ENTEROTOXIGENIC *ESCHERICHIA COLI* O169:HUT FROM A DIARRHEAL PATIENT: PHYLOGENETIC GROUP AND ANTIMICROBIAL SUSCEPTIBILITY

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Abstract. Enterotoxigenic *Escherichia coli* (ETEC) is one of the most common pathogenic *E. coli* pathotypes causing diarrhea in children worldwide. Its enterotoxins, LT and ST, including colonization factors mainly are responsible for human pathogenesis. From 239 rectal swabs of diarrheal patients at Hat Yai and Pattani Hospitals during August 2013 and May 2014, five isolates from only a single *E. coli* sample demonstrated the possession of *estA1*, encoding porcine heat-stable enterotoxin (STp). These isolates all belonged to serotype O169:H Untypeable (HUT) and carried *astA*, encoding enteroaggregative heat-stable enterotoxin 1. A PCR-based phylogenetic group investigation classified them as members of the virulent *E. coli* phylogenetic group D. The isolates were resistant to cephalothin, penicillin G, streptomycin, tetracycline and vancomycin. Confirmation of their clonality was conducted by enterobacterial repetitive intergenic consensus sequence PCR typing, which revealed that these ETEC were derived from the same clone. This is the first report of ETEC O169:HUT in southern Thailand.

Keywords: enterotoxigenic *Escherichia coli*, EAST1, *elt*, *est*, ETEC O169, Thailand

INTRODUCTION

Diarrheal diseases play a major role as a public health concern accounting for approximately 10% of children deaths worldwide, making it the second leading cause of mortality among children under 5 years of age (CDC, 2013). Among the six diarrheagenic *Escherichia coli* categories (Nataro and Kaper, 1998), enterotoxigenic *E. coli* (ETEC) has been reported to be the cause of approximately 800,000 deaths every year (Turner *et al*, 2006) and the most common cause of diarrhea in developing countries (WHO, 1999).

The diseases caused by ETEC are triggered mainly by two types of enterotoxins, namely, 18-19 amino acids heat-stable enterotoxin (ST) peptide and 84 kDa oligomeric heat-labile enterotoxin (LT) protein, the latter being closely related structurally, functionally and immunologically to cholera toxin (CT) produced by *Vibrio cholerae* O1. Additional virulence factors, which

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also play pivotal roles in ETEC pathogenesis, are colonization factors (CFs). To date, at least 22 CFs have been described among human ETEC (Gaastra and Svennerholm, 1996). The CFs are mainly fimbrial or fibrillar proteins (Evans *et al*, 1978; Gaastra and Svennerholm, 1996) and are responsible for the establishment of bacterial colonization in the small bowel. The close proximity of the bacteria to intestinal epithelial cells concomitant with the production of enterotoxins lead to the production of diarrheal stools caused by an imbalance of the bowel absorptive capacity (Qadri *et al*, 2005).

Outbreaks caused by ETEC of a wide variety of ETEC serotypes have been reported worldwide (Beatty *et al*, 2004; Konishi *et al*, 2011; Harada *et al*, 2013). However, there is still inadequate knowledge regarding ETEC in Thailand, particularly from southern Thailand. Most recently, Phetkhajorn *et al* (2014) reported that a significant proportion of raw meat, in particular chicken, sold in fresh food markets in southern Thailand, harbored ETEC. Kalnauwakul *et al* (2007) surveyed the presence of diarrheagenic *E. coli* in Songklanagarind Hospital, Hat Yai, Songkhla Province from 1999 to 2000 and demonstrated the presence of ETEC in 2.5% of investigated isolates. In order to obtain a more up-to-date picture of ETEC epidemiological situation in southern Thailand, we have conducted a survey of ETEC infection in diarrheal patients from Hat Yai and Pattani Hospitals, including examination of serotypes, virulence factors, phylogenetic groups, antibiogram patterns and their genetic relationships.

MATERIALS AND METHODS

Bacterial strains

A total of 748 *E. coli* isolates from 239 rectal swabs were collected from diarrheal patients attending Hat Yai (119 samples) and Pattani (120 samples) Hospitals during August 2013 and May 2014 and cultured on MacConkey agar. Three to five colonies per sample were selected and grown in 3 ml of Tryptic Soy broth (TSB) for 18 hours prior to be kept as stocks at -80°C (a final glycerol concentration of 10%) for further analysis. The protocols were approved by the ethics committee of the Faculty of Medicine, Prince of Songkla University (EC code 56-225-19-2-3).

Investigation of ETEC virulence genes

PCR was employed to detect *E. coli uidA*, the core virulence genes, *est*, *elt* and *cfaB* (encoding CFA/I colonization factor), *est* subtypes, *estA1* and *estA2-4* (encoding STh and STp, respectively), *astA* (encoding enteroaggregative heat-stable enterotoxin 1, carried by ETEC pathogenic to humans) and *hlyA* (encoding an α-hemolysin). DNA templates were prepared by the boiling method as described previously (Pannuch *et al*, 2014). In brief, a single colony was inoculated into 3 ml of TSB and incubated at 37°C for 6 hours with shaking. One ml aliquot of bacterial culture was boiled for 10 minutes, immersed in ice for 5 minutes and centrifuged at 11,000*g* for 10 minutes. The supernatant was diluted 1:10 in sterile deionized water and used as PCR template. PCR was performed 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, 3 mM $MgCl_2$, 0.5 U Go*Taq* DNA polymerase (Promega, Madison, WI) and 2 µl of DNA template. Amplicons were analyzed by 1.0% agarose-gel electrophoresis and visualized by ethidium bromide staining using WSE-5200 Printpraph 2M gel imaging system (Tokyo, Japan).

DNA sequencing of *est*

Fragment of *est* was run in 1.0% aga-

rose gel and purified using Gel/PCR DNA fragment extraction kit (Geneaid, New Taipei City, Taiwan). DNA sequencing was performed in an ABI Prism 377 sequencer (Applied Biosystems, Foster City, CA) using JW14 as a primer. Nucleotide sequences were analyzed for similarity using Basic Local Alignment Search Tool (BLAST) from National Center for Biotechnology Information (NCBI).

Serotype determination

O and H antigen serotyping were carried out by an agglutination assay using *E. coli* specific antisera (set1 and set2) (Denka Seiken, Tokyo, Japan). In brief, for O antigen typing, a single colony of each bacterial isolate was inoculated in 3 ml of TSB and incubated at 37ºC for 6 hours with aeration. Bacteria were harvested by sedimentation and re-suspended in physiological saline and heated at 121ºC for 15 minutes. The cell suspension was sedimented and the pellet was re-suspended in 0.5 ml of physiological saline. The agglutination pattern was observed on glass slide where the suspension was reacted with the specific anti-serum. For H antigen typing (formalin-kill-based method), bacteria were induced to fully express H antigen by passing through a 0.3% semiliquid media (TSB-based media) 3 times, followed by culturing in 10 ml of TSB at 37ºC for 6 hours. The bacterial culture was mixed with an equal volume of physiological saline containing 1% (v/v) formalin to preserve the H antigen. A 180 µl aliquot of formalin-treated bacterial suspension was mixed with an equal volume of the specific anti-serum in a U-shaped bottom glass tube and incubated at 50ºC for 1.5 hours. A cotton-wool-like agglutination product is regarded as positive while a homogeneous suspension as negative. Formalin-killed bacteria in physiological saline were used as negative control.

Phylogenetic group examination

Three specific PCR reactions were carried out to investigate *E. coli* phylogenetic group, *chuA*, *yjaA*, and TspE4.C2 fragment (Clermont *et al*, 2000). Uni-plex PCRs were performed as described above using primers listed in Table 1 and the following thermocycling (in T100™ Thermal Cycler; Bio-rad, Hercules, CA) conditions: 95ºC for 3 minutes; 35 cycles of 94ºC for 50 seconds, 54ºC for 50 seconds, and 72ºC for 30 seconds; with a final step of 72ºC for 5 minutes. The amplicons were analyzed as described above.

Antimicrobial susceptibility test

Investigation of antimicrobial susceptibility of ETEC was performed by a disk diffusion method (CLSI, 2014) using ceftazidime (30 µg), cephalothin (30 µg), chloramphenical (30 µg), fosfomycin (200 µg), gentamicin (10 µg), kanamycin (30 µg), penicillin G (10 µg), streptomycin (10 µg), tetracycline (30 µg), and vancomycin (30 µg) (Oxiod, Hamshire, UK). Vancomycin susceptibility was judged using the criterion of CLSI (2007).

ETEC genotyping

Enterobacterial repetitive intergenic consensus sequence typing (ERIC)-PCR was conducted to demonstrate the clonality of five *est*-positive ETEC isolates. PCR was carried out in a 25 µl reaction consisting of 0.2 µM of ERIC2 primer, 0.2 mM dNTPs, 1X Go*Taq* DNA polymerase buffer, 3 mM $MgCl_2$, 1.25 U Go*Taq* DNA polymerase (Promega, Madison, WI) and 2 µl of DNA template. ERIC2 primer was listed in Table 1. Thermocycling conditions were as follows: 95ºC for 3 minutes; 30 cycles of 94ºC for 3 seconds, 92ºC for 30 seconds, and 50ºC for 1 minute; followed by a final heating at 65ºC for 8 minutes. The amplicons were analyzed by 1.5% agarose gel-electrophoresis for 2 hours at

Table 1
Primers used in the study.

Gene	Virulence factor	Primer name	Sequence (5′ to 3′)	Amplicon size (bp)	Reference
est	ST	JW14	ATTTTTACTTTCTGTATTAGTCTT	190	Stacy-Phipps *et al*, 1995
		JW7	CACCCGGTACAAGGCAGGATT		
elt	LT	TW20	GGCGACAGATTATACCGTGC	450	Stacy-Phipps *et al*, 1995
		JW11	CGGTCTCTATATTCCCTGTT		
estA1	STp	STp-F	AGGTAACATGAAAAAGCTAATGTTG	209	Nazarian *et al*, 2014
		STp-R	AGGCAGGATTACAACAAAGTTCA		
estA2-4	STh	STh-F	TGCTAAACCAGTAGAGTCTTCAAAAG	157	Nazarian *et al*, 2014
		STh-R	TAATAGCACCCGGTACAAGCAG		
cfaB	CFA/I	CFA/I-F	ACAATGTTTGTAGCAGTGAGTGC	450	Nazarian *et al*, 2014
		CFA/I-R	AGATACTACTCCTGAATAGTTTCCTG		
chuA	Heme transport	chuA1	GACGAACCAACGGTCAGGAT	279	Clermont *et al*, 2000
		chuA2	TGCCGCCAGTACCAAAGACA		
yjaA	Unknown	yjaA1	TGAAGTGTCAGGAGACGCTG	211	Clermont *et al*, 2000
		yjaA2	ATGGAGAATGCGTTCCTCAAC		
TspE4.C2	Unknown	TspE4.C2-1	GAGTAATGTCGGGGCATTCA	152	Clermont *et al*, 2000
		TspE4.C2-2	CGCGCCAACAAAGTATTACG		
hlyA	HlyA	HlyA-F	AACAAGGATAAGCACTGTTCTGGCT	1,177	Yamamoto *et al*, 1995
		HlyA-R	ACCATATAAGCGGTCATTCCCGTCA		
astA	EAST1	EAST11a	CCATCAACACAGTATATCCGA	111	Yamamoto and Echeverria, 1996
		EAST11b	GGTCGCGAGTGACGGCTTTGT		
uidA	β-glucoronidase	uidA-F	ATCACCGTGGTGACGCATGTCGC	486	Heninger *et al*, 1999
		uidA-R	CACCACGATGCCATGTTCATCTGC		
Repetitive sequence		ERIC2	AAGTAAGTGACTGGGGTGAGCG	Variable	Versalovic *et al*, 1991

100V and visualized as described above.

RESULTS

ETEC virulence genes

Of the 748 isolates from 239 rectal swab samples, only one sample (0.4%) from a 29-year old male foreigner at Hat Yai Hospital was positive for ETEC (as indicated by the presence of *E. coli*-specific *uidA* and *est*). Five isolates from this sample carried *est*, which was confirmed by DNA sequencing (98% similarity with ETEC strain H10407**)**. Further subclassification of *est* gene showed that they were members of STp group (Table 2). The isolates also contained *astA* encoding EAST1 which was reported in enteroaggregative *E. coli* (EAEC) and other *E. coli* pathotypes (Table 2). The presence of *elt*, *cfaB* and *hlyA* were not detected.

Serotype

Using *E. coli* antisera specific to 43 somatic O and 22 H antigen types (flagella antigen), the *E. coli* isolates belonged to serotype O169:H Untypeable (HUT).

Phylogenetic group

Based on the presence of *chuA* but not *yjaA* and TspE4.C2 fragment, all 5 ETEC isolates could be classified as members of *E. coli* phylogenetic group D, while the control ETEC strain KETE, displayed the PCR product of *yjaA* and TspE4.C2 fragment but not for *chuA*, belonging to *E. coli* phylogenetic group B1 (Table 3).

Antimicrobial susceptibility

All 5 isolates had the same antimicrobial resistant pattern against cephalothin, penicillin G, streptomycin, tetracycline, and vancomycin (Table 2). For vancomycin susceptibility test, no clear zone was observed when using vancomycin disk.

ETET genotype

According to the virulence gene pattern and phylogenetic group analysis of all five ETEC isolates including antimicrobial susceptibility tests, these isolates were presumably derived from the same clone. This was confirmed by ERIC2-PCR demonstrating indistinguishable genetic profiles (Fig 1).

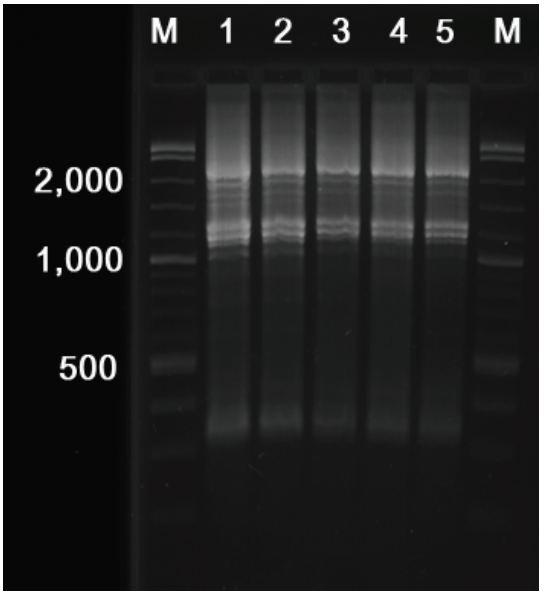

Fig 1–Enterobacterial repetitive intergenic consensus sequence 2-PCR typing of five *est*-positive ETEC isolates. Experimental protocols are described in Materials and Methods. Lane M, DNA size markers (bp) (2-log DNA, New England Biolabs, Ipswich, MA); lanes 1-5, *est*-positive ETEC strain PSU192-196, respectively.

DISCUSSION

In last decade, the determination of ETEC pathotype has been shown to be associated with bacterial serotypes. Evidence has shown in Bangladesh that ETEC strains can be classified into certain serotypes. During the investigation of ETEC strains from Cholera Research Hospital in Dhaka, in 1976, 59/69 (86%) of ST- and

Table 2
Virulence genes, serotypes and antibiogram of five ETEC isolates from Hat Yai Hospital, southern Thailand.

Strain	Serotype	Virulence gene					Antibiogram
		[a]*est*	*elt*	*cfaB*	*astA*	*hlyA*	*uidA*	
PSU192	O169:HUT	+	-	-	+	-	+	KF, P, S, TE, Va
PSU193	O169:HUT	+	-	-	+	-	+	KF, P, S, TE, Va
PSU194	O169:HUT	+	-	-	+	-	+	KF, P, S, TE, Va
PSU195	O169:HUT	+	-	-	+	-	+	KF, P, S, TE, Va
PSU196	O169:HUT	+	-	-	+	-	+	KF, P, S, TE, Va
[b]ETEC strain KETE	NA	+	+	+	+	-	+	NA

KF, cephalothin; P, penicillin G; TE, tetracycline; S, streptomycin; Va, vancomycin; NA, not applicable; [a]STp group; [b]Control strain.

LT-producing ETEC were shown to be owned by 4 serogroups, O6, O8, O78, and O115. The two most common serogroups for ST were O78 and O128 (Merson *et al*, 1979). A retrospective study from Wolf *et al* (1997) also showed that the common *E. coli* serotypes O6, O78, O8, O128, and O153 accounted for over half of the 954 ETEC strains. However, other studies have reported that clinical ETEC consisted of a large number of serotypes (Stoll *et al*, 1983; Peruski *et al*, 1999). Thus, serotyping is considered not a reliable method to classify the being of ETEC.

Among the serotypes associated with ETEC, O169 serogroup has been increasingly associated with diarrheal cases. Since 1984, *E. coli* O169:H8 has appeared as one of ETEC strains (Orskov *et al*, 1984). ETEC O169:H41 has become one of the most prevalent ETEC in Japan since the first outbreak by this serogroup in 1991 (Harada *et al*, 2013). This serotype has also played a role in the western hemisphere such as in the United States where 10 of 16 outbreaks between 1996 and 2003, were reported (Beatty *et al*, 2004). Our study was able to isolate ETEC O169:HUT exhibiting multi-drug resistant phenotype from a foreigner with diarrhea. This patient may have lacked adequate immunity against ETEC, resulting in an establishment of infection. Ratchtrachenchai *et al* (2004) reported the detection of ETEC O169 in 6/78 ETEC strains collected from 15 hospitals across Thailand between 1996 and 2000. To the best of our knowledge, this is the first report of ETEC O169:HUT from southern Thailand.

The five *est*-positive ETEC in this study carried *astA*, coding for the enteroaggregative heat-stable enterotoxin 1, EAST1. This enterotoxin was first reported in EAEC strain 17-2 isolated from a Chilean child with diarrhea (Nataro *et al*,

Table 3
Phylogenetic group of five ETEC isolates from Hat Yai Hospital, southern Thailand.

Isolates	Phylogenetic group	*chuA* gene	*yjaA* gene	TSPE4.C2
PSU192	Group D	+	-	-
PSU193	Group D	+	-	-
PSU194	Group D	+	-	-
PSU195	Group D	+	-	-
PSU196	Group D	+	-	-
[a]ETEC strain KETE	Group B1	-	+	+

[a]Control strain.

1987). As EAST1 is present in a wide variety of diarrheagenic *E. coli* (Savarino *et al*, 1996; Paiva de Sousa and Dubreuil, 2001), the presence of *astA* in ETEC in this study is not surprising. Although it is generally believed that EAST1 is responsible for diarrheal illnesses, Ruan *et al* (2012) have demonstrated the lack of EAST1 ability in stimulation of intracellular cAMP and cGMP productions in T-84 cells (human colorectal carcinoma cells) and its incapability of causing diarrhea in 5 day-old young gnotobiotic pigs. Furthermore, no synergistic action of EAST1 toward LT and ST was found. The discrepancy of the role of EAST1 as an etiological agent of diarrhea needs further investigation.

E. coli strains fall into four main phylogenetic groups, namely, A, B1, B2, and D (Selander *et al*, 1987; Herzer *et al*, 1990). As for extra-intestinal *E. coli*, virulent strains chiefly belong to phylogenetic group B2 and D, while the less virulent and commensal strains are associated with phylogenetic group B1 and A (Bingen *et al*, 1996; Boyd and Hartl, 1998; Clermont *et al*, 2000). The 5 ETEC isolates from this study belonged to phylogenetic group D. Thus, this may suggest a pathogenic potential of these isolates, but other factors, such as the status of the host immune system, also play a role in determining the extent of pathological outcome.

In summary, the data from previous and current studies regarding the successful ETEC detection confirmed the existence of this bacterial pathotype in this area, especially, the presence of ETEC serotype O169 which has been reported to be the prominent serotype causing illnesses in several countries worldwide. These isolated strains exhibited one of the core ETEC virulence factor, *est* gene including the gene coding for EAST1. Furthermore, their multi-drug resistant pattern may halt the therapeutic processes, resulting in delayed patient's recovery. Moreover, the finding of clinical ETEC strain in this study suggests ETEC contamination in foods and/or water in high dose. The potential vehicle origin which plays a role in the infection in this area needs further investigation and the surveillance of ETEC from foods/water should be performed periodically to prevent prospective outbreaks.

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