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
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, antibiotic 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 11,000g 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 GoTaq DNA polymerase buffer, 3 mM MgCl₂, 0.5 U GoTaq 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% agar-
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 37°C for 6 hours with aeration. Bacteria were harvested by sedimentation and re-suspended in physiological saline and heated at 121°C 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% semi-liquid media (TSB-based media) 3 times, followed by culturing in 10 ml of TSB at 37°C 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 50°C 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: 95°C for 3 minutes; 35 cycles of 94°C for 50 seconds, 54°C for 50 seconds, and 72°C for 30 seconds; with a final step of 72°C 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 GoTaq DNA polymerase buffer, 3 mM MgCl₂, 1.25 U GoTaq DNA polymerase (Promega, Madison, WI) and 2 µl of DNA template. ERIC2 primer was listed in Table 1. Thermocycling conditions were as follows: 95°C for 3 minutes; 30 cycles of 94°C for 3 seconds, 92°C for 30 seconds, and 50°C for 1 minute; followed by a final heating at 65°C for 8 minutes. The amplicons were analyzed by 1.5% agarose gel-electrophoresis for 2 hours at
Table 1

<table>
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<th>Gene</th>
<th>Virulence factor</th>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
<th>Amplicon size (bp)</th>
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<td>est</td>
<td>ST</td>
<td>JW14</td>
<td>ATTTTTACTTTTCTGATATTAGTCTT</td>
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<td>elt</td>
<td>LT</td>
<td>TW20</td>
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<td>ERIC2</td>
<td>AAGTAAGTGACTGCGGTGAGCG</td>
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<td>Versalovic et al, 1991</td>
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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).

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
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. Ratchrachenchai 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.

<table>
<thead>
<tr>
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<td>Group D</td>
<td>+</td>
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<td>Group D</td>
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<td>-</td>
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<td>PSU196</td>
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<td>aETEC strain KETE</td>
<td>Group B1</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
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aControl 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 inability 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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REFERENCES


Ruan X, Crupper SS, Schultz BD, Robertson DC, Zhang W. Escherichia coli expressing EAST1 toxin did not cause an increase of cAMP or cGMP levels in cells, and no diarrhea in 5-day old gnotobiotic pigs. PlosOne 2012; 7: e43203.


