DETECTION AND MOLECULAR CHARACTERIZATION OF ENTEROAGGREGATIVE *ESCHERICHIA COLI* FROM DIARRHEAL PATIENTS IN TERTIARY HOSPITALS, SOUTHERN THAILAND

Kannika Sukkua[1], Wichien Patungkaro[2] and Pharanai Sukhumungoon[1,3]

[1]Department of Microbiology, Faculty of Science, Prince of Songkla University; [2]Department of Pathology, Hat-Yai Hospital; [3]Food Safety and Health Research Unit, Prince of Songkla University, Hat Yai, Songkhla, Thailand

Abstract. Enteroaggregative *Escherichia coli* (EAEC) is a member of diarrheagenic *E. coli*, which plays an important role in human infections in developing countries. Investigations of EAEC in diarrheal patients at Hat-Yai and Pattani Hospitals, southern Thailand identified 5 EAEC strains, which were classified into serogroups, O44 and O127a. All 5 EAEC contained the global transcriptional regulator gene, *aggR*, and possessed fimbrial subunit genes, *aggA* and *aafA*, and EAEC-related toxin genes, *astA* and *pet*. PCR-based phylogenetic group assay revealed that the EAEC belonged to groups B1 and D. Low levels of clump formation were observed for all EAEC strains. Scanning electron microscopy showed an absence of fimbrial structure. DNA profiles generated by BOX- and ERIC2-PCR exhibited correspondingly identical patterns among all 5 EAEC strains, suggesting that they evolved from the same bacterial clone. Thus, there exists a low but still significant prevalence of EAEC-related diarrheal illnesses in the southern region of Thailand.

Keywords: *Escherichia coli*, BOX-PCR, diarrhea, EAEC O44, EAEC O127a, ERIC2-PCR

INTRODUCTION

Among the six distinct *Escherichia coli* pathotypes, enteroaggregative *E. coli* (EAEC) has been identified to play a role in persistent diarrhea in childhood (Cravioto *et al*, 1991; Fang *et al*, 1995). The first description of EAEC was by Nataro *et al* (1987) reporting an aggregative adherent pattern of *E. coli* isolated from a Chilean child with diarrhea. The aggregative adherence is characterized by a stacked-brick appearance of bacteria to human laryngeal HEp-2 cancer cells, followed by pathological processes mediated by enterotoxins (Nataro and Kaper, 1998).

Diarrhea caused by EAEC is a problem for children in both developing and developed countries worldwide (Cravioto *et al*, 1991; Knutton *et al*, 2001). Travelers to developing regions are at risk for EAEC infections because of the absence of specific immunity, which may be due to restricted exposure to EAEC (Cennimo *et al*, 2007). Diagnostic methods developed to detect EAEC from clinical and environ-

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

mental samples include hybridization detection of EAEC-specific gene, *aatA*, on pCVD432, a 60 KDa plasmid containing EAEC virulence genes, formalin-preserved HEp-2 cell assay to observe aggregative adherence pattern (Cennimo *et al*, 2007) and PCR-based detection of *aggR* coding for the major transcription regulator of EAEC gene expression (Tsukamoto, 1996).

Pathogenesis caused by EAEC infection is complex and involves multiple factors. Besides the ability to adhere to intestinal mucosa by means of adherence factors, such as fimbriae AAF/I (coded by *aggA*) and fimbriae AAF/II (coded by *aafA*), this is followed by enhanced mucus production resulting in thick mucus deposition and EAEC encrustation. EAEC also possesses other virulence factors, namely, enteroaggregative heat-stable enterotoxin 1 (EAST1) (encoded by *astA*) and plasmid-encoded toxin (encoded by *pet*) (Vila *et al*, 1998). These factors lead to persistent colonization, malabsorption and eventually damage of intestinal cells (Nataro and Kaper, 1998).

This current study investigated seroprevalence of EAEC from diarrheal patients in tertiary hospitals, southern Thailand. Serotype, antimicrobial susceptibility profile, degree of virulence, and genetic relationship of the strains also were assessed.

MATERIALS AND METHODS

Bacterial strains

For clinical samples, *E. coli* from rectal swab samples collected from Hat-Yai and Pattani Hospitals, southern Thailand between August, 2013 and June, 2014, were cultured by inoculating bacteria on MacConkey agar (MAC) (Becton, Dickinson, Sparks, MD) and incubating at 37ºC for 18 hours. Three to five pink isolated colonies/sample were selected and cultured in 3 ml of tryptic soy broth (TSB) (Becton, Dickinson, Sparks, MD) for 18 hours. They were screened for the presence of *aggR* by PCR. PCR template was prepared by a boiling method (Pannuch *et al*, 2014). In brief, an individual colony was inoculated into 3 ml of TSB and incubated at 37ºC for 6 hours with shaking. One ml aliquot of bacterial culture was harvested and washed with 0.1 M phosphate buffer saline, pH 7.4 prior to boiling for 10 minutes, then immediately immersed on ice for 5 minutes, centrifuged at 11,000*g* for 10 minutes and supernatant diluted 10-fold in sterile deionized water. The cultures of isolated colonies were kept at -80ºC (final 10% glycerol concentration) for further investigations. Research protocols were approved by the Ethics Committee of the Faculty of Medicine, Prince of Songkla University, Thailand (EC no. 56-225-19-2-3).

PCR assay of EAEC *aagA*, *aafA*, *pet* and *astA*

PCR template was prepared as described above. Identification of *E. coli* was performed by PCR amplification of *uidA* (Heninger *et al*, 1999). PCR was conducted in a 25 µl reaction consisting of 0.4 µM each primer pair (Table 1), 0.1 mM dNTPs, 1X Go*Taq* DNA polymerase buffer, 0.5 U Go*Taq* Flexi DNA polymerase (Promega, Madison, WI) and 2 µl of DNA template. Thermocycling (conducted in T100™ Thermal Cycler, Bio-Rad, Hercules, CA) conditions were as follows: 95ºC for 3 minutes; followed by 35 cycles of 94ºC for 1 minute, 47ºC for 50 seconds (for *aggA* and *aafA*) or 50ºC for 50 seconds (for *pet* and *astA*) or 60ºC for 50 seconds (for *uidA*), and 72ºC for 1 minute; and a final step at 72ºC for 5 minutes. Amplicons were analyzed by 1.5% agarose gel-electrophoresis, stained with ethidium bromide and visu-

alized using WSE-5200 Printpraph 2M gel imaging system (ATTO, Tokyo, Japan).

PCR-based classification of diarrheagenic *E. coli* pathotypes

In order to demonstrate whether *E. coli* containing *aggR* carried other genes for other *E. coli* pathotypes, the following genes were subjected to PCR amplification: *est*/*elt* for ETEC (Stacy-Phipps *et al*, 1995), *ipaH* for enteroinvasive *E. coli* (EIEC) (Sethabutr *et al*, 1993), *bfpA* (Gunzburg *et al*, 1995) and *eae* (Gannon *et al*, 1993) for EPEC, stx_1, stx_2 (Sukhumungoon *et al*, 2011) and *eae* for EHEC, and *daaE* (Vidal *et al*, 2005) for diffusely adherent *E. coli* (DAEC). Primers used are listed in Table 1. PCR mixtures were as described above and thermocycling conditions were as follows: 95ºC for 3 minutes followed by 35 cycles of 94ºC for 1 minute, 40ºC (for *est*), 50ºC (for *elt*, *aggR*, and stx_2), 55ºC (for stx_1, *eae*, *bfp* and *daaE*), or 60ºC (for *ipaH*) for 1 minute, and 72ºC for 1 minute or 1.15 minutes for *eae*; with a final step at 72ºC for 5 minutes. Amplicons were analyzed as described above.

Serotype determination

O antigen serotyping was carried out using an agglutination assay with *E. coli*-specific antisera (set 1) (Denka Seiken, Tokyo, Japan), as previously described (Sirikaew *et al*, 2014). In brief, a single colony was grown in 5 ml of TSB at 37ºC for 6 hours with shaking. Bacterial cells were harvested and re-suspended in physiological saline and heated at 121ºC for 15 minutes, centrifuged at 900*g* for 20 minutes and re-suspended in 0.5 ml of physiological saline. Agglutination pattern was observed on a glass slide by reacting the bacterial suspension with the specific antisera.

Clump formation assay

Clump formation was carried out as described previously (Albert *et al*, 1993). In short, an individual colony grown on MAC (Becton, Dickinson, Sparks, MD) was inoculated into 5 ml of Mueller Hinton broth (MHB) (Becton, Dickinson, Sparks, MD) and incubated at 37 ºC with 100 rpm shaking for 20 hours, after which bacterial clumps were visible as a scum at the surface of broth culture. Enterohemorrhagic *E. coli* (EHEC) O157:H7 strain EDL933 was used as negative control and clinical methicillin-resistant *Staphylococcus aureus* strain PSU20, an unrelated strain, for clump formation negative control (Sukhumungoon *et al*, 2014).

Phylogenetic group determination

PCR-based phylogenetic group examination was performed using three separate PCR amplification of *chuA*, *yjaA* and TspE4.C2 fragment (Clermont *et al*, 2000). The primers were listed in Table 1. PCR assay was performed as described above but with the following thermocycling conditions: 95ºC for 3 minutes; 35 cycles of 94ºC for 50 seconds, 54ºC for 50 seconds, and 72ºC for 30 seconds; with a final heating at 72ºC for 5 minutes. Amplicons were analyzed by 1.0% agarose gel-electrophoresis as described above.

Antimicrobial susceptibility test

Investigation of antimicrobial susceptibility of EAEC isolates was performed by disk diffusion method (CLSI, 2014) using 10 antimicrobial agents: amikacin (30 μg), ceftriaxone (30 μg), cephalothin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), cotrimoxazole (25 μg), gentamicin (10 μg), imipenem (10 μg), streptomycin (10 μg), and tetracycline (30 μg) (Oxoid, Hampshire, UK).

Hemolysis assay

EAEC isolates were cultured on blood agar to monitor the erythrocyte lysis ability. In brief, an individual colony was

Table 1
Oligonucleotides used in this study.

Gene	Virulence factor	Primer name	Sequence (5′ to 3′)	Amplicon size (bp)	Reference
aggR	AggR, a transcriptional activator of AAF/I	AggR-1 AggR-2	CAGAATACATCAGTACACTG GAAGCTTACAGCCGATATAT	433	Tsukamoto, 1996
aggA	Fimbriae AAF/I	aggA-F aggA-R	TTAGTCTTCTATCTAGGG AAATTAATTCCGGCATGG	457	Vila *et al*, 2000
aafA	Fimbriae AAF/II	aafA-F aafA-R	TGCGATTGCTACTTTATTAT ATTGACCGTGATTGGCTTCC	242	Vila *et al*, 2000
astA	EAST1	EAST11a EAST11b	CCATCAACACAGTATATCCGA GGTCGCGAGTGACGGCTTTGT	111	Yamamoto and Echeverria, 1996
pet	Plasmid encoded toxin	pet-F pet-R	ACTGGCGGACTCATTGCTGT GCGTTTTTCCGTTCCCTATT	832	Vila *et al*, 2000
uidA	β-Glucoronidase	uidA-F uidA-R	ATCACCGTGGTGACGCATGTCGC CACCACGATGCCATGTTCATCTGC	486	Heninger *et al*, 1999
daaE	F1845 fimbriae	daaF-F daaF-R	GAACGTTGGTTAATGTGGGGTAA TATTCACCGGTCGGTTATCAGT	542	Vidal *et al*, 2005
bfpA	Bundle forming pili	EP-1 EP-2	AATGGTGCTTGCGCTTGCTGC GCCGCTTTATCCAACCTGGTA	326	Gunzburg *et al*, 1995
eae	Intimin	AE-19 AE-20	CAGGTCGTCGTGTCTGCTAAA TCAGCGTGGTTGGATCAACCT	1,087	Gannon *et al*, 1993
stx1	Shiga toxin 1	EVT-1 EVT-2	CAACACTGGATGATCTCAG CCCCCTCAACTGCTAATA	350	Sukhumungoon *et al*, 2011
stx2	Shiga toxin 2	EVS-1 EVS-2	ATCAGTCGTCACTCACTGGT CCAGTTATCTGACATTCTG	404	Sukhumungoon *et al*, 2011
ipaH	Enteroinvasive mechanism	ipaIII ipaIV	GTTCCTTGACCGCCTTTCCGATACCGTC GCCGGTCAGCCACCCTCTGAGAGTAC	603,619	Sethabutr *et al*, 1993
elt	Heat-labile enterotoxin	TW20 JW11	GGCGACAGATTATACCGTGC CGGTCTCTATATTCCCTGTT	450	Stacy-Phipps *et al*, 1995
est	Heat-stable enterotoxin	JW14 JW7	ATTTTTACTTTCTGTATTAGTCTT CACCCGGTACAAGGCAGGATT	190	Stacy-Phipps *et al*, 1995
chuA	Heme transport	chuA1 chuA2	GACGAACCAACGGTCAGGAT TGCCGCCAGTACCAAAGACA	279	Clermont *et al*, 2000
yjaA	Unknown	yjaA1 yjaA2	TGAAGTGTCAGGAGACGCTG ATGGAGAATGCGTTCCTCAAC	211	Clermont *et al*, 2000
TspE4.C2	Unknown	TspE4.C2-1 TspE4.C2-2	GAGTAATGTCGGGGCATTC A CGCGCCAACAAAGTATTACG	152	Clermont *et al*, 2000

grown in 5 ml of TSB as described above, streaked on human erythrocyte agar and incubated at 37ºC for 24 hours to monitor hemolysis around the bacterial colony.

Scanning electron micrograph (SEM) procedure

Bacteria cultured in MHB for 20 hours were harvested by sedimenting at 5,000*g* for 5 minutes at 4ºC and processed as described by Chusri *et al* (2012). In short, each sample was treated with 2.5% glutaraldehyde in cacodylate buffer for 90 minutes, washed twice with cacodylate buffer and dehydrated using a series of analytical-grade ethanol. Samples then were coated with gold and examined by scanning electron microscopy (Quanta, Hillsboro, OR) at magnification of 3,000x, 30,000x and 80,000x.

DNA profiling

DNA profiling of EAEC isolates was carried out by BOX- and ERIC2-PCR (Versalovic *et al*, 1991). PCR of 25 µl contained 0.2 µM each specific primer (Table 1), 0.2 mM dNTPs, 1X Go*Taq* DNA polymerase buffer, 3.0 mM $MgCl_2$, 1.25 U Go*Taq* DNA polymerase (Promega, Madison, WI) and 50 ng of DNA template (prepared using a glass fiber matrix spin column; Geneaid, New Taipei City, Taiwan). Thermocycling condition was as follow: 95ºC for 3 minutes; followed by 30 cycles of 94ºC for 3 seconds, 92ºC for 30 seconds, 50ºC for 1 minute, and 65ºC for 8 minutes. Amplicons were analyzed by 1.5% agarose gel-electrophoresis at 90 V for 2 hours and visualized as described above.

RESULTS

Detection and characterization of EAEC

A total of 376 isolates (332 from 105 rectal swab samples at Hat-Yai Hospital, and 44 from 44 rectal swab samples at Pattani Hospital), were screened for the presence of *aggR*, and 5 isolates from 3/149 (2%) samples were positive, without the presence of other indicative genes of other *E. coli* pathotypes and hence these isolates were identified as EAEC (Table 2). All 5 EAEC strains harbored *aggA*, 2 strains (PSU263 and PSU264) did not contain *aafA* or *pet* and 4 strains (PSU280, PSU281, PSU263, PSU264) possessed *astA*. The 5 EAEC strains were classified into 2 serogroups, namely, O127a (PSU263 and PSU264) and O44 (PSU280, PSU281, and PSU294) and belonged to 2 phylogenetic groups, namely, B1 (*chuA*$^-$, TspE4.C2$^+$; PSU263 and PSU264) and D (*chuA*$^+$, *yjaA*$^-$; PSU280, PSU281 and PSU294) (Table 2). Hemolytic activity assay revealed that all strains produced gamma hemolysis.

Antimicrobial susceptibility

PSU263, PSU264 and PSU294 were resistant to ceftriaxone and cephalothin, PSU280 was resistant to streptomycin and PSU281 was susceptible to all antimicrobial agents (Table 2).

Clump formation and SEM

The 5 EAEC strains produced approximately 2 mm thick bacterial scum on the surface of MHB within 20 hours of incubation (Fig 1), which did not increase after 24 hours of incubation (data not shown), indicating that these strains were weakly positive for clump formation. *E. coli* O157:H7 strain EDL933 and methicillin-resistant *Staphylococcus aureus* strain PSU20, were used as negative controls (Fig 1).

Using 3 representative strains, PSU263, PSU280, and PSU294, SEM revealed aggregative patterns on bacterial substratum (Fig 2A, B and C; left panel). Unexpectedly, fimbriae were not clearly visible, although the putative fimbrial or pilus basement was observed (Fig 2A,

Table 2
Characteristics of EAEC strains from 149 diarrheal patients at Hat-Yai and Pattani Hospitals, Thailand, collected during August, 2013 - June, 2014.

Sample no.	Strain	Hospital[a]	Date of isolation	Sex[b]/age (years)	EAEC virulence gene					O-serotype	Hemolysis	Phylogenetic group	Antibiogram[c]
					aggR	*aggA*	*aafA*	*astA*	*pet*				
16	PSU280	HY	22 Sep 2013	F/44	+	+	+	+	+	O44	γ	D	S
	PSU281	HY	22 Sep 2013	F/44	+	+	+	+	+	O44	γ	D	SA
28	PSU294	HY	26 Sep 2013	M/56	+	+	+	-	+	O44	γ	D	CRO, KF
41	PSU263	HY	11 Jun 2014	F/1	+	+	-	+	-	O127a	γ	B1	CRO, KF
	PSU264	HY	11 Jun 2014	F/1	+	+	-	+	-	O127a	γ	B1	CRO, KF

[a]HY, Hat-Yai Hospital. [b]F, female; M, male. [c]CRO, ceftriaxone; KF, cephalothin; S, streptomycin; SA, susceptible to all antimicrobial agents. tested.

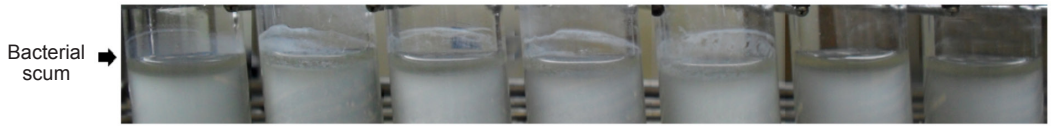
right panel). A putative F pilus was observed in PSU294 (Fig 2C, right panel). There were appearances of knob-like particles (approximately 0.2 μm in diameter) (Fig 2C, central panel), which may be bits of bacterial cell debris or residues of gold particles from the coating process.

Genotyping by BOX-and ERIC2-PCR

Both BOX- and ERIC2-PCR produced consistent results. EAEC O127a strains PSU263 and PSU264, obtained from the same patient, demonstrated identical DNA profile (Fig 3). Interestingly, EAEC O44 strains PSU280 and PSU281 from a 44 year-old female had identical DNA profile as that of EAEC O44 strain PSU294 from a 56 year-old male (Fig 3), indicating infection by the same bacterial clone.

DISCUSSION

EAEC is one of the causes of diarrhea in developing countries (Cennimo *et al*, 2007). The infection is defined by an aggregative adherence characteristic on HEp-2 cells (Nataro *et al*, 1987). In Thailand, Yamamoto *et al* (1991) isolated from a child with diarrhea EAEC O127a:H2 strain TL100 that is capable of adhering to human and animal mucosa, showed an aggregative-pattern adherence to HeLa cells, formed a predominant scum on the surface of L broth and contained fimbrial structures as seen under both SEM and transmission electron microscopy. In our study, EAEC O127a strains PSU263 and PSU264, isolated from a 1 year-old female child with diarrhea, produced a lower degree of clump formation and lacked of distinct fimbrial structures. Similar results were obtained with EAEC O44 strains PSU280, PSU281 and PSU294. This property is not due to the absence of fimbrial subunit genes as all strains possessed at least 2 fimbrial genes, *aggA* and *aafA*. The



Strain	EAEC PSU263	EAEC PSU264	EAEC PSU280	EAEC PSU281	EAEC PSU294	EHEC EDL933	MRSA PSU20
Degree of clump formation	+	+	+	+	+	-	-

Fig 1–Clump formation produced by EAEC strains. Bacteria were cultured in MHB at 37°C, with shaking (100 rpm) for 20 hours. An enterohemorrhagic *E. coli* O157:H7 strain EDL933 and methicillin-resistant *Staphylococcus aureus* strain PSU20 are negative controls.

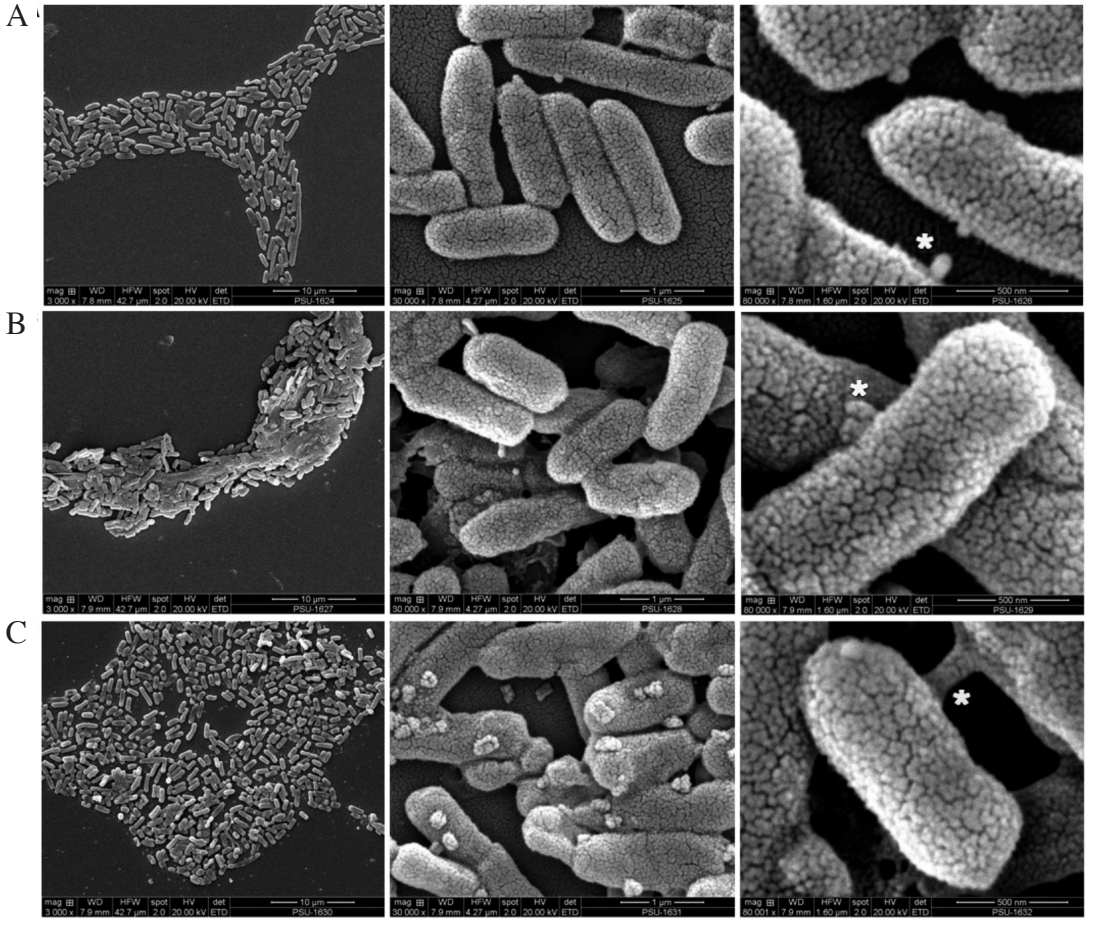


Fig 2–Scanning electron micrographs of representative EAEC strains. Row A, PSU263 (O127a). Row B, PSU280 (O44). Row C, PSU294 (O44). Left panels, 3,000× magnification; central panels, 30,000× magnification; right panels, 80,000× magnification. *(A, right panel) putative fimbrial or pilus basement. *(B and C, right panel) putative F pilus.

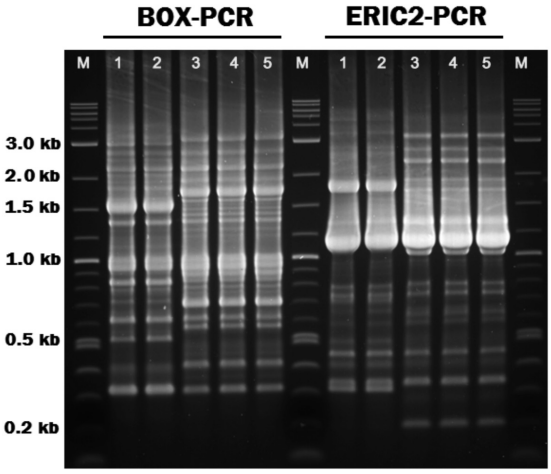


Fig 3–BOX- and ERIC2-PCR- based DNA profiles of EAEC strains. The profiles were generated as described in Materials and Methods. Lane M, DNA size markers; lane 1, EAEC strain PSU263 (O127a); lane 2, PSU264 (O127a); lane 3, PSU280 (O44); lane 4, PSU281 (O44); lane 5, PSU294 (O44).

absence of fimbriae may be attributed to inappropriate growth conditions for their synthesis. Based on our findings we suggest EAEC O127a is an important EAEC serotype that circulates in Thai environment for a long period of time. Although cell adhesion assay is important to identify this pathogen, this technique is expensive and is not routinely available. Molecular technique such as PCR detection of the core EAEC strains is widely performed with high sensitivity and specificity (Nguyen *et al*, 2005).

Although EAEC O44 strains, PSU280 and PSU281 (from the same patient) and PSU294 (from another patient) were isolated at different time periods, they demonstrated the same DNA profile; despite the latter lacking *astA* and exhibiting a slightly different antimicrobial susceptibility profile. This phenomenon may be explained by loss of plasmid-bearing *astA* and horizontal transfer of drug resistant genes (Ménard and Dubreuil, 2002). We suggest that the infections in these 2 patients were caused by strains evolved from the same bacterial clone.

Kalnauwakul *et al* (2007) investigating diarrheagenic *E. coli* from 530 stool and rectal swab samples collected at Songklanagarind Hospital, southern Thailand between April, 2001 and December, 2002, found that 5.8% were EAEC, of which 22 (4.15%) and 9 (1.7%) were obtained from children and adults, respectively. This prevalence of EAEC in diarrheal patients is comparable to our study of 2% EAEC infections.

In summary, EAEC infection in southern part of Thailand was infrequent, being isolated from 3/149 patients with diarrhea, each infected with either EAEC serotype O127a that was described over last two decades ago in Thailand or EAEC serotype O44 that was first described from a patient in Peru in 1987 (Nataro *et al*, 1987). These results indicate the potential of EAEC to cause illness, posing a small but still a significant health risk in this region of Thailand.

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