]
Heat-labile toxin 2 (LT2)	LT2-F LT2-R	ATA TCA TTT TCT GTT TCA GCA AA CAA TAA AAT CAT CTT CGC TCA TG	720	Kong *et al.* [18]
Verotoxin 1 (VT1) Verotoxin 2 (VT2)	VT-F VT-R	GAA CGA AAT AAT TTA TAT GTG CCT GAT GAT GGC AAT TCA GTA	523 520	Kong *et al.* [18]
Attachment and Effacement (*eaeA*)	AE22 AE20-2	ATT ACC ATC CAC ACA GAC GGT ACA GCG TGG TTG GAT CAA CCT	397	Fratamico and Strobaugh [18]

PCR amplification and analysis of PCR products

Initially, detection of alkaline phosphatase gene (housekeeping gene of *E. coli*) by a monoplex PCR for every isolate was carried in a total volume of 25 µl containing 2.5 µl of 5x Colorless GoTaq® Flexi Buffer (Promega), 1.0 mM of Magnesium Chloride ($MgCl_2$), 0.14 mM of deoxynucleotide triphosphates (dNTPs), 0.5 U of *Taq* polymerase (GoTaq® DNA Polymerase by Promega), 0.22 µM of Pho primer and 5.0 µl of DNA template. The amplifications were performed using a Mastercycler personal by Eppendorf. Cycling conditions were as follows: initial denaturation at 94°C for 2 minutes, followed by 35 cycles of three steps consisting denaturation at 94°C for 1 minute, primer annealing at 56°C for 1 minute and DNA extension at 72°C for 1 minute and followed by a final extension at 72°C for 10 minutes.

Prior to combining all five primer sets to detect the six virulence genes in a single multiplex PCR, each pair of primers was optimized singly in separate PCR assays with three positive control bacterial strains and a negative control using sterile distilled water as template. The PCR mixture reaction and amplifications were similarly carried out as above.

A multiplex PCR using DNA templates from positive control strains was optimized by varying the concentrations of primers (0.07 to 0.8 µM) and dNTPs (180 to 200 µM). Two different sources of *Taq* DNA polymerase from different commercial vendors were also evaluated. Other parameters such as annealing temperature (56°C), concentrations of $MgCl_2$ (3.0 mM) and concentration of *Taq* DNA polymerase (1.5 U) were kept constant. Then, the optimized multiplex PCR assay was carried out in a total volume of 50 µl containing 5.0 µl of 5X Colorless GoTaq® Flexi Buffer, 3.0 mM of $MgCl_2$, 0.2 mM of dNTPs, 1.5 U of *Taq* polymerase (GoTaq® DNA Polymerase by Promega), 5.0 µl of DNA template and primer mix consists of 0.70 µM of VT primer, 0.60 µM of LT2 primer and 0.07 µM each of LT1 primer, ST primer and AE primer. The cycling conditions were carried out as mentioned above.

All the amplified PCR products were analyzed by horizontal agarose gel electrophoresis. 5µl of the PCR products were electrophoresis on 1.5% agarose gels (Promega) in Tris-borate-EDTA buffer along with a 100bp DNA ladder (Promega). Fragments were visualized by UV illumination and photographed after ethidium bromide staining.

Determination of virulence genes *in E. coli* isolates and water samples

The optimized mPCR protocol was applied to determine the presence of the six virulence genes in the *E. coli* isolates. In addition, the PCR assay was also applied directly to water samples collected from seven sites in Petaling Jaya. Briefly, microbial cells in water samples (200 ml) were harvested by centrifugation 13,000 x *g* for 20 minutes and deposited pellet cells were incubated in 5 ml Luria-Bertani broth for 6 hours in water bath shaker at 37°C. Cells were harvested by centrifugation at 12,000 x *g* for 2 minutes and washed five times with double distilled water. The cell pellet was resuspended in 100 ul sterile water and boiled at 95°C for 2 min. An aliquot of 5 µl of the cell lysate was used as DNA template.

RESULTS

Confirmation of *E. coli* isolates

All isolates used in the study were visualized as blue-black colonies (2-3mm diameter) with green metallic sheen on Eosin Methylene Blue Agar (Levine) Agar. The observation indicated a presumptive identification of *E. coli* for all 87 isolates. Next, a monoplex PCR detecting alkaline phosphatase gene (*phoA* gene) was performed on all *E. coli* strains. All isolates showed positive result for the presence of *phoA* gene (903 bp), thus confirming their identity as *E. coli* (Figure 1).

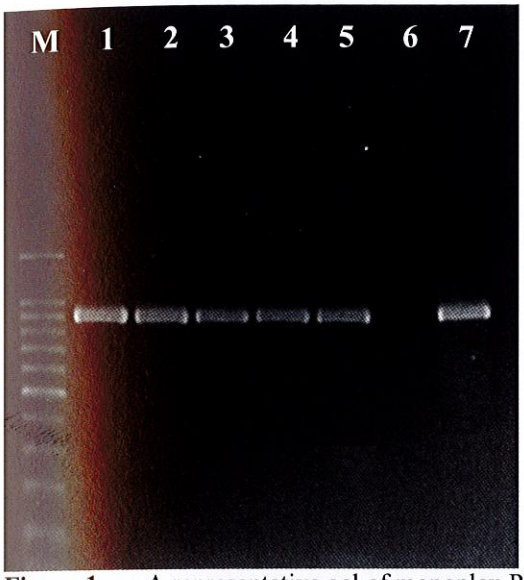

Figure 1. A representative gel of monoplex PCR for *phoA* gene (housekeeping gene of *E. coli*) from the *E. coli* isolates and positive control strains. Lane M, 100bp DNA ladder; Lane 1, EC 356 (isolated from environmental water); Lane 2, EC 368 (isolated from food); Lane 3, EC 369 (isolated from food); Lane 4, EC 7 (human isolate); Lane 5, EC 9 (human isolate); Lane 6, negative control; Lane 7, SA 53 (positive control).

Optimization of Multiplex PCR

Prior to the optimization of multiplex PCR, a monoplex PCR for each primer set was carried out with a negative control, three positive control bacterial strains and a total DNA mixture containing all these bacterial control strains. The results were examined and confirmed that no spurious results or unspecific bindings have occurred (data not shown). Each pair of primer used was specific to their corresponding target gene. LT1, LT2, VT, AE and ST primer sets were designed to simultaneously detect six virulence-associated genes and amplify these genes at predicted product size. The five pairs of primer were selected such that the predicted sizes of the amplification products of each target gene would be different to permit size discrimination by gel electrophoresis.

The amount and quality of *Taq* polymerase, annealing temperature, and concentrations of primers, dNTPs and $MgCl_2$ used are key parameters in developing a specific and sensitive amplification of target gene sequences by multiplex PCR assay [19]. Attempts at achieving the optimal multiplex PCR assay by ascertaining the best source of *Taq* polymerase, concentrations of primers, dNTPs and $MgCl_2$ and annealing temperature were successful.

The performance of two different commercial *Taq* polymerase enzymes in multiplex PCR was tested under the same PCR cycling conditions. They were GoTaq® Flexi DNA Polymerase from Promega and *Taq* DNA polymerase from Vendor X. The results showed that the PCR performed by the GoTaq® Flexi DNA Polymerase (Promega) gave stable PCR results with more intense bands (Lane 5 of Figure 2A) and lower incidence of non-specific bands. However, more unspecific bands were obtained when *Taq* DNA polymerase from vendor X was used (Lanes 1 and 3 of Figure 2B) Also the bands were less intense and sharp indicating of weak amplification (Lane 5 of Figure 2B). As a result, GoTaq® Flexi DNA Polymerase (Promega) was chosen to carry out the subsequent PCR assays.

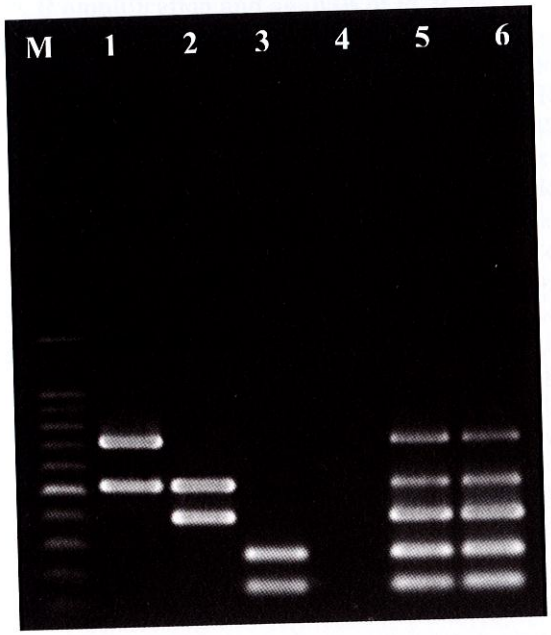

Figure 2A. Representative gels showing multiplex PCR performed by different sources of *Taq* DNA polymerase. GoTaq® Flexi DNA Polymerase (Promega) (Lane M, 100bp DNA ladder; Lane 1, SA 53; Lane 2, O157; Lane 3, ATCC 35401; Lane 4, negative control; Lane 5 and 6, positive control).

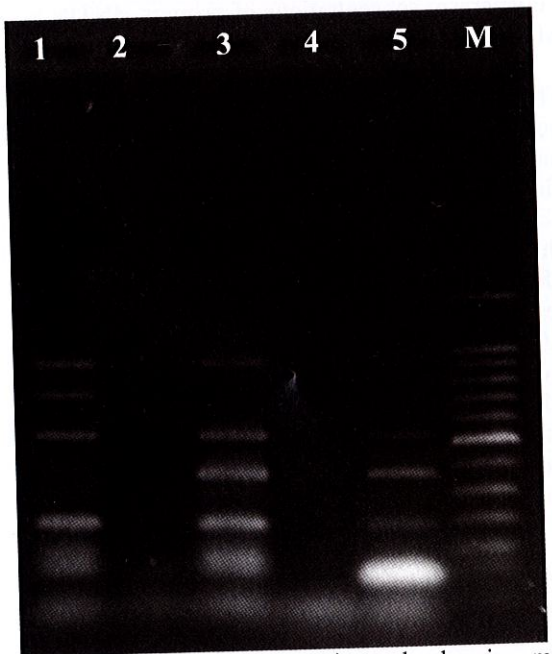

Figure 2B. Representative gels showing multiplex PCR performed by different sources of *Taq* DNA polymerase. *Taq* DNA polymerase from vendor X (Lane 1, SA 53; Lane 2, ATCC 35401; Lane 3, O157; Lane 4, negative control; Lane 5, positive control; Lane M, 100bp DNA ladder).

In the optimization of multiplex PCR assay, concentrations of primers (0.07 to 0.8 μM) and dNTPs (180 to 200 μM) were evaluated. When 0.80 μM each of VT primer and LT2 primer and 0.20 μM each of LT1 primer, ST primer and AE primer were used, intense primer-dimers were noticed in multiplex PCR results (Figure 3A). High primer concentrations that allow mis-priming caused the spurious products to compete with the target sequences for primers. Consequently, target genes showed weak amplifications that yielded weak bands (VT and LT2 genes, Lane 5 of Figure 3B). Hence, concentrations of primer sets with intense bands showed strong amplifications can be decreased and concentrations of primer sets producing faint bands (weak amplifications) can be increased or maintained to reduce the effect of primer-dimers phenomenon. When primer concentrations were reduced (0.70 μM each of VT primer and LT2 primer and 0.14 μM each of LT1 primer, ST primer and AE primer), primer-dimers were still visible (Figure 3B). Only when 0.70 μM of VT primer, 0.60 μM of LT2 primer and 0.07 μM each of LT1 primer, ST primer and AE primer were used, no primer-dimers were observed in the PCR results (Figure 3C). Intense amplicons with a clean background were obtained with the optimized primer concentrations (Figure 3C). Hence, these primers combination was found to be optimal for the multiplex PCR.

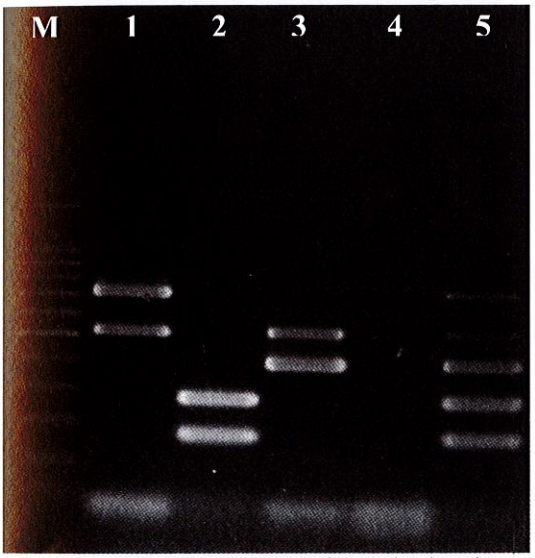

Figure 3 A. Representative gels showing multiplex PCR results using three different concentrations of primers. (A) primer combination consists of 0.80 μM each of VT primer and LT2 primer and 0.20 μM each of LT1 primer, ST primer and AE primer.

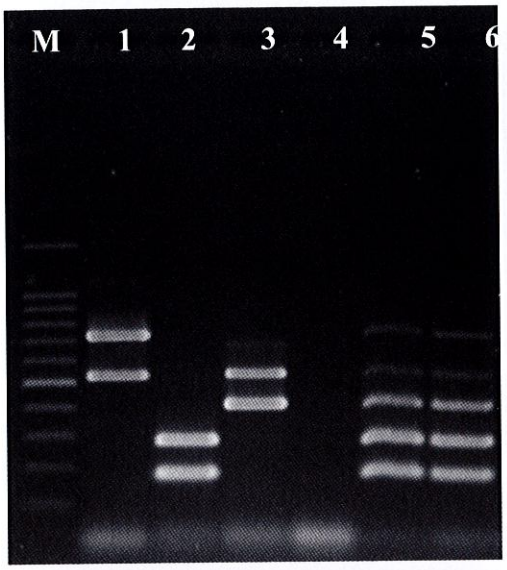

Figure 3B. Representative gels showing multiplex PCR results using three different concentrations of primers. (B) primer combination consists of 0.70 µM each of VT primer and LT2 primer and 0.14 µM each of LT1 primer, ST primer and AE primer.

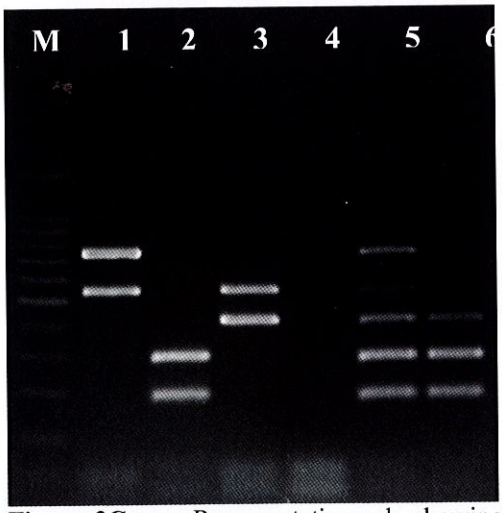

Figure 3C. Representative gels showing multiplex PCR results using three different concentrations of primers. (C) primer combination consists of 0.70 µM of VT primer, 0.60 µM of LT2 primer and 0.07 µM each of LT1 primer, ST primer and AE primer. Lane M, 100bp DNA ladder. Lane 1, SA 53; Lane 2, ATCC 35401; Lane 3, O157; Lane 4, negative control; Lane 5 and 6, positive control.

Bands of variable intensities were obtained for positive control strains at 180 µM of dNTPs. The LT2 and VT bands were markedly less intense compared to the other three bands (ST, LT1 and *eaeA*) for positive control (Lane 5 of Figure 4A). Attempts at amplifying all five genes equally in a single reaction were performed by adjusting the concentration of dNTPs. As shown in Lane 5 of Figure 4B, 200 µM of dNTPs produced equally intense bands for positive control. Hence, 200 µM were adopted as the efficient concentrations of dNTPs.

Determination of incidence of virulence genes in E. *coli* isolates and environmental water sample using optimized multiplex PCR

Using the optimized multiplex protocol described above, detection of virulence genes of 87 *E. coli* isolates was carried out. The results demonstrated that only one *E. coli* isolate (EC 375, isolated from beef satay) showed the presence for *eaeA* gene (Figure 5). One of seven environmental water samples showed the presence for ST, LT1, VT and *eaeA* genes (data not shown).

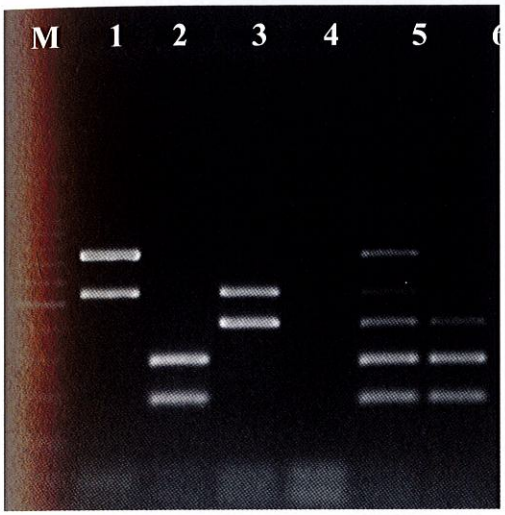

Figure 4A. Representative gels showing multiplex PCR results using different dNTPs concentrations. (A) 180 µM of dNTPs (bands of variable intensities were observed for positive control, Lanes 5 and 6) (Lane M, 100bp DNA ladder; Lane 1, SA 53; Lane 2, ATCC 35401; Lane 3, O157; Lane 4, negative control; Lane 5 and 6, positive control).

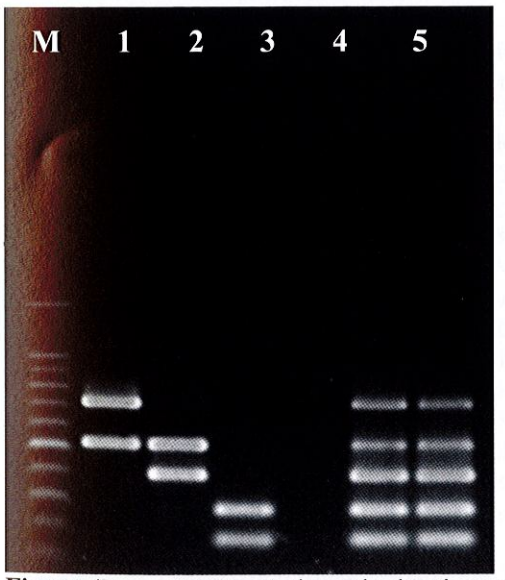

Figure 4B. Representative gels showing multiplex PCR results using different dNTPs concentrations. (B) 200 µM of dNTPs (bands of equal intensities were observed for positive control, Lanes 5 and 6) (Lane M, 100bp DNA ladder; Lane 1, SA 53; Lane 2, O157; Lane 3, ATCC 35401; Lane 4, negative control; Lane 5 and 6, positive control).

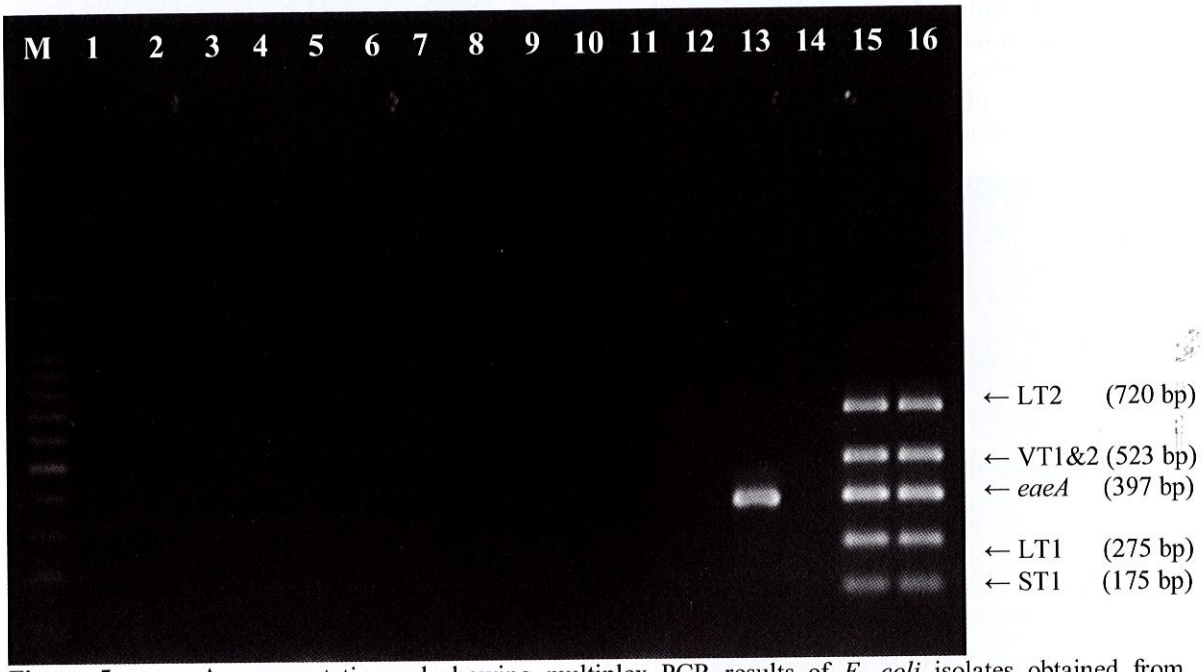

Figure 5. A representative gel showing multiplex PCR results of *E. coli* isolates obtained from environmental water, foods and humans. Lane M, 100bp DNA ladder; Lane 1, EC 360; Lane 2, EC 361; Lane 3, EC 362; Lane 4, EC 363; Lane 5, EC 379; Lane 6, EC 380; Lane 7, EC 381; Lane 8, EC 384; Lane 9, EC 311; Lane 10, EC 312; Lane 11, EC 318; Lane 12, EC 319; Lane 13, EC 375 ; Lane 14, negative control; Lane 15 and 16, positive control.

DISCUSSION

The main objective of the study was to develop a reliable method to detect six virulence genes of pathogenic *E. coli*. Optimization of a multiplex PCR method was a tedious but necessary step towards minimizing the cost and labor involved in detecting multiple targets simultaneously. The multiplex PCR technique was improved by ascertaining the best source of *Taq* DNA polymerase and adjusting the concentrations of primers and dNTPs as well as other cycling parameters to their optimal levels.

DNA polymerases are enzymes which catalyze the synthesis of long polynucleotide chains from dNTPs using one target parental strand as a template for the synthesis of a new complementary strand [19]. Therefore, *Taq* DNA polymerase plays an important role in gene amplification of PCR assay. Broude *et al.* [20] had compared the efficiency of two commercial *Taq* DNA polymerases and analyzed the PCR products. They reported that different *Taq* DNA polymerases display different specificity in multiplex PCR [20]. Preferential amplification of one target gene over another is a known phenomenon in multiplex PCR. This explains the different efficiency and uniformity of amplifications performed by the two *Taq* DNA polymerases tested in this study (GoTaq® Flexi DNA Polymerase by Promega and *Taq* polymerase by vendor X) in the study.

Primers are an important factor in determining the reliability and specificity of PCR assays. Formation of unwanted products will affect the reliability of the PCR result by interfering with the interpretation of electrophoresis results [19]. Primer pairs should be designed for complementariness with other primers to avoid the formation of primer-dimers. Primer-dimers formation, which is an amplification artifact, is due to extension of one primer by *Taq* DNA polymerase using other primer or itself as a template, resulting in a short incorrect product [19]. Therefore, the performance of each primer was optimized to verify the specificity of the multiplex

PCR assay, in order to ensure the primers to be of significant affinities only for their target genes. Primer concentration is another critical parameter for successful multiplex PCR [21]. Primer combination with suitable primer ratios has to be ascertained to ensure the efficiency of multiplex PCR assays. Preferential amplification of one target sequence over another can be overcome by decreasing the concentration of primers with stronger amplification at the same time with an increase of the concentration of the primers with weaker amplification can be applied [22].

The multiplex PCR assay employs five pairs of primers include ST, LT1, LT2, VT and AE primers. There is a region where two different stretches of DNA sequences within VT1 and VT2 genes share 100% sequence similarity. VT primer in the study is able to detect this region. Therefore, the VT primer was expected to amplify both or either of the genes from corresponding DNA sequence of EHEC members [18]. On the other hand, the AE primers target at two DNA domains that are highly conserved in the *eaeA* genes of EHEC and EPEC strains. The primers were assumed to bind the targeted gene fragment from DNA of either of the groups [23]. In short, the multiplex PCR was successfully designed and optimized to detect six virulence genes of *E. coli* by five pairs of primers.

The five pairs of primers in use were targeted at virulence genes of different category of diarrheagenic *E. coli*: ST1, LT1 and LT2 are enterotoxin genes of ETEC [24], VT1 and VT2 are verotoxin genes of EHEC [6] and *eaeA* is attachment and effacement gene of EHEC and EPEC [8]. In the study, three positive control strains carrying known target genes were used and tested with all the primers. As shown in the PCR results, SA 53 carried the LT2 and VT genes, ATCC 35401 carried the LT1 and ST genes and O157 carried the VT and *eaeA* genes. The results are consistent with previous reports in Kong's study [18].

In the study, one food isolate, which is EC 375 was positive for *eaeA* gene. Since *eaeA* gene is present in EHEC and EPEC, further studies have to be carried out to confirm the identity of EC 375. EC 375 can be O and H serogrouped with commercial sera to examine its serotype [25] or use of another primer sets which detect Shiga toxins and BFP gene (*bfpA*) as reported by Al Haj *et al.* [17]. They have developed a multiplex PCR using four sets of primers to detect diarrheagenic *E. coli* belonging to Shiga toxin producing *E. coli* (STEC) (*eae, stx1* and *stx2*) and EPEC (*eae* and *bfpA*).

No virulence genes associated with diarrhea were detected in the human *E.coli* isolates. This may due to the fact that the isolates studied were involved in extra-intestinal infections and not enteric and were recovered from various parts of the human body (except stools). Therefore, these human isolates may not acquire the targeted diarrhea associated virulence genes. Previous studies done by Catalina *et al.* [26] have demonstrated diarrhea-causing *E. coli* strains can be isolated from stools of diarrhea patients. In their study, eleven of 58 hospitalized diarrhea patients had diarrhea-causing *E. coli* in their stools. Of the eleven multiplex PCR-positive specimens, five were Shiga toxin producing *E. coli* (STEC) and four were ETEC, EPEC and EIEC.

In the study of Kong *et al.* [18], ST, LT1, VT and *eaeA* genes were detected in all four seawater samples by using the multiplex PCR. The observation was consistent with the multiplex PCR results seen in one of our water samples harboring pathogenic *E. coli* isolates with ST, LT1, VT and *eaeA* genes.

In the study, screening the alkaline phosphastase gene (*phoA*) of each isolate was performed by monoplex PCR using the Pho primer before the further investigation of virulence genes by multiplex PCR. *phoA* encodes for a hydrolase enzyme which is responsible for removing phosphate groups from molecule [27]. It is a housekeeping gene present in all *E. coli* strains [18]. Kong *et al.* [28] identified *E. coli* strain by its housekeeping gene (*phoA*) from other water borne bacteria. In order to enhance the utility of the developed assay to identify *E. coli* and detect its virulence genes in the same reaction, a set of primer that targets for the alkaline phosphastase gene (*E. coli* housekeeping gene, *phoA*) may be incorporated to the multiplex PCR.

The virulence mechanism in *E. coli* is coded by repertoire of virulence genes. The simple mPCR assay developed in this study only targets a subset

of these genes. Although PCR assays have become a useful technique to detect pathogenic potential (pathotype) of *E. coli*, these assays could not quantify the amplification product because agarose gel electrophoresis and ethidium bromide staining were used for visualizing PCR amplified products. Real-time PCR assays have been developed for detection and quantification of pathogen-specific gene products. Sharma and Dean-Nystrom [29] used a multiplex real-time PCR assay to detect and quantify *E. coli* O157:H7. On the other hand, applications of nucleic acid microarrays also can be employed in multi-gene detection. In the study of Call *et al.* [30], O157:H7 strains were detected by using multiplex PCR and nuclei acid microarrays composed of oligonucleotide probes complementary to four virulence loci (intimin, Shiga-like toxins I and II and hemolysin A).

CONCLUSION

The multiplex PCR assay developed in the study provides a promising and rapid option for detection of six virulence determinants of pathogenic *E. coli*. Therefore, the method would be particularly useful for the assessment of health risks that may be associated with the exposure to diarrhea-causing *E. coli*. In this study, the utility of multiplex PCR assay developed was demonstrated by using reference positive control strains as well as foods, water and humans isolates. One food isolate was detected with the presence of *eaeA* gene and one environmental water sample was detected with the presence of ST, LT1, VT and *eaeA* genes. The findings reported in the study highlights the need of consistent surveillance to ensure that our food and water to be consumed are clean and safe. Further precaution and prevention can be taken if only the cause of disease outbreaks is identified.

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