Research Journal of Biological Sciences 3 (11): 1327-1332, 2008
ISSN: 1815-8846

Development of a PCR Primer and a Marker Band for Detection of *E. coli* from Various Sources Based on Arbitrary Primer Set

[1]Nagi A. AL-Haj, [1]Mariana N. Shamsudin, [2]Raha A. Rahim and [3]Zamri Ishak
[1]Department of Microbiology and Parasitology, Faculty of Medicine and Heath Sciences,
[2]Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences,
University Putra Malaysia, 43400 Serdang Selangor, Malaysia
[3]Biotechnology Center, Mardi, P.O. Box 12301, Malaysia

Abstract: Although, PCR methods aimed on the detection of genes associated with the pathogenicity of *Escherichia coli* have been reported, tests allowing the direct identification of this serotype are rare. In this study the Random Amplified Polymorphic DNA (RAPD) fingerprinting technique allowed genetic diversity assessment of 25 *E. coli* isolates of various sources. A highly significant finding from the DNA fingerprinting is the display of a predominant band at a size of 308 bp when arbitrary OPAE-10 primer was used. After sequencing this fragment primer called *secD* was designed to be used as PCR primer. *secD* primer pairs was highly specific to detect all isolates including *E. coli* O157: H7.

Key words: Random Amplified Polymorphic DNA (RAPD), uropathogenic *E. coli* (UPEC), Neonatal Meningitis-associated *E. coli* (MNEC), University Putra Malaysia (UPM), primer, sequence

INTRODUCTION

Until fairly recently, there was a common perception that pathogenicity traits in *Escherichia coli* are more the exception than the rule and *E. coli* was generally regarded as part of the normal lower intestinal flora (Schmidt *et al*., 2001). However, an increasing number of categories of pathogenic *E. coli* isolates have been identified over the past few decades, which has led to the current situation in which there are now at least 11 recognized pathotypes of *E. coli* in humans (Kaper *et al*., 2004). Pathogenic *E. coli* strains are divided into pathotypes on the basis of their distinct virulence properties and the clinical symptoms of the host (Muhldorfer and Hacker, 1994).

Three main types of clinical syndrome can result from infection with one of these pathotypes: Enteric and diarrheal diseases, urinary tract infections and sepsis/meningitis. The *E. coli* pathotypes responsible for intestinal infections include enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli*, enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli*, diffusely adherent *E. coli*, necrotoxic *E. coli* and cell-detaching *E. coli*. Three additional *E. coli* pathotypes, collectively called ExPEC (Russo and Johnson, 2003), are responsible for extraintestinal infections. ExPEC is composed of uropathogenic *E. coli* (UPEC) isolates that cause urinary tract infections, neonatal meningitis-associated *E. coli* (MNEC) and *E. coli* strains that cause septicemia (Bekal *et al*., 2003; Kaper *et al*., 2004). These pathotypes are defined by the presence of combinations of virulence and virulence-related genes; conversely, the pathotype of an uncharacterized strain can be inferred from its virulence gene profile (Kaper *et al*., 2004). Number of PCR-based assays has been developed for the detection of *E. coli* O157: H7. Most of these PCR primers were designed for the specific amplification of virulence genes, including genes encoding Shiga-like toxin I or II (*sltI* and *sltII*), intimin gene (AL-Haj *et al*., 2007a, b; Willshaw *et al*., 1994; Sandhu *et al*., 1996; Ellingson *et al*., 2005) and haemolysin gene (hlyA) (Paton and Paton, 1998). Many investigators have recently developed multiplex PCR system for the simultaneous amplification of several virulent genes in a single PCR assay (AL-Haj *et al*., 2007a, 2008; Cebula *et al*., 1995; Fagan *et al*., 1999).

However, the multiplex PCR method has some limitations in use since it is difficult to apply to the detection of the target cells in mixtures with significantly different bacteria ratios or in stool and food samples Hsih and Tsen (2001). Prior to tests, investigators require

Corresponding Author: Dr. Nagi Ahmed AL-Haj, Department of Microbiology and Parasitology,
Faculty of Medicine and Heath Sciences, Universitiy Putra Malaysia, 43400 Serdang Selangor, Malaysia

isolation of the tested strain to confirm that alldetected genes are present in the same strain (Perelle *et al*., 2002). Random amplified polymorphic DNA RAPD-PCR is an alternative approach for molecular typing and probe designing and that no prior DNA sequence information is required (Fani *et al*., 1993). RAPD typing has been used for interspecies differentiation of *Listeria* sp., *Campylobacter* sp. (Farber and Addison, 1994; Mazurier *et al*., 1992) and moreover, intraspecies differentiation of *Bacillus cereus*, *Lactobacillus sake* and *Lactobacillus plantarum* (Stephen, 1996).

In this study, we performed a series of RAPD-PCR assays for of *E. coli* isolates from various sources, as clinical as well as environmental. One common RAPD fragment with 310 bp length, termed as *secD*, was generated in RAPD pattern using primer M13. After cloning and sequencing this *secD* fragment, a PCR primer set was developed for the specific detection of *E. coli* strains including the O157: H7.

MATERIALS AND METHODS

Sources of isolates: Twenty five *E. coli* isolates as clinical, sea water, river, food and animal were studied in University Putra Malaysia (UPM), 2005. The clinical (pus, 2 urine and 2 stool) samples were studied from the specific culture of Microbiology Laboratory there is takes were originally provided from Kula Lumpur Hospital (HKL), which were marine and river isolates were collected from Costrica beach, Sunggi linggi river Nergeri Sembilan State. The food (raw milk, chess, yogurt and 2 raw meats) sample was selected randomly from different restaurant in Seri Serdange area, Selangor state. The last sample of animal source (deer, pig, goat and 2 chickens) was provided by bacteriology department, Faculty of veterinary University Putra Malaysia (UPM).

RAPD of *E. coli* isolates: Genomic analysis of *E. coli* isolates was done by RAPD-PCR analysis using 15 single primers (Table 1). GenomicDNA (10-20 ng) was used as a template RAPD analysis A data matrix of 1' s and 0' s (presence and absence of RAPD bands, respectively) was recorded and analyzed by RAPD istance software program (Amstrong *et al*., 1998). The genetic distance was generated automatically by the software whereas the percent of similarity were calculated according to Nei and Li's (1979) theory.

RAPD PCR: RAPD was carried out with twenty commercially available random 10-mer primers OPQ-01 to OPQ-20 (Operon Technologies, USA) (Table 1). Basically, only one primer was used in one reaction. PCR for

Table 1: Sequences of primers screened for RAPD procedure

No.	Primer designation	Sequence 5-3
1	OPAE-01	TGAGGGCCGT
2	OPAE-04	AATCGGGCTG
3	OPAE-05	CCTGTCAGTG
4	OPAE-06	GGGGAAGACA
5	OPAE-07	GTGTCAGTGG
6	OPAE-08	CTGGCTCAGA
7	OPAE-09	TGCCACGAGG
8	OPAE-10	CTGAAGCGCA
9	OPAE-11	AAGACCGGGA
10	OPAE-12	CCGAGCAATC
11	OPAE-13	TGTGGACTGG
12	OPAE-14	GAGAGGCTCC
13	OPAE-15	TGCCTGGACC
14	OPAE-16	TCCGTGCTGA
15	OPAE-18	CTGGTGCTGA

RAPD-PCR analysiswas done using the thermal cycler (Biometra-Trio Thermoblock. Germany) in a volume of 25 Fl containing 80 mM MgCl2, PCR buffer, 3.75 mM dNTP mix (MBI Fermentas), 10 picomoles of each RAPD primers, 100 ng of template and 1Unit of Taq polymerase (BST Taq, Biosyntech Sdn. Bhd, Malaysia). After a hot start at 95EC for 2 min, the DNA was subjected to 35 cycles of denaturing at 94 EC for 2 min, annealing at 35EC for 2 min and extension at 72 EC for 2 min. A final extension step was done for 10 min 72 EC. The PCR products were separated on 1.5% (wt/vol) agarose gels in 1 × TAE (0.04 M Tris acetate, 0.001 M EDTA (8.0 pH) buffer at 70 V for 3 h. After being stained with ethidiumbromide (2.5Fl of a 10 mg mL^{-1} solution), the gels were photographed with Alph-Immegar 2200™ camera under UV light.

Cloning and sequencing of RAPD fragment: Specific fragment of the primer 10 OPEA (Operon Technologies, USA) with products 310-pb was cut and purified (Fig. 1) and cloned in a Topo vector 2.1 and transformed into *E. coli* competent cells. An aliquot of 6 μL Topo-cloning reaction was prepared with 2 μL of fresh PCR product (amplified RAPD fragment), 2 μL of sterile water, 1 μL salt solution and 1 μL of PCR Topo vector 2.1. The mixture was mixed gently and incubated for 5 min at room temperature of 25 EC. For the best possible results, the mixture was not left for more than 5 min as the transformation and cloning efficiencies may decrease. Subsequently, the cocktail mixture was quick spined at room temperature, placed on ice and the one shot transformation was carried out immediately. The specific RAPD fragment was cloned with a Topo 2.1 TA cloning kit (Invitrogen Technologies) according to the instructions of the manufacturer. Nucleotide sequences of the RAPD fragment band was determined by sequencing commercially (Research Biolab) the plasmid containing the RAPD fragment with M13 forward and reverse primers.

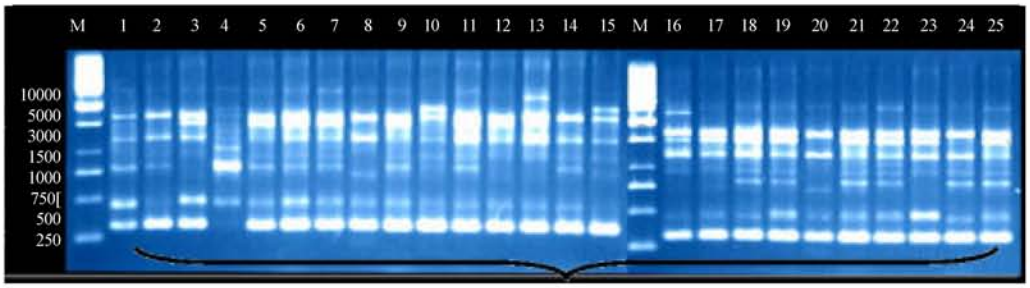

Fig. 1: Genomic fingerprint of RAPD Primer OPAE-10. M. Marker ladder 1kb bp 1-5 clinical (2 stool, 2urine and pus), 6-10 marine, 11-15 river, 16-20 (food raw milk, chess, yogurt and 2 raw meats), 21-25 animal (deer, pig, goat and 2 chicken)

Table 2: Designed primer used for amplification of the *secD* gene

Sequence (5'-3')	Target gene	Amplicon (bp)	Reference
F-ATCGTGGTGATTGTCATCG R-ACGCCCATTAATGCTTCAC	*secD*	244	Present study

The M13 forward and reverse primers were provided in the Topo TA cloning kit (Invitrogen The M13 forward and reverse primers were provided in the Topo TA cloning kit (Invitrogen Technologies). DNA homology analysis of sequenced RAPD fragment was carried out with BLAST programs.

Primer designing specific PCR: Based on the DNA sequences of RAPD fragment, PCR primer was designed through Work Bench Biology 3.2 (Table 2). The PCR conditions for specificity testing of *E. coli* O157: H7 strains were the same as those for RAPD PCR, except that the $MgCl_2$ concentration was 1.5 mM. DNA amplification steps performed were initial denaturation at 94°C for 2 min, followed by 30 cycles of amplification steps consisting of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and elongation at 72°C for 2 min. The amplification was stopped following with a single final extension at 72°C for 7 min. The PCR products were analyzed by 1.5% TAE agarose gel electrophoresis was photographed with Alph-Immegar 2200™ camera under UV light.

RESULTS AND DISCUSSION

RAPD: In this study, the Random Amplified polymorphic DNA (RAPD) technique was performed according to Van Coppenolle *et al*. (1993) with minor modifications to fingerprint 25 *E. coli* isolates utilizing 20 different arbitrary primers. The primer namely, OPAE 10 produced DNA fingerprinting patterns with discriminatory power and potential epidemiological and diagnostic markers display. The potential markers are delineated as thick and bright bands constantly present in the profiles of all 5 isolates from each source. In addition to the development of markers, the RAPD method elucidated genetic diversity

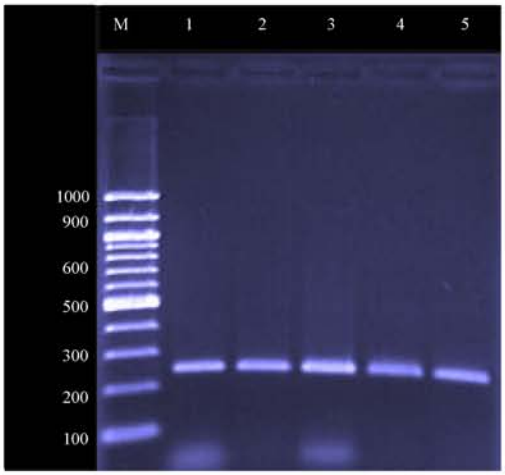

Fig. 2: Amplified *secD* gene using *secD* primers from the genomic DNA of three genera isolates. The *secD* gene positive isolates had a single band presented in the region between the ladders of 200 bp-300 bp (Lane M). The actual band position was at a 244 bp for isolates 1-clinical *E. coli (*pus), 2-sea water,3- river water 4-*E. coli* 0157: H7 of raw milk, 5-*E. coli* gout sample

of *E. coli* isolates from the different sources. In detecting genetic markers to differentiate isolates of various sources, no single primer can differentiate all sources but one primer, OPAE-10 (Fig. 1) displayed a prominent band at 308 bp in the isolates from all sources. The 308 bp band is proposed to be highly suitable as epidemiological or species-specific marker for *E. coli* of various strains. The RAPD technique is a PCR-based discrimination method in which short arbitrary primers anneal to multiple random target sequences Welsh and (McClelland, 1990) and result in patterns of diagnostic value. Therefore, RAPD fragments with the same length among tested strains are not necessarily homologous to each other (Oakey *et al*., 1998). Thus, further confirmatory assay is needed.

gi|91070629|gb|CP000243.1|*Escherichia coli* 100%

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Query  1
GAAGCGCACCAAAGAACACAAAAAGCTGATGGACTCCATCGCTAAAGGTGATGAAGTGCT  60
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GAAGCGCACCAAAGAACACAAAAAGCTGATGGACTCCATCGCTAAAGGTGATGAAGTGCT  501665
Sbjct  501606
Query  61
GACGAACGGTGGCCTGGTCGGTCGCGTAACCAAAGTAGCGGAAAACGGCTACATTGCTAT  120
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GACGAACGGTGGCCTGGTCGGTCGCGTAACCAAAGTAGCGGAAAACGGCTACATTGCTAT  501725
Sbjct  501666
Query  121
CGCACTGAATGACACCACTGAAGTAGTTATTAAACGTGACTTCGTAGCTGCCGTTCTGCC  180
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CGCACTGAATGACACCACTGAAGTAGTTATTAAACGTGACTTCGTAGCTGCCGTTCTGCC  501785
Sbjct  501726
Query  181
GAAAGGCACCATGAAGGCGCTGTAATTAAAATTTTTCCCTAAGGGAATTGCCGTGTTAAA  240
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
GAAAGGCACCATGAAGGCGCTGTAATTAAAATTTTTCCCTAAGGGAATTGCCGTGTTAAA  501845
Sbjct  501786
Query  241
CCGTTATCCTTTGTGGAAGTACGTCATGCTGATCGTGGTGATTGTCATCGGTCTGCTGTA  300
||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
CCGTTATCCTTTGTGGAAGTACGTCATGCTGATCGTGGTGATTGTCATCGGTCTGCTGTA  501905
Sbjct  501846

Query  301     TGCGCTTC  308
                ||||||||
Sbjct  501906  TGCGCTTC  501913
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Fig. 2: Analysis of RAPD fragment gene sequence by BLASTN alignment package, Comparison between published *secD* Query sequence and RAPD product gene from this study (sbjct)

DNA sequencing of diagnostic marker from the RAPD-PCR fingerprint: The plasmid from the positive clone upon sequencing commercially by an automated sequencing method and analysed by computer assisted BLASTN analysis (Fig. 2) to gave 100% homology to *E. coli* Protein-export membrane, protein *secD* Operon (positions 501606-501913) (accession number: CP000243. This molecular marker was obtained through RAPD; it was named as RapD10 marker. From the Blast analysis the RAPD marker being highly similar (100%) homology could be considered as a gene in the region of the *E. coli* genome. Moreover, the Blast analysis showed that this RapD10 fragment low homologues to other Gram negative genera such as *Salmonella* sub sp. *enterica* 89% (positions 203363-203544), (accession number: AL627266.1). Therefore, this molecular marker was considered as diagnostic marker for *E. coli*.

Amplification of *secD* gene from *E. coli* and *Salmonella* sp.: RAPD marker was reamplified from *E .coli* isolates. A single band of approximately 244 bp was amplified among all *E. coli* isolates (Fig. 2).

Findings of this study suggest that the sequencing of the RAPD fragment enable the development of important tool in typing of *E. coli* isolates. No variation in the nucleotide sequences among the *E. coli* suggests that

selection of the RAPD fragment as a candidate for studying the epidemiology of *E. coli* as well as a diagnostic marker for this organism is possible and was demonstrated in the dot blot reaction of the study. Therefore, typing of *E. coli* based on the sequences of RAPD fragment may contribute to a better understanding of the epidemiology of *E. coli*. Finally, it is recommended that this marker could be effectively used as a potential diagnostic marker to determine the *E coli* isolates from various sources. Recently, Perelle *et al.* (2002) developed a specific PCR method for the detection of *E. coli* O157: H7. The target for amplification is a small inserted locus (SIL_{O157}) which was screened out using a 387-bp RAPD fragment derived from a similar approach as that described here with little difference in the number of primers used. Several primer pairs designed from SIL_{O157} have also been evaluated for the detection specificity to O157: H7 serotype. Their results indicated that certain primer pair showed good specificity to stx-producing O157: H7 serotype (Perelle *et al.*, 2002). However, these serotypes are not prevalent in enterohemolytic outbreaks. There was no significant homology obtained with the Blast program between our Q3H1 fragment and the 387-bp fragment or even the whole sequences of SIL O157 reported by Perelle *et al.* (2002). Therefore, *secD* is a novel fragment which potentially can be used for the specific detection of *E. coli* O157: H7. More extensive field-testing is neededbefore large-scale microbialsource-tracking studies can be initiated. The marker may provide a cost-effective, quantitative and accurate method for determining sources ofgenetically diverse *E. coli* strains for use in water qualityanalyses and host point determinations.

CONCLUSION

Results shown above indicate that *secD* is universal to *E. coli* strains including the most common pathogens O157: H7. The PCR primer pairs, *secD* designed from it also showed specificity for these strains. Besides, PCR primer may serve as highly discriminative fragment in probes diagnostic format like DNA microarray.

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