FIRST REPORT IN THAILAND OF A *STX*-NEGATIVE *ESCHERICHIA COLI* O157 STRAIN FROM A PATIENT WITH DIARRHEA

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Abstract. *E. coli* serotype O157 is well known to cause serious illnesses in humans. However, there has been no case report to date of this serotype in Thailand. In this study, we report for the first time *E. coli* O157 (designated as PSU120) isolated from a stool sample among 228 diarrheal swab samples at Hat Yai Hospital, Songkhla Province, Thailand. This PSU120 was identified as being *stx*-negative and lacked *eae* but carried *escV*, a marker for the locus of enterocyte effacement. Of the five reported integration sites frequently occupied by *stx* phages, the *sbcB* and *yehV* loci were occupied, suggesting that PSU120 is active in horizontal genetic transfer. Antimicrobial susceptibility assay revealed that *E. coli* O157 strain PSU120 was resistant to cephalothin, erythromycin, methicillin and vancomycin. Using pulsed-field gel-electrophoresis to compare the genetic relatedness of *E. coli* O157 strain PSU120 to two other *E. coli* O157 strains, namely, the well-established EHEC strain EDL933 and PSU2, a surrogate of *E. coli* O157:H7 whose genotype stx_1^-, stx_2^+, eae^+ is frequently obtained from the environment in this area during the last decade, revealed 88.6% in similarity. We suggest that PSU120 was originally stx^+ but lost the gene after establishing infection.

Keywords: *Escherichia coli* O157, diarrhea, Shiga toxin, Thailand

INTRODUCTION

Shiga toxin-producing *Escherichia coli* (STEC) is a food-borne pathogen, which is frequently associated with food poisoning outbreaks in several countries around the world (Michino *et al*, 1999; Dundas *et al*, 2001; Rangel *et al*, 2005). More than 40 serotypes of STEC have been reported to be involved with severe forms of the disease in humans (Paton and Paton, 1996), including hemorrhagic colitis (HC) (Riley *et al*, 1983) and hemolytic uremic syndrome (HUS) (Karmali *et al*, 1983).

STEC is capable of producing Shiga toxin 1 (Stx1), Shiga toxin 2 (Stx2) or

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both. Stx is the principal virulence factor in STEC. It is internalized into eukaryotic cells by binding to a cellular receptor globotriaosylceramide (Gb3) (de Sablet *et al*, 2008) and once Stx is present in the cytoplasm, it exerts the elimination of an adenine residue from 28S ribosomal RNA, resulting in inhibition of cytoplasmic proteins biosynthesis and cell death (Nataro and Kaper, 1998). The most important STEC serotype causing outbreaks worldwide is O157:H7. Typical EHEC O157:H7 carries one of the *stx* genes (stx_1 and stx_2) or both genes and *eae* (encoding intimin which influences the establishment of bacterial adherence to the intestinal epithelial cells) (Gannon *et al*, 1993). Besides the antigenic determinants in the bacterial cell wall, the somatic O-antigen, *E. coli* also possesses flagella which are associated with the bacterial movement. Flagella organelle is constituted by flagella proteins which carry the antigenic determinants for the H-antigens and are used to be one of *E. coli* epidemiological data. To date, at least 53 H-antigen types in *E. coli* have been documented (Wang *et al*, 2000) and with the epidemiological data of *E. coli* O157, H7 antigen was the most frequently observed to be accompanied with the somatic O157 antigen.

It has been demonstrated that the genes coding for Stxs are found in lysogenic lambdoid bacteriophages (Schmidt, 2001). In stx_2 phage genome, stx_2 gene is located downstream of *Q* gene which codes for Q protein responsible for the late antitermination activity. Strong antitermination activity is demonstrated by the possession of Q_{933} type, contributing to the large amount of Stx production while the possession of Q_{21} leads to the weak antitermination activity and low amount of Stx (LeJeune *et al*, 2004; Koitabashi *et al*, 2006). Thus, the presence of Q_{933} type is able to use as a virulence marker in *E. coli* O157:H7 (LeJeune *et al*, 2004).

Infection by enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 was first reported in 1982 in the United States (Karmali *et al*, 1983; Riley *et al*, 1983). Many types of food have been described as being etiologic agents of *E. coli* O157:H7 infection, including ground beef, raw milk, unpasteurized apple cider, and salad, which lead to sporadic cases and outbreaks (Michino *et al*, 1999; Hilborn *et al*, 2000; Dundas *et al*, 2001; Jay *et al*, 2004; Guh *et al*, 2010; Xiong *et al*, 2012). However, infection by this pathogen has rarely been reported from developing countries.

We report, for the first time, the isolation of *E. coli* O157 strain from stool of a diarrheal patient in southern Thailand and the investigation of its principal virulence genes including other important characteristics, as well as its genomic properties by pulsed-field gel-electrophoresis.

MATERIALS AND METHODS

Bacterial strains

A total of 560 *E. coli* isolates from 228 diarrheal rectal swabs were collected from patients admitted to Hat Yai Hospital, Songkhla Province and Pattani Hospital, Pattani Province, Thailand from October 2012 to September 2013. Swabs were inoculated on Sorbitol MacConkey agar to obtain isolated colonies. Both sorbitol fermenters (SF) and sorbitol-non-fermenters (SNF) were selected and kept stock at -80ºC for further investigations. Further characterization of the positive isolate on the extra differential medium, CHROMagar O157 (CHROMagar, Paris, France) was carried out. The protocol was approved by the Ethics Committee of the Faculty of Medicine, Prince of Songkla

University, Thailand (EC code 56-225-19-2-3).

Detection of virulence genes

The principal virulence genes, stx_1, stx_2, *eae*, *escV* [marker for type III secretion system (T3SS) located in the locus of enterocyte effacement, LEE], and *hlyA* were investigated by PCR using oligonucleotide primers (Table 1). In brief, PCR amplification was performed in a 25 µl reaction mixture consisting of 0.08 mM dNTPs, 0.4 µM each primer pair, 3.0 mM $MgCl_2$, 0.5 U Go*Taq* DNA polymerase (Promega, Madison, WI), 1X Go*Taq* green buffer and 2.0 µl of DNA template. Thermal cycling (conducted using T100™ Thermal cycler, Bio-Rad, Hercules, CA) consisted of a preheat step at 95ºC for 3 minutes followed by 35 cycles of 94ºC for 1 minute, annealing temperature for each primer pair (Table 1) for 1 minute, and 72ºC for 1 minute. The amplicons were separated by 1.0% agarose gel-electrophoresis, stained with ethidium bromide and visualized using UV-transilluminator system (Syngene, Los Altos, LA).

Serotyping of *E. coli* O157 strain

E. coli O157 genotyping was performed by PCR using O157-F and O157-R oligonucleotide primers (Table 1) (Maurer *et al*, 1999). PCR amplification was performed in a 25 µl reaction mixture and analyzed as described above. This was confirmed by agglutination assay using anti-O157 antibody (Denka Seiken, Tokyo, Japan). The presence of *fliC*H7 was investigated by PCR as described above using the oligonucleotide primers shown in Table 1.

Investigation of Q_{933} and Q_{21}

PCR amplification of Q_{933} and Q_{21} were performed using oligonucleotide primer pair qEf-1/qEr-2, and qDf-1/qDr-2, respectively (Koitabashi *et al*, 2006). *E. coli* O157:H7 strain EDL933 (O'Brien *et al*, 1983) and *E. coli* O157:H7 strain Thai-12 (Vuddhakul *et al*, 2000), were used as the positive controls for detecting Q_{933} and Q_{21}, respectively. The amplification was carried out in a total volume of 20 µl, composed of 0.1 mM dNTPs, 2.5 mM $MgCl_2$, 0.1 µM each primer pair, 1X Go*Taq* DNA polymerase buffer, and 0.5 U Go*Taq* DNA polymerase. Thermal cycling conditions were as follows: 95ºC for 2 minutes; 35 cycles of 94ºC for 30 seconds, 55ºC for Q_{933} or 51ºC for Q_{21}, for 30 seconds, and 72ºC for 2 minutes; and a final step of 72ºC for 5 minutes and standing at 10ºC. Amplicons were analyzed as described above.

PCR amplification of insertion locus

PCR was performed for amplification of each insertion locus using specific primers (Table 1). In brief, a single colony of each strain was grown in 3 ml of Luria-Bertani (LB) broth (Difco, Sparks, MD) for 18 hours at 37ºC with shaking. One ml aliquot of culture was boiled for 10 minutes and immediately immersed on ice for 5 minutes prior to centrifugation at 11,000*g* for 5 minutes. The supernatant was used as DNA template in subsequent PCR. This was performed using Go*Taq* Flexi system (Promega, Madison, WI). The amplicons were analyzed as described above. If the PCR exhibited no amplicon, it was attributed to an insertion of a prophage, resulting in a fragment too large to be amplified by PCR approach (Mellmann *et al*, 2008).

Antimicrobial susceptibility test

E. coli O157 was determined for antimicrobial susceptibility by a disk diffusion method (CLSI, 2013). Eleven antimicrobial agents (Oxoid, Hamshire, UK) were used: ampicillin (10 µg), ceftazidime (30 µg), cephalothin (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), methicillin

Table 1
Oligonucleotides used in the study.

Primer	Sequence (5'to 3')	Gene	Annealing temperature	Amplicon size (bp)	Reference
EVT-1	CAACACTGGATGATCTCAG	stx_1	55	350	Sukhumungoon
EVT-2	CCCCCTCAACTGCTAATA				*et al*, 2011
EVS-1	ATCAGTCGTCACTCACTGGT	stx_2	50	404	Sukhumungoon
EVS-2	CCAGTTATCTGACATTCTG				*et al*, 2011
AE-19	CAGGTCGTCGTGTCTGCTAAA	*eae*	55	1,087	Gannon
AE-20	TCAGCGTGGTTGGATCAACCT				*et al*, 1993
O157-F	CGTGATGATGTTGAGTTG	*rfb*O157	50	400	Maurer *et al*,
O157-R	AGATTGGTTGGCATTACTG				1999
FlicH7-F	GCGCTGTCGAGTTCTATCGAGC	*fli*CH7	57	625	Gannon
FlicH7-R	CAACGGTGACTTTATCGCCATTCC				*et al*, 1997
escV-F	GGCTCTCTTCTTCTTTATGGCTG	*escV*	45	534	Müller *et al*,
escV-R	CCTTTTACAAACTTCATCGCC				2006
hlyA-F	AACAAGGATAAGCACTGTTCTGGCT	*hlyA*	55	1,177	Yamamoto
hlyA-R	ACCATATAAGCGGTCATTCCCGTCA				*et al*, 1995
QEf-1	ATGCGGATCCACACTGGCGATAAAGAAGGG	Q_{933}	55	567	Koitabashi
QEr-2	ATGCGGATCCTCGACTGCGTGGCAATGTAA				*et al*, 2006
QDf-1	ATGCGGATCCAAATCTCACATTGATTCAGG	Q_{21}	51	561	Koitabashi
QDr-2	ATGCGGATCCATAGTGTTGCTCATTTGCTC				*et al*, 2006
Z2577-F	AACCCCATTGATGCTCAGGCTC	Z2577	53	909	Koch *et al*, 2003
Z2577-R	TTCCCATTTTACACTTCCTCCG				
*sbc*B1	CATGATCTGTTGCCACTCG	*sbcB*	50	1,800	Ohnishi *et al*, 2002
*sbc*B2	AGGTCTGTCCGTTTCCACTC				
EC10	GCCAGCGCCGAGCAGCACAATA	*yecE*	60	400	DeGreve *et al*,
EC11	GGCAGGCAGTTGCAGCCAGTAT				2002
*wrb*A1	ATGGCTAAAGTTCTGGTG	*wrbA*	47	600	Toth *et al*, 2003
*wrb*A2	CTCCTGTTGAAGATTAGC				
Primer A	AAGTGGCGTTGCTTTGTGAT	*yehV*	50	340	Shaikh and Tarr,
Primer B	AACAGATGTGTGGTGAGTGTCTG				2003

(5 μg), streptomycin (10 μg), tetracycline (30 μg), and vancomycin (30 μg). Vancomycin susceptibility was judged by the criterion of CLSI (2007). Clear zone was measured using a Vernier caliper. *E. coli* ATCC 25922 was used as a control microorganism.

Pulsed-field gel electrophoresis

E. coli O157 genome was digested with FastDigest *Xba*I restriction endonuclease (Thermo Scientific, Rockford, IL) at 37°C for 1 hour. The DNA fragments were separated by 1.0% agarose gel-electrophoresis in 0.5X TBE buffer using CHEF DR III system (Bio-rad, Hercules, CA) at 14°C. Electrophoresis was performed at 6 V/cm, field angle 120°. The initial and final switch time was 2.2 and 54.2 seconds, respectively. Overall run time was 19 hours. After electrophoresis, the gel was stained with ethidium bromide and recorded. The 48.5 kb Lambda phage was used as a molecular size standard marker. Dendrogram was constructed using unweighted

Table 2
Characteristics of *E. coli* O157 strain PSU120.

Source of sample	Virulence gene					O157/non-O157	Q_{933}/Q_{21}	Insertion site occupied	Antibiogram pattern
	stx_1	stx_2	*eae*	*escV*	*hlyA*				
48 year-old male	-	-	-	+	-	+/-	-/-	*sbcB*, *yehV*	E, KF, MET, Va

E, erythromycin; KF, cefalothin; MET, methicillin; Va, vancomycin.

pair-group method of arithmetic average (UPGMA) (BioNumerics software version 7.0, Applied Maths, Sint-Martens-Latem, Belgium).

RESULTS

Five hundred sixty *E. coli* isolates were obtained including SF and SNF, from stool swabs of 228 diarrheal patients who visited Hat Yai Hospital, Songkhla Province, South Thailand. One isolate (designated PSU120) was found to be *E. coli* O157 and the investigation of *fliC*H7 showed that PSU120 carried H-antigen other than H7 type. PSU120 was isolated from a 48 year-old man on 17 May 2013, and no other enteric pathogen was detected by standard examinations from the diarrheal sample of this patient, indicating the diarrhea resulted from infection solely by PSU120. PSU120 was a SF isolate and formed a blue colony on CHROMagarO157 which reflected an atypical characteristic. The isolate lacked stx_1, stx_2, *eae* and *hlyA* and was negative also for the presence of Q_{933} and Q_{21}, antiterminators of *stx* phages, probably supporting the absence of the *stx* genes (Table 2). A marker for T3SS, *escV*, located in the LEE was determined and displayed the presence of this gene. Of the 11 antimicrobial agents tested, PSU120 strain was resistant to cephalothin, erythromycin, methicillin and vancomycin (Table 2). Five *E. coli* O157 genetic loci which have frequently been reported to be integrated by *stx*-phages, were examined for the observation of *stx*-phage occupancy. The results demonstrated that *sbcB* and *yehV* were found to be occupied by prophages while *yecE*, *wrbA*, and Z2577 were not (Table 2).

Comparison of genetic relatedness among clinical, *E. coli* O157:H7 strain EDL933 and PSU120, and environmental

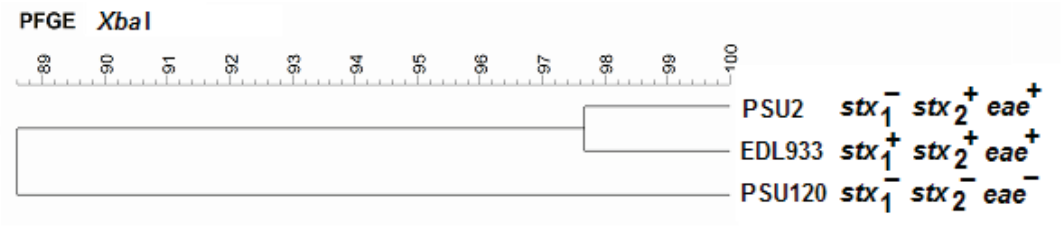

Fig 1–Pulsed-field gel-electrophoresis -based dendrogram of *E. coli* O157 isolated from clinical and environmental samples in southern Thailand. Genomes of *E. coli* O157 strains were digested with *Xba*I and separated as described in Materials and Methods. The hierarchical clustering was constructed using unweighted pair-group method of arithmetic average (UPGMA) and BioNumerics software version 7.0 (Applied Maths, Belgium).

O157:H7 strain PSU2 by pulsed-field gel-electrophoresis was performed. Dendrogram constructed based upon the unweighted pair-group method of arithmetic average using BioNumerics software version 7.0 revealed that the environmental *E. coli* O157:H7 strain PSU2 and EHEC strain EDL933 showed similar DNA fingerprints with their similarities being 97.6% by a pulsed-field gel-electrophoretic analysis (Fig 1). These strains and PSU120 relatively displayed the closely relatedness (88.6% similarity). Thus, it was suggested the high degree of similarity among these O157 strains.

DISCUSSION

Despite that *E. coli* O157:H7 strain PSU120 was the sole possible enteric pathogen of the diarrheal patient, the absence of the principal virulence determinants, stx_1 and stx_2, makes understanding of the role of PSU120 difficult. Prevalence of stx^+ *E. coli* O157 in marketed beef in the study area (Vuddhakul *et al*, 2000; Sukhumungoon *et al*, 2011) and a similar example in the United States suggesting such strains may be descended from EHEC O157 by the loss of the *stx* gene during infection (Mellmann *et al*, 2008). *E. coli* O157:H7 strain PSU2 with stx_1^--stx_2^+-eae^+ genotype represents the most frequently found environmental *E. coli* O157 strains isolated in this area for a decade and the well-established EHEC strain EDL933 (stx_1^+-stx_2^+-eae^+) showed 97.6% similarity in DNA profiles based on pulsed-field gel-electrophoresis. These strains and PSU120 displayed the closely relatedness (88.6% similarity) (Fig 1). In addition, in PSU120, *sbcB* and *yehV* were found to be occupied by prophages, while *yecE*, *wrbA*, and Z2577 were not, suggesting PSU120 is capable of horizontal genetic transfer. Thus, we are tempted to speculate PSU120 was originally stx^+ but lost the gene after establishing infection.

Vancomycin resistance in *E. coli* has not been reported frequently. However, *E. coli* O157 isolates collected from 200 children in Baghdad, Iraq, were shown to be resistant to vancomycin as well as erythromycin and polymyxin B (Shebib *et al*, 2003). Osaili *et al* (2013) detected 40 O157:H7 isolates which were resistant to vancomycin from slaughtered cattle in Amman City, Jordan, with MIC more than 512 μg/ml. Dissemination of vancomycin resistance can be occurred through horizontal gene transfer. In enterococci, the most common resistance genotype is *vanA*, which is harbored by the transpos-

able element, Tn*1546*, allowing the spread of vancomycin resistance to other bacteria through integration of Tn*1546*-containing *vanA* into conjugative plasmid (Woodford, 2001).

The very infrequent infection by *E. coli* O157 is probably related to strong acquired immunity to the O157 antigen, for which cross-reacting antigen in other bacterial species in the environment are known (Vuddhakul *et al*, 2000; Voravuthikunchai *et al*, 2005). In one study carried out in the central part of Thailand in 1997 and 1998, an *E. coli* O157:H7 strain harboring stx_1 and stx_{2v} was isolated from a stool specimen obtained from a healthy two-year old child and exhibited no toxicity to Vero cells (Leelaporn *et al*, 2003). A large number of diarrheal specimens were investigated by standard method and by immunomagnetic beads specific to O157 antigen in southern Thailand, but EHEC O157 was not detected (Kalnauwakul *et al*, 2007). To further examine the hypothesis that there is no patient infected by EHEC O157 in Thailand, the study was carried out in southern Thailand. It is generally presumed that there is more possibility of isolating EHEC O157 from clinical specimens in northern than southern Thailand because people in northern Thailand consume traditional foods containing raw meat (beef or buffalo), whereas the people in southern Thailand are mostly Muslims who thoroughly cook meat (beef and goat). Nevertheless, *E. coli* O157 carrying *stx* and *eae* are present at high frequencies in beef sold in markets in southern Thailand (Vuddhakul *et al*, 2000; Sukhumungoon *et al*, 2011). It is not surprising that we reported here for the first time the isolation of an *E. coli* O157 strain from stool of a patient with diarrhea admitted to a hospital in Thailand. Although the isolate was *stx*-negative, it carried important pathogenicity marker gene, the *escV* gene, a marker for Type III secretion system. At any rate, we concluded that this is the first report on human infection by *E. coli* O157 in Thailand.

ACKNOWLEDGEMENTS

This work was supported in part by a grant from Prince of Songkla University. MT is a recipient of Prince of Songkla University scholarship grant No. SCI560349S. We thank Prof Varaporn Vuddhakul for kindly providing *E. coli* O157:H7 strain Thai-12 and anti-O157 antibody.

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