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Development of SCAR Primers Based on a Repetitive DNA Fingerprint for *Escherichia coli* Detection

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The present study aimed to use enterobacterial repetitive intergenic consensus (ERIC) fingerprints to design SCAR primers for the detection of *Escherichia coli*. The *E. coli* strains were isolated from various water sources. The primary presumptive identification of *E. coli* was achieved using MacConkey agar. Nineteen isolates were selected and confirmed to be *E. coli* strains based on seven biochemical characteristics. ERIC-PCR with ERIC 1R and ERIC 2 primers were used to generate DNA fingerprints. ERIC-PCR DNA profiles showed variant DNA profiles among the tested *E. coli* strains and distinguished all *E. coli* strains from the other tested bacterial strains. A 350 bp band that predominated in five *E. coli* strains was used for the development of the species-specific SCAR primers EC-F1 and EC-R1. The primers showed good specificity for *E. coli*, with the exception of a single false positive reaction with *Sh. flexneri* DMST 4423. The primers were able to detect 50 pg and 10^0 CFU/ml of genomic DNA and cells of *E. coli*, respectively.

Keywords: *E. coli*, ERIC-PCR, SCAR primer, detection

Introduction

The Gram-negative bacterium *Escherichia coli* has been studied extensively. *E. coli* is used as an indicator of human health hazards (Vinten *et al*., 2004) and is the microbiological parameter that is most frequently monitored during surveillance of drinking and bathing water (Heijnen and Medema, 2009). *E. coli* is a bacterium that resides in the intestines of warm-blooded animals, and it has proven value in the detection of fecal contamination in water [Organization for Economic Cooperation and Development (OECD), 2003].

Many selective media can be used for the presumptive detection of *E. coli*. These culture methods are sensitive but may be time-consuming (Heijnen and Medema, 2009). The development of new techniques for the rapid detection and accurate identification of *E. coli* is therefore important.

In recent years, molecular technologies to detect and identify this microorganism using techniques such as PCR, nucleic acid hybridization (McKillip and Drake, 2000; Brasher *et al*., 2002; Tsai *et al*., 2006), PCR-ELISA (Daly *et al*., 2002; Kuo *et al*., 2010) and protein profile (PAGE) analysis (Abdallah, 2005) have developed rapidly. One of the detection techniques, species-specific molecular markers, can successfully be used to develop sequence characterized amplified region (SCAR) markers to detect many bacterial strains. For example, Kim *et al*. (2005) successfully used a subspecies-specific DNA probe and PCR primers to identify *Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586. Additionally, Kumer *et al*. (2011) used an amplicon of 304 bp from the *opc* gene as a specific genetic marker for the specific detection of *N. meningitidis* in patients with bacterial meningitis.

Among the available species-specific molecular markers, the markers derived from repetitive element polymorphism (rep)-PCR (Versalovic *et al*., 1991; Martin *et al*., 1994; Versalovic *et al*., 1994) can be used to identify many bacteria (Cherif *et al*., 2002). Rep-PCR primers have targets within repetitive regions in the bacterial genome. Therefore, they can produce a PCR product profile that is generally specific to a given strain. Brumlik *et al*. (2001) used rep-PCR to distinguish 105 *B. anthracis* strains from related species within the *B. cereus* group. In another case, enterobacterial repetitive intergenic consensus (ERIC)-PCR was used to detect and classify outbreak isolates of *Vibrio parahaemolyticus* (Khan *et al*., 2002). Subsequently, dos Anjos Borges *et al*. (2003) reported rep-PCR to be a powerful technique for the characterization of *E. coli* isolates from polluted water sites. The present study aimed to generate DNA fingerprints from *E. coli* isolated from various water sources to develop SCAR primers for the detection of *E. coli*.

Materials and Methods

Sample collection and isolation of *E. coli*

A total of 19 water samples from waste water (WW), stagnant water (SW), streaming water (STW), treated water (TW), and domestic animal water (DAW) were collected from Maha Sarakham Province, Thailand. *E. coli* was isolated from all water samples by the dilution plating method. MacConkey agar was used to select *E. coli* isolates. Isolates obtained from the cultural examination of all samples were further confirmed as *E. coli* based on colony characteristics, morphology, Gram reaction, and biochemical properties. After isolation, the *E. coli* was stored in NA medium at room temperature. No strain was subcultured more than twice before examination.

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Biochemical property tests

Gram staining was performed on the isolated *E. coli* prior to testing as described by Kanungo (2009). Several biochemical tests were performed including the indole test, methyl red test, Voges-Proskauer test, citrate utilization test, motility test, Triple Sugar Iron Agar test and lysine decarboxylase test. These tests were performed as described by Delost (1997).

Bacterial genomic DNA extraction

Total genomic DNA was extracted from 19 *E. coli* isolates and nine reference strains of bacteria (*E. coli* ATCC 25122, *E. coli* ATCC 25922, *E. coli DH5α, Vibrio cholerae* ATCC 14035, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 25923, *Shigella flexneri* DMST 4423, *Sh. dysenteriae* DMST 15111, and *Salmonella typhi* DMST 5784). The latter three species were obtained from the Culture Collection for Medical Microorganisms Department of Medical Sciences Thailand. Genomic DNA extraction for Gram-positive and Gram-negative bacteria were performed using alkaline lysis method (Sambrook *et al.*, 1989). Briefly as follow, 1 ml of culture was added to a microcentrifuge tube and centrifuged at 10,000 rpm for 2 min. The supernatant was discarded, and the pellet was suspended in 0.5 ml STE buffer (sodium-tris-EDTA). Then, 10 µl 10 N NaOH was added, and the solution was mixed by inverting 20 times before incubation at 95°C (in a water bath) for 2 min. After the incubation, 0.5 ml of phenol chloroform was added and the solution was incubated at room temperature for 5 min. The mixture was then centrifuged at 13,000 rpm for 5 min. The top aqueous layer was transferred to a new microcentrifuge tube, and 0.1 vol. of 3 M sodium acetate and 2.5 vol. of chilled absolute ethanol were added. This mixture was stored at -20°C for 2 h, and then centrifuged at 13,000 rpm for 20 min. The supernatant was then discarded, and the DNA pellet was air-dried and dissolved in 100 µl of TE buffer.

AAGTAAGTGA CTGGGGTGAG CGAACGCAGC CAAGG**CAGAG**
GCGGCTTGAA GGATGAAGTG TATATAAAGA ATGTCGCCAG
AAATAAACGG GCATACGGCC CGGGGATCTC TGCGCCCTGA
CGTTCACAAA CTGCATATAT CTGATAGACG TGAAACAGGA
GTCATAATGA ATTTTCATCA TCTGGCTTAC TGGCGGGATA
AAGCGTTAAG TCTCGCCATT GAAAACCGCT TATTTATTTA
ACGGTGAATA TACTGCTGCG GCGGAAAATG AAACCTTTG**A**
AACCGTTGAT CCGCTCACCC CAGTCACTTA CTT

Fig. 1. Nucleotide sequence from 5′ to 3′ of the species-specific band derived from *E. coli* isolate SW3. The black boldface underlined sequence indicates the EC-F1 primer while the red boldface underlined sequence indicates the EC-R1 region.

ERIC-PCR primers, conditions, and nucleotide sequencing

Genomic DNA was subjected to PCR using the primers ERIC lR (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) (Versalovic *et al.*, 1991; Louws *et al.*, 1994). The temperature profiles of the PCR cycles were as follows: initial denaturation at 95°C for 3 min, followed by 30 cycles at 95°C for 1 min, 45°C for 1 min, and 72°C for 2 min, and a final extension reaction at 72°C for 10 min. The amplification was carried out in a thermal cycler (Applied Biosystems, USA) in a 25 µl of polymerase chain reaction (PCR) mixture containing 100 ng of total DNA, 0.2 mM of each dNTP, 1.25 units of *Tag* DNA polymerase (Invitrogen, USA) in 5 µl of 10× *Tag* buffer, 1.5 mM $MgCl_2$, 0.4 mM of each primer, and 13.5 µl sterile deionized water. A 5 µl aliquot of each amplification reaction was analyzed using electrophoresis on a 1% agarose gel and run in 0.5× TBE buffer, pH 8.3 (Sambrook *et al.*, 1989). The gel was stained with ethidium bromide and photographed using a gel documentation (Bio-Rad, USA). A 100 base pair marker (Real Biotech Corporation, Taiwan) was included on the gel. A specific band obtained only from *E. coli* was selected and purified with a Gel/PCR DNA Fragments extraction kit (Real Biotech Corporation) and

Table 1. Biochemical characterization of 19 isolates of *E. coli* in comparison with a reference strain of *E. coli*

Strain/Isolate	Gram stain reaction	Indole test	Methyl red test	Voges-Proskauer test	Citrate utilization test	Lysine decarboxylase test	TSI
ATCC25122	-	+	+	-	-	+	A/Ag
SW1	-	+	+	-	-	+	A/Ag
SW2	-	+	+	-	-	+	A/Ag
SW3	-	+	+	-	-	+	A/Ag
SW4	-	+	+	-	-	+	K/Ag
SW5	-	+	+	-	-	+	K/Ag
SW6	-	+	+	-	-	+	A/Ag
WW1	-	+	+	-	-	+	A/Ag
WW2	-	+	+	-	-	-	A/Ag
WW3	-	+	+	-	-	+	A/Ag
WW4	-	+	+	-	-	+	K/Ag
WW5	-	+	+	-	-	-	A/Ag
WW6	-	+	+	-	-	+	A/Ag
STW1	-	+	+	-	-	+	K/Ag
STW2	-	+	+	-	+	-	A/Ag
TW1	-	+	+	-	-	+	A/Ag
TW2	-	+	+	-	-	+	K/Ag
DAW1	-	+	+	-	-	+	A/Ag
DAW2	-	+	+	-	-	+	K/Ag
DAW3	-	+	+	-	-	+	A/Ag

cloned into the pGEM-T-Easy vector (Promega, USA) according to the protocols provided by the manufacturers. The plasmids were transformed into competent *E. coli* JM109 using the TSS method (Chung and Miller, 1993). The nucleotide sequencing was performed by the Biomolecular Analysis Service Unit at the Department of Biochemistry, Faculty of Medicine, Khon Kaen University, Thailand.

Primer specificity and sensitivity test

The specific primers EC-F1 (5′-CAGAGGCGGCTTGAAG GATG-3′) and EC-R1 (5′-GGGTGAGCGGATCAACGGT TT-3′) were designed based on the sequence of the specific band (Fig. 1). PCR was performed to validate the primer specificity and determine the detection limits of the PCR primers. PCR specificity was evaluated by testing the 19 *E. coli* isolates and seven bacterial strains (*E. coli* ATCC 25122, *Sh. flexneri* DMST 4423, *Sh. dysenteriae* DMST 15111, *S. typhi* DMST 5784, *V. cholerae* ATCC 14035, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 25923). For the PCR sensitivity test, the lower limit of detection was defined as the smallest amount of bacterial genomic DNA or number of bacterial cells detectable by PCR. This was determined by serial dilution of the genomic DNA and *E. coli* SW3 cells. The genomic DNA and bacterial cell dilutions ranged from 5 µg to 50 fg and from 10^8 to 1 CFU/ml in 10-fold dilution intervals.

Amplification was performed using a thermal cycler (Applied Biosystems) in 25 µl polymerase chain reaction (PCR) mixtures containing 0.2 mM of each dNTP, 1.25 units of *Tag* DNA polymerase (Invitrogen, USA) in 5 µl of 10× *Tag* buffer, 2 mM $MgCl_2$ and 0.2 mM of each primer. The genomic DNA and bacterial cells were then added to a PCR tube. The temperature profile for the PCR cycles were as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles at 94°C for 1 min, 58°C or 60°C or 62°C for 1 min, and 72°C for 1 min, and a final extension reaction at 72°C for 10 min. A 5 µl aliquot of each amplification reaction was analyzed by electrophoresis on a 1% agarose gel.

Results and Discussion

Sample collection, isolation and biochemical property tests of *E. coli*

Water samples obtained from different locations in Maha Sarakham Province were cultured on nutrient agar medium and yielded 126 isolates of bacteria. Only 19 isolates produced smooth, white, round colonies that were be suspected as *E. coli*. Their identities were confirmed as *E. coli* by examining their morphology, evaluating the colony color on MacConkey agar and testing their biochemical characteristics. Nineteen isolates that produced pink colored colonies indicative of lactose fermentation, were selected for further testing.

The biochemical test results were similar for all 19 isolates (Table 1), and all strains were Gram-negative. In the indole test, all isolates showed red coloration after the addition of Kovac's reagent. All of the tested isolates were methyl red-positive and Voges-Proskauer-negative, respectively. In the

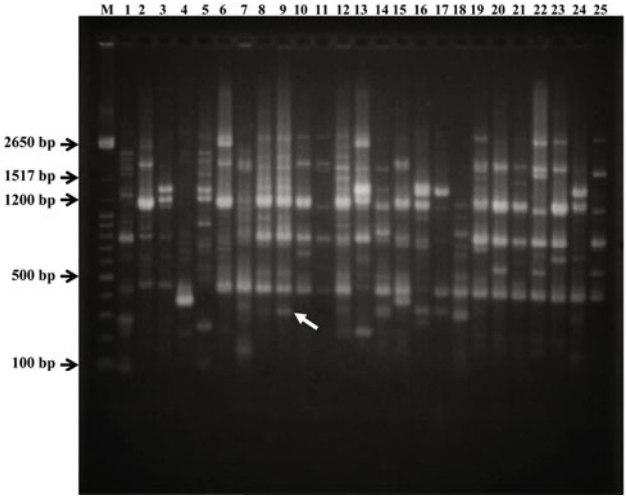

Fig. 2. ERIC-PCR DNA fingerprint of 19 isolates of *E. coli* compared with 6 reference strains of bacteria on a 1% agarose gel electrophoresis: *E. coli* ATCC 25122 (1), *E. coli* DH5α (2), *E. coli* ATCC 25922 (3), *S. typhi* DMST 5784 (4), *Sh. flexneri* DMST 4423 (5), *V. cholerae* ATCC 14035 (6), *E. coli* isolates SW1-6 (7–12), WW1-6 (13–18), DAW1-3 (19–21), STW1-2 (22–23), and TW1-2 (24–25). The arrow indicates the species-specific band used to develop the species-specific SCAR primer.

citrate utilization test, all samples produced a green color after 24 h incubation, except isolate STW2. However, the isolates showed different utilization patterns in the TSI and lysine decarboxylase tests. The results obtained were the same as those described by Mahon and Manuselis (2000). These results confirmed that all 19 isolates were *E. coli*.

ERIC-PCR primers, conditions and nucleotide sequencing

ERIC-PCR analysis of the 19 *E. coli* isolates and six references strains was preformed with the primers ERIC lR and ERIC2. The generated fingerprints were evaluated for overall band-

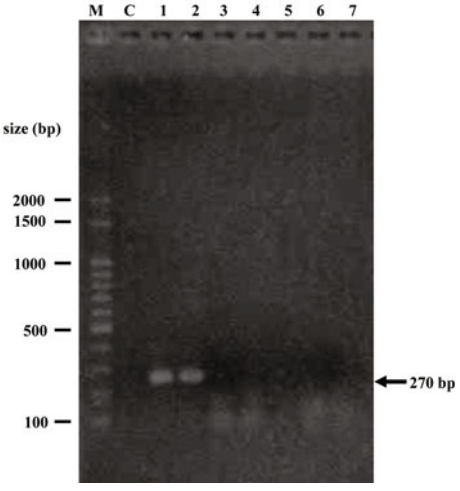

Fig. 3. Specificity test of the EC-F1 and EC-R1 primer pair. 100 ng of each sample of bacterial genomic DNA was used as the PCR templates. The PCR products were electrophoresed on a 1% agarose gel: negative control (C), *E. coli* ATCC 25122 (1), *Sh. flexneri* DMST 4423(2), *Sh. dysenteriae* DMST 15111 (3), *S. typhi* DMST 5784 (4), *V. cholera* ATCC 14035 (5), *P. aeruginosa* ATCC 27853 (6) and *S. aureus* ATCC 25923 (7).

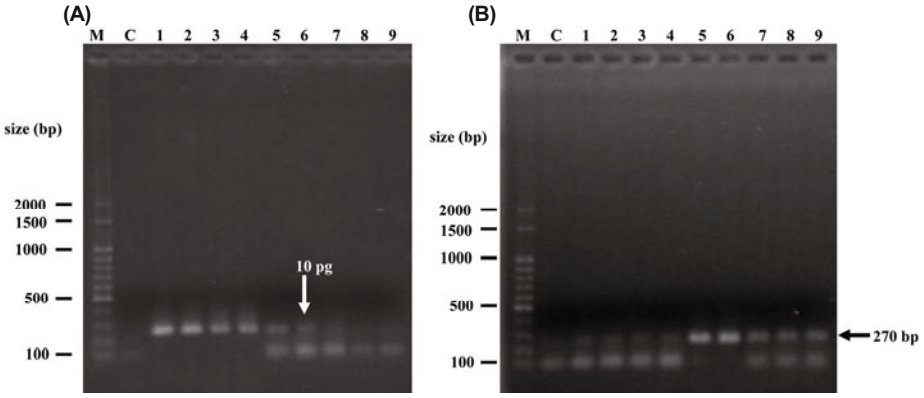

Fig. 4. Sensitivity test of the EC-F1 and EC-R1 primer pair. PCR products were electrophoresed on 1% agarose gel. A, Genomic DNA of *E. coli* isolate SW3 serially diluted 10-fold, from 5 µg to 50 fg. B, Cell suspension of *E. coli* isolate SW3 serially diluted 10-fold from 10^8 to 1 CFU/ml.

ing pattern clarity. The primers showed polymorphism amplification patterns and consistently produced 6–10 bands of 0.2–2.7 kb. One band with a size of 350 bp was predominant in the fingerprints of the five *E. coli* isolates, SW3, SW6, WW2, WW4, and WW6 (Fig. 2), while no corresponding bands were present in the other tested isolates. This band was purified from isolate SW3 and cloned into pGEMT-easy for nucleotide sequencing. The fragment, which was found to consist of 313 nucleotides, was subjected to DNA similarity searches (BLAST) to the GenBank database. The entire fragment was found to share 83–100% homology at the nucleic acid level with regions of the recently sequenced *E. coli* genome. Therefore, this band was used for the development of species-specific SCAR primer for *E. coli* detection.

Primer specificity and sensitivity test

The specific primers EC-F1 and EC-R1 were designed from the 313 nucleotide DNA fragment. The optimal annealing temperature of this primer pair was 60°C. The data obtained from the specificity test showed that the primers EC-F1 and EC-R1 were specific for *E. coli* ATCC 25122 with the exception of *Sh. flexneri* DMST 4423 (Fig. 3). This result was consistent with the evidence from the nucleotide search in the National Center for Biotechnology Information data bank (www.ncbi.nih.gov/blast). A BLAST search in NCBI showed that the sequence of this DNA fragment had 98–100% homology to *E. coli* and *Sh. flexneri* strains (not shown). These results are similar to those of Fukushima *et al.* (2002), who found that *E. coli* is closely related to *Shigella*. It has been proposed that *E. coli* and *Shigella* should be considered a single species based on their genetic relationships (Lawrence *et al.*, 1991; Paradis *et al.*, 2005). These two bacteria are listed as the same species in Bergey's Manual of Systematic Bacteriology (Brenner, 1984). Based on a BLAST search, *Sh. dysenteriae* has 97% homology to this DNA fragment but was not amplified by these two primers. This confirms that the two primers should not detect other pathogenic and non-pathogenic bacteria with low DNA homology.

The EC-F1 and EC-R1 primer pair was able to detect genomic DNA and *E. coli* SW3 cells at concentrations as low as 50 pg and 10^0 CFU/ml, respectively (Fig. 4). This sensitivity is comparable to those of the culture-based amperometric method, reverse transcription multiplex TaqMan PCR and PCR-ELISA, which were previously reported by Pérez *et al.* (2001), Tsai *et al.* (2006), and Kuo *et al.* (2010), respectively. Moreover, the primers amplified DNA from *E. coli* ATCC 25122, *E. coli* ATCC 25922, and the 19 *E. coli* isolates without producing any non-specific bands (Fig. 5). These data indicate that the EC-F1 and EC-R1 primers could be useful for the detection of *E. coli*. For increased efficiency, this species-specific SCAR primer could be used in combination with MacConkey agar or Eosin Methylene Blue (EMB) agar methods to differentiate *E. coli* from *Sh. flexneri*.

From this study, we also concluded that *E. coli* is found in different water sources, which is important because the contamination of water sources by *E. coli* may indicate a threat to public health. The reliable detection and/or iden-

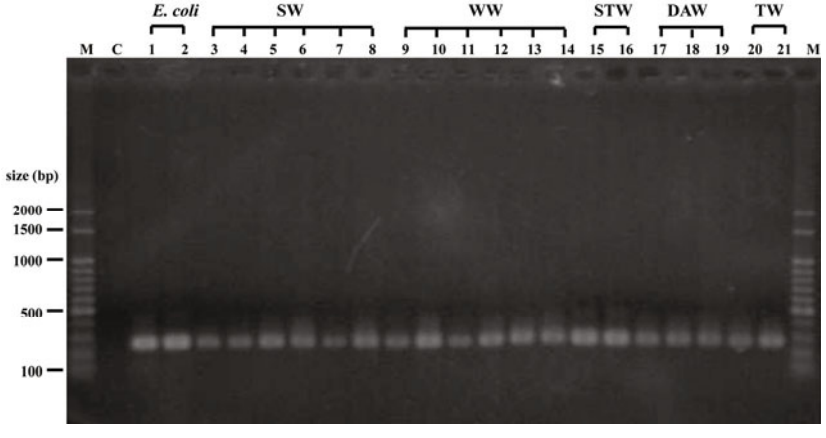

Fig. 5. PCR performed with primers EC-F1 and EC-R1. 100 ng of each sample of bacterial genomic DNA was used as a PCR template. The PCR products were electrophoresed on 1% agarose gel: negative control (C), *E. coli* ATCC 25122 (1), *E. coli* ATCC 25922 (2), *E. coli* isolates SW1-6 (3–8), WW1-6 (9–14), STW1-2 (15–16) DAW1-3 (17–19) and TW1-2 (20–21).

tification of *E. coli* from such environments could be performed by culture on MacConkey agar or EMB agar followed by use of the newly designed SCAR primers (EC-F1 and EC-R1 primer).

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