

REPETITIVE SEQUENCE BASED-PCR PROFILING OF *ESCHERICHIA COLI* O157 STRAINS FROM BEEF IN SOUTHERN THAILAND

Pharanai Sukhumungoon^{1,2}, Rujira Tantadapan² and Pattamarat Rattanachuy³

¹Food Safety and Health Research Unit, ²Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla; ³Department of Pre-Clinic, Faculty of Science and Technology, Prince of Songkla University, Pattani Campus, Pattani, Thailand

Abstract. Beef and its products are potential vehicles of *Escherichia coli* O157, the most important serotype implicated in many large outbreaks of diarrheal infection in humans worldwide. There is a need for rapid detection of contaminated food in order to implement appropriate and effective control measures. In this study, repetitive sequence (rep)-PCR, using three different primers, BOXA1R, ERIC2 and (GTG)₅, singly and in combinations, were employed to compare the genetic relatedness among *E. coli* O157 group with other diarrheagenic *E. coli* strains as controls. Although a combination of BOXA1R + ERIC2 + (GTG)₅ primers generated a rep-PCR profile containing the highest number of amplicon bands among the DEC strains tested, dendrogram (at 80% similarity) exhibited the lowest DEC classification of 5 clusters, whereas that from BOXA1R or BOXA1R + (GTG)₅ rep-PCR profiling produced 8 clusters. Nevertheless, focusing *E. coli* O157 strains were grouped into 4 clusters irrespective of the rep-PCR profiles analyzed, and all 14 but two, PSU60 and PSU132, *E. coli* O157 strains isolated from beef in southern Thailand during 2012 to 2014 fell into a single cluster. Thus, rep-PCR profiling generated with BOXA1R or BOXA1R + (GTG)₅ is sufficient for distinguishing among DEC strains, including *E. coli* O157 in southern Thailand.

Keywords: *Escherichia coli* O157, BOXA1R, ERIC2, (GTG)₅, repetitive sequence-PCR, Thailand

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is a food-borne pathogen associated with outbreaks of food poisoning worldwide (Michino *et al*, 1999; Rangel *et al*, 2005). STEC carries *stx*₁ or *stx*₂ or

both encoding Stx isoforms, which upon release, bind to the specific receptor, globotriaosylceramide (Gb3) (de Sablet *et al*, 2008) and then are internalized into target cells where they cause the elimination of an adenine residue from 28S ribosomal RNA of eukaryotic ribosome, resulting in inhibition of protein biosynthesis (Nataro and Kaper, 1998). Although over 40 serotypes of STEC have been reported to cause severe forms of diseases (Paton and Paton, 1996), *E. coli* O157:H7 (*E. coli* O157) is the most important serotype implicated

Correspondence: Pharanai Sukhumungoon, Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand.
Tel: +66 (0) 74 288322; Fax: +66 (0) 74 446661
E-mail: pharanai82@gmail.com

in many large outbreaks, causing massive economic losses and public health costs (Michino *et al*, 1999; Xiong *et al*, 2012). Enterohemorrhagic *E. coli* (EHEC) is defined by the possession of *stx* together with *eae* coding for intimin, a crucial protein responsible for bacterial attachment to host cells (Nataro and Kaper, 1998).

As cattle are reservoir hosts of *E. coli* O157, beef and its products constitute a potential vehicle for transmission of *E. coli* O157 infection (Rangel *et al*, 2005). Ground beef frequently has been reported to be implicated in a number of outbreaks worldwide (Rangel *et al*, 2005).

Establishment of the association of *E. coli* O157 with food and clinical symptoms is needed to be uncovered rapidly in order to limit the spread of the infectious agent. An important technique in tracking bacterial strains is repetitive sequence-PCR (rep-PCR) as enterobacterial repetitive intergenic consensus (ERIC), repetitive BOX elements (BOX) and repetitive poly-trinucleotide (GTG)₅ sequences are conserved in several specific bacterial species including *E. coli* (Versalovic *et al*, 1994). Profiles generated by PCR amplification of these DNA regions have proven to be reliable and reproducible for individual bacteria strains (Versalovic *et al*, 1994). Furthermore, the protocol to generate the DNA profiles is simple and not time consuming.

Thus, we employed rep-PCR to compare the above mentioned repetitive sequences in *E. coli* O157 from beef in southern Thailand during 2012-2014 in order to elucidate their genetic relatedness.

MATERIALS AND METHODS

Diarrheagenic *E. coli* (DEC) strains

Fourteen *E. coli* O157 (PSU2-PSU6,

PSU53-PSU60, PSU132) isolates from beef during 2012 to 2014 and a clinical *stx*-negative *E. coli* O157 isolated from a diarrheal patient (PSU120), Hat-Yai Hospital, Songkhla Province, Thailand, were studied (Table 1). *E. coli* O157 strain EDL933, a clinical EHEC O157 from USA was used as a control strain, and four diarrheagenic *E. coli* pathotypes, namely, STEC O8 strain PSU1, enteropathogenic *E. coli* (EPEC) O111 strain PE-27, enterotoxigenic *E. coli* (ETEC) O169 strain PSU192, and enteroaggregative *E. coli* (EAEC) O44 strain PSU280, were also included for comparative purposes.

Rep-PCR profile and dendrogram construction

An individual bacterial colony grown on tryptic soy agar (TSA) (Becton, Dickinson, San Jose, CA) was inoculated into 5 ml of tryptic soy broth (TSB) (Becton, Dickinson) and incubated at 37°C for 6 hours with shaking. One ml aliquot of bacterial culture was subjected to genomic DNA (gDNA) extraction process using a glass fiber matrix spin column (Geneaid, New Taipei City, Taiwan). Quantification and intactness of extracted gDNA were performed using 0.8% agarose gel-electrophoresis with standard DNA size markers (2-log DNA Marker; NEB, Ipswich, MA). Rep-PCR was performed using primers ERIC2 (5'- AAGTAAGTGACTGGGGT-GAGCG-3') (Versalovic *et al*, 1991), BOXA1R (5'- CTACGGCAAGGCGAC-GCTGACG-3') (Versalovic *et al*, 1994), and (GTG)₅ (5'- GTGGTGGTGGTGGTG-3') (Versalovic *et al*, 1991). In addition, various combinations of these specific primers were performed, namely, BOXA1R + (GTG)₅, BOXA1R + ERIC2, (GTG)₅ + ERIC2, and (GTG)₅ + ERIC2 + BOXA1R. PCR was carried out in a 25- μ l reaction mixture composed of 1.0 μ M each primer

Table 1
Diarrheagenic *E. coli* strains used in the study.

Pathotype	Strain	Year of isolation	Origin	Genotype	Serotype	Reference
EHEC	PSU2	2012	Beef, Thailand	<i>stx</i> ₁ ⁻ <i>stx</i> ₂ ⁺ <i>eae</i> ⁺	O157	Sukhumungoon <i>et al</i> , 2013
	PSU3	2012	Beef, Thailand	<i>stx</i> ₁ ⁻ <i>stx</i> ₂ ⁺ <i>eae</i> ⁺	O157	Sukhumungoon <i>et al</i> , 2013
	PSU4	2012	Beef, Thailand	<i>stx</i> ₁ ⁻ <i>stx</i> ₂ ⁺ <i>eae</i> ⁺	O157	Sukhumungoon <i>et al</i> , 2013
	PSU5	2012	Beef, Thailand	<i>stx</i> ₁ ⁻ <i>stx</i> ₂ ⁺ <i>eae</i> ⁺	O157	Sukhumungoon <i>et al</i> , 2013
	PSU6	2012	Beef, Thailand	<i>stx</i> ₁ ⁻ <i>stx</i> ₂ ⁺ <i>eae</i> ⁺	O157	Sukhumungoon <i>et al</i> , 2013
	PSU53	2012	Beef, Thailand	<i>stx</i> ₁ ⁻ <i>stx</i> ₂ ⁺ <i>eae</i> ⁺	O157	Sukhumungoon <i>et al</i> , 2013
	PSU54	2012	Beef, Thailand	<i>stx</i> ₁ ⁻ <i>stx</i> ₂ ⁺ <i>eae</i> ⁺	O157	Sukhumungoon <i>et al</i> , 2013
	PSU55	2012	Beef, Thailand	<i>stx</i> ₁ ⁻ <i>stx</i> ₂ ⁺ <i>eae</i> ⁺	O157	Sukhumungoon <i>et al</i> , 2013
	PSU56	2012	Beef, Thailand	<i>stx</i> ₁ ⁻ <i>stx</i> ₂ ⁺ <i>eae</i> ⁺	O157	Sukhumungoon <i>et al</i> , 2013
	PSU57	2012	Beef, Thailand	<i>stx</i> ₁ ⁻ <i>stx</i> ₂ ⁺ <i>eae</i> ⁺	O157	Sukhumungoon <i>et al</i> , 2013
	PSU58	2012	Beef, Thailand	<i>stx</i> ₁ ⁻ <i>stx</i> ₂ ⁺ <i>eae</i> ⁺	O157	Sukhumungoon <i>et al</i> , 2013
	PSU59	2012	Beef, Thailand	<i>stx</i> ₁ ⁻ <i>stx</i> ₂ ⁺ <i>eae</i> ⁺	O157	Sukhumungoon <i>et al</i> , 2013
	PSU60	2012	Beef, Thailand	<i>stx</i> ₁ ⁻ <i>stx</i> ₂ ⁺ <i>eae</i> ⁺	O157	Sukhumungoon <i>et al</i> , 2013
	EDL933	1982	Human, USA	<i>stx</i> ₁ ⁺ <i>stx</i> ₂ ⁺ <i>eae</i> ⁺	O157	Riley <i>et al</i> , 1983
	PSU120	2014	Human, Thailand	<i>stx</i> ₁ ⁻ <i>stx</i> ₂ ⁻ <i>eae</i> ⁺	O157	Thempachana <i>et al</i> , 2014
	EAEC	PSU132	2014	Beef, Thailand	<i>stx</i> ₁ ⁻ <i>stx</i> ₂ ⁺ <i>eae</i> ⁺	O157
PSU280		2013	Human, Thailand	<i>aggR</i> , <i>aggA</i> , <i>aafA</i> , <i>pet</i> , <i>astA</i>	O44	Sukkua <i>et al</i> , 2015
EPEC	PE-27	n.d.	n.d.	<i>bfp</i> , <i>eae</i>	O111	Reid <i>et al</i> , 1999
ETEC	PSU192	2014	Human, Thailand	<i>est</i> , <i>astA</i>	O169	Sirikaew <i>et al</i> , 2014
STEC	PSU1	2012	Beef, Thailand	<i>stx</i> ₁ ⁺ <i>stx</i> ₂ ⁺ <i>eae</i> ⁺	O8	Sukhumungoon <i>et al</i> , 2013

n.d., no data.

pair, 0.2 mM dNTPs, 1X GoTaq DNA polymerase buffer, 3.0 mM MgCl₂, 1.25 U GoTaq DNA polymerase (Promega, Madison, WI) and 50 ng of gDNA. Thermocycling (conducted in T100 Thermal Cycler; Bio-rad, Hercules, CA) conditions were as follows: 95°C for 3 minutes; followed by 30 cycles of 94°C for 3 seconds, 92°C for 30 seconds, 40°C for 1 minute for (GTG)₅ or 50°C for 1 minute for BOXA1R, ERIC2 and BOXA1R + ERIC2 or 45°C for (GTG)₅ + BOXA1R, (GTG)₅ + ERIC2 and (GTG)₅ + ERIC2 + BOXA1R, and 65°C for 8 minutes. Amplicons (15 µl) were analyzed by 1.5% agarose gel-electrophoresis (Invitrogen, Carlsbad, CA) for 1 hour at 100 V, staining with ethidium bromide and visualizing using WSE-5200 Printgraph 2M gel imaging system (ATTO, Tokyo, Japan). Dendrograms were constructed using unweighted pair-group method of arithmetic average (UPGMA) (BioProfile software, Waltham, MA).

RESULTS

Rep-PCR profiles

Among *E. coli* O157 strains, rep-PCR profiles generated by a combination of (GTG)₅ + ERIC2 + BOXA1R primers gave the highest numbers of DNA bands (ranging from 22 to 26), with sizes of ≈120-3,000 bp (Fig 1G and Table 2), while the lowest number (11) of DNA bands was generated using ERIC2 primer, with sizes of 350-3,500 bp (Fig 1C). BOXA1R or (GTG)₅ primer alone showed distinctly higher number of DNA bands compared to ERIC2 primer (Table 2). Corresponding results were obtained when all DEC strains were analyzed (Fig 1).

Dendrograms

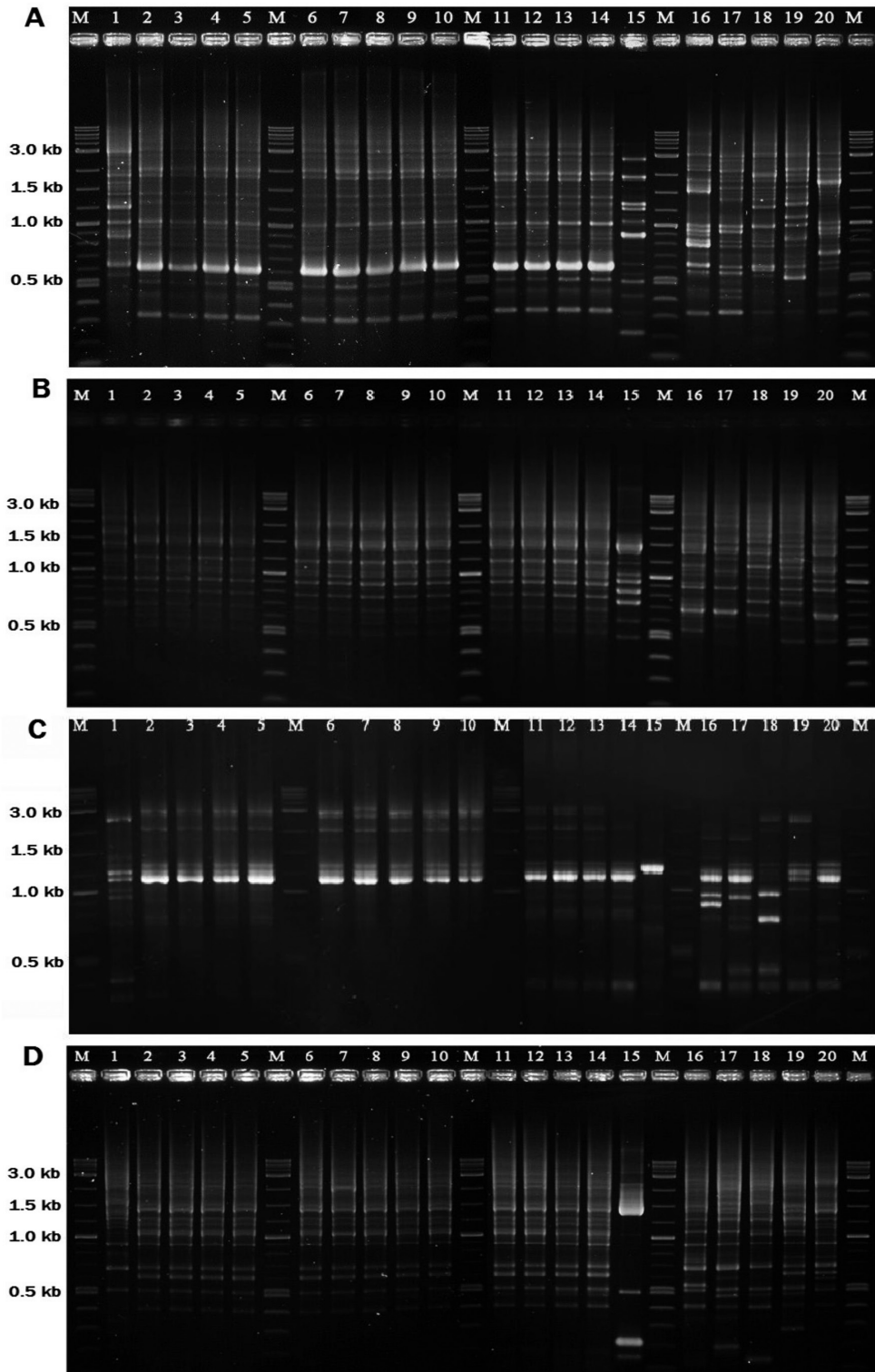
Dendrograms were analyzed at 80% similarity. Rep-PCR profiles generated us-

ing BOXA1R and BOXA1R + (GTG)₅ primers provided the highest discriminatory power, classifying all DEC strains into 8 distinguishable clusters (Fig 2). ERIC2, BOXA1R + ERIC2, and (GTG)₅ + ERIC2 rep-PCR produced 7 clusters, (GTG)₅ generated 6 clusters and, surprisingly, only 5 clusters were obtained from (GTG)₅ + ERIC2 + BOXA1R primer combination. All primers used presented a similar set of members in such specific clusters. For instance, STEC O8 strain PSU1, EHEC O157 strain PSU60 and EHEC O157 strains from beef together with EHEC O157 strain EDL933 always belonged to different clusters using every primer and primer combination.

The 16 *E. coli* O157 strains could be classified into 4 distinct clusters (for example by BOXA1R classifying into cluster II, III, V, and VI) using rep-PCR profiles produced from any of the primers, singly or in combination (Fig 2). All *E. coli* O157 strains from beef samples of southern Thailand (PSU2-PSU6, PSU53-PSU59, PSU132) belonged to cluster II except EHEC O157 strain PSU60 that was located distantly related. One of the factors affecting the divergence of this PSU60 strain is the presence of Q₉₃₃ (coding for Q₉₃₃ protein responsible for strong anti-termination activity resulting in downstream gene expression) derived from bacteriophage 933 together with Q₂₁ in its chromosome while the other O157 strains possessed only Q₂₁ (data not shown).

The *stx*-negative *E. coli* O157 strain PSU120 and PSU132 (*stx*₁⁻, *stx*₂⁻, *eae*⁻), which exhibited different rep-PCR profiles, fell into different cluster, such as cluster V and VI by BOXA1R and cluster IV and III by (GTG)₅, respectively (Fig 2). It was clear also that these *stx*-negative *E. coli* O157 lineages had genetically

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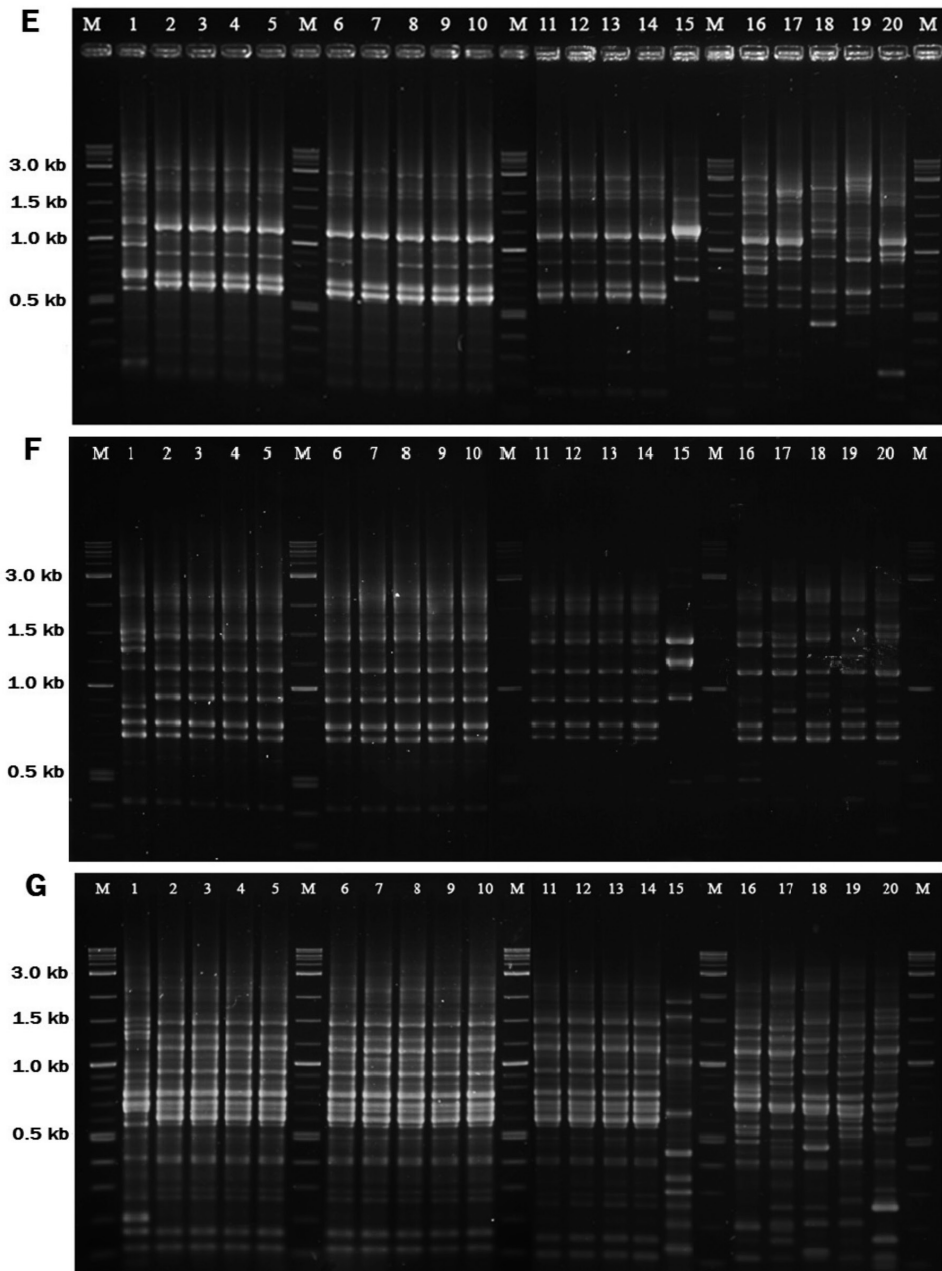


Fig 1—Rep-PCR amplicon profile of *E. coli* O157 strains isolated from beef and humans. (A) BOXA1R, (B) $(GTG)_5$, (C) ERIC2, (D) BOXA1R + $(GTG)_5$, (E) BOXA1R + ERIC2, (F) $(GTG)_5$ + ERIC2, and (G) BOXA1R + ERIC2 + $(GTG)_5$. PCR protocols and primer sequences are described in Materials and Methods. Lane M, DNA size markers; lane 1, STEC O8 strain PSU1; lanes 2-15, EHEC O157 strains PSU2, PSU3, PSU4, PSU5, PSU6, EDL933, PSU53, PSU54, PSU55, PSU56, PSU57, PSU58, PSU59, and PSU60; lane 16, EPEC O111 strain PE-27; lane 17, *E. coli* O157 strain PSU120; lane 18, *E. coli* O157 strain PSU132; lane 19, ETEC O169 strain PSU192; lane 20, EAEC O44 strain PSU280.

Table 2
Rep-PCR amplicon patterns of diarrheagenic *E. coli* (DEC) strains.

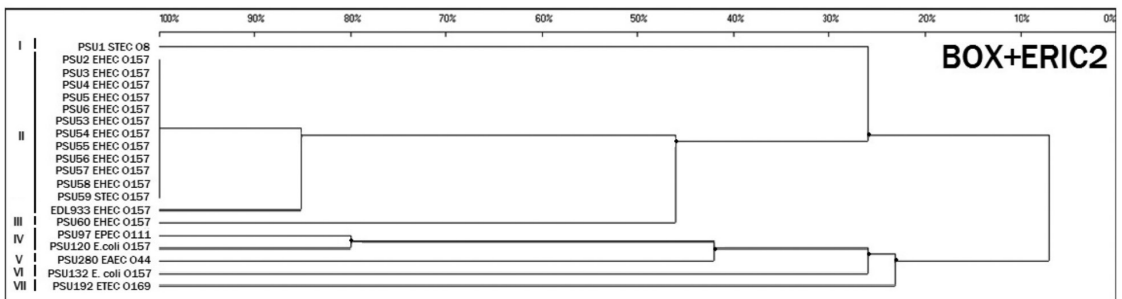
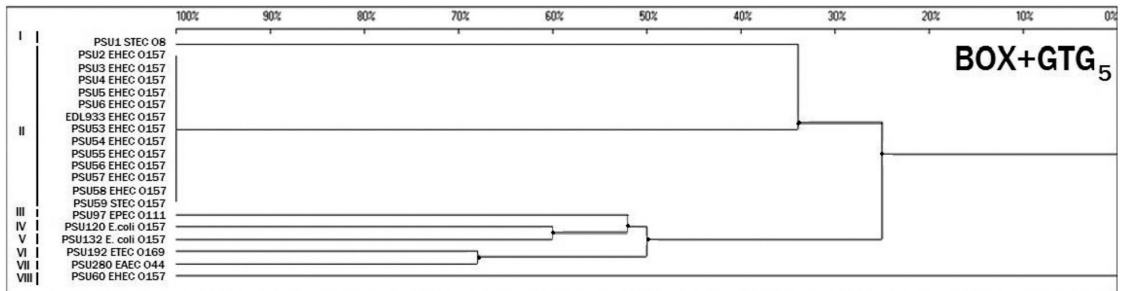
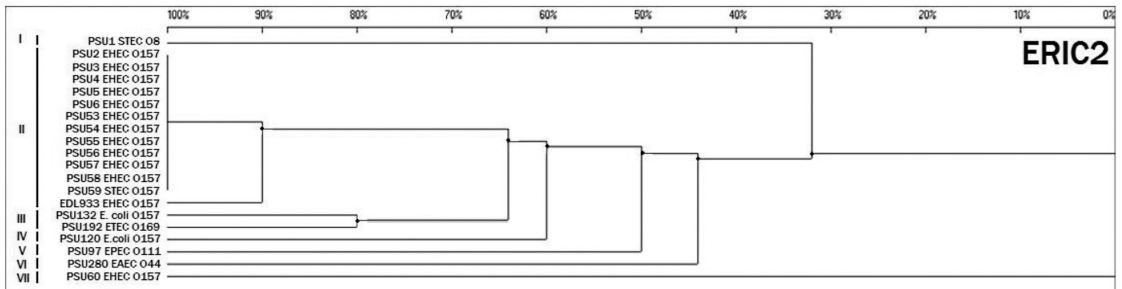
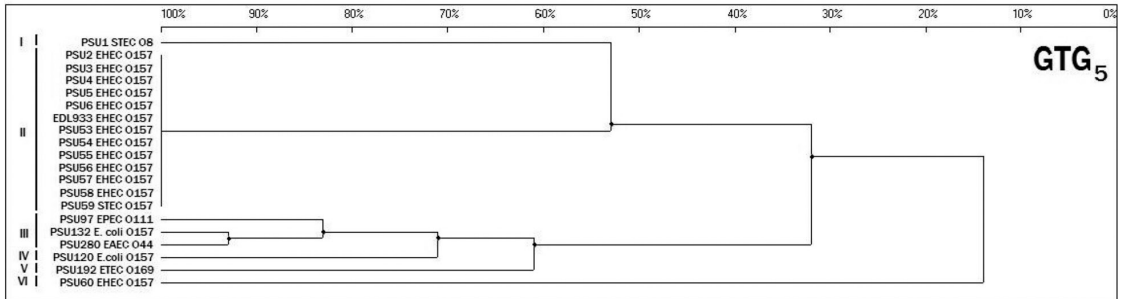
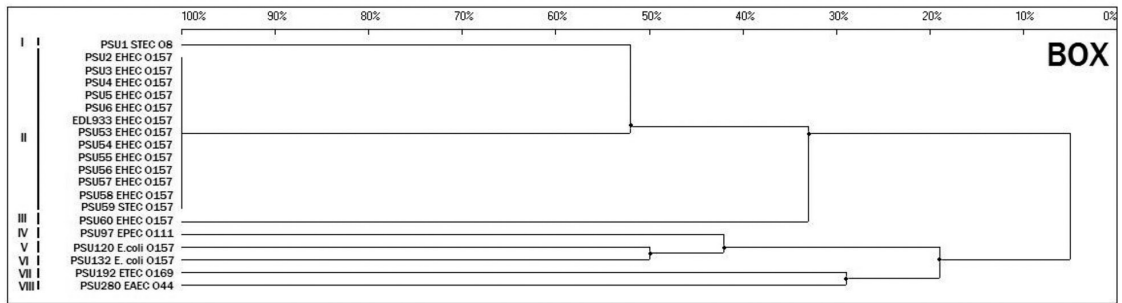
Primer	DEC strains						<i>E. coli</i> O157 strains					
	No. of bands			Size of band (bp)			No. of bands			Size of band (bp)		
	Lowest	Highest	Average	Smallest	Largest	Lowest	Highest	Average	Smallest	Largest		
BOXA1R	13	21	16	200	4,000	14	21	15	≈320	4,000		
(GTG) ₅	12	20	15	500	3,000	13	16	15	500	3,000		
ERIC2	9	13	11	350	3,500	9	12	11	350	3,500		
BOXA1R + (GTG) ₅	11	20	16	150	3,000	11	20	16	150	3,000		
BOXA1R + ERIC2	14	20	15	150	3,500	14	17	15	150	3,500		
(GTG) ₅ + ERIC2	8	18	16	300	4,000	8	17	16	≈420	4,000		
(GTG) ₅ + ERIC2 + BOXA1R	22	27	26	100	3,000	22	26	26	≈120	3,000		

diverged from the *stx*₁⁻, *stx*₂⁺, *eae*⁺ O157 strains, as demonstrated by the observation that BOXA1R and BOXA1R + ERIC2 showed < 10% similarity in genetic relatedness between these two groups.

DISCUSSION

In this study using a cut-off percentage of 80% similarity, rep-PCR profiling employing BOXA1R and BOXA1R + (GTG)₅ primers provided the highest discriminatory power, which differed from the work of Mohapatra *et al* (2007) showing that (GTG)₅ rep-PCR profiling is better than that of BOX and ERIC for differentiating fecal *E. coli* isolated from human, poultry, and wild bird fecal material regardless of their serotypes. Studies from Turkey indicated comparable efficacy between ERIC and (GTG)₅ rep-PCR for *E. coli* O157 differentiation (Adiguzel *et al*, 2012). In addition, ERIC rep-PCR has been shown to have a greater ability than (GTG)₅ and BOXA1R primers in discriminating among *Yersinia ruckeri* strains (Huang *et al*, 2013). These differences may vary laboratory-to-laboratory due to the differences in bacterial species, PCR conditions and analysis tools used. Recently, a commercially automated rep-PCR system, DiversiLab (bioMérieux), has been developed, which has a potential to be standardized for routine bacterial and fungal typing inter- and intra-laboratories, providing the advantages in terms of discriminatory power and reliability (Healy *et al*, 2005). Nevertheless, utilization of rep-PCR for investigation of outbreak should be carefully interpreted with the conjunction of epidemiological data.

Although the combination of (GTG)₅ + ERIC2 + BOXA1R primers gave the highest average number DNA band in rep-PCR, its discriminatory power as



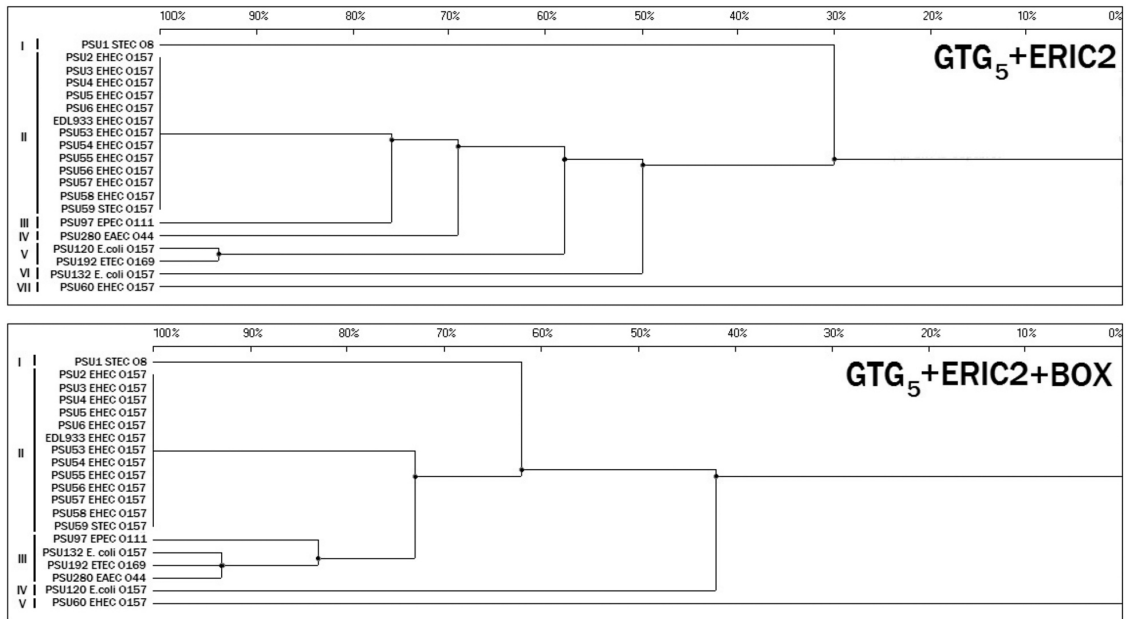


Fig 2–Dendrograms of *E. coli* O157 isolated from beef and standard DEC strains based on rep-PCR profiles. Dendrograms are constructed using unweighted pair-group method of arithmetic average (UPGMA) (BioProfile software). The data are analyzed at cut-off of 80% similarity. Primers used in rep-PCR are indicated in the diagram. BOX = BOXA1R.

shown by dendrogram analysis (80% similarity) ranked the lowest among the 7 primers (single and in combination) utilized. It is assumed that the combination of the three primers in the same PCR reaction does not perturb the amplification reactions of each other, and no significant primer dimers are generated so as to compromise the overall rep-PCR efficiency. Our results are in agreement with those of Yoke-Kqueen *et al* (2013) who showed that among rep-PCR profiling of *Vibrio parahaemolyticus* strains carrying *tdh* and *trh* using ERIC, RAPD, and BOX primers and their combinations, ERIC + RAPD combination produces the highest discriminatory power regardless of percent similarity.

In conclusion, based on rep-PCR pro-

filings with BOXA1R, ERIC2, and (GTG)₅ primers, alone and in combinations, revealed all but two of 14 *E. coli* O157 strains isolated from beef in southern Thailand are derived from the same clone, whereas *stx*-negative *E. coli* O157 is not closely related genetically. In addition, combinations of rep-PCR primers may not be always increase the discriminatory power for *E. coli* O157 differentiation, but only one or two appropriate primers are sufficient for strain typing. As regards to genotyping of *E. coli* O157 from beef in southern Thailand, rep-PCR employing BOXA1R primer is sufficient enough, with the discriminatory power and clear amplicons profile, and should be used for investigating genetic relatedness of *E. coli* O157 in future work.

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