EXAMINATION OF DIARRHEAL STOOLS IN HAT YAI CITY, SOUTH THAILAND, FOR ESCHERICHIA COLI O157 AND OTHER DIARRHEAGENIC ESCHERICHIA COLI USING IMMUNOMAGNETIC SEPARATION AND PCR METHOD

Sineenart Kalnawakul¹, Manthana Phengmak¹, Urairat Kongmuang¹, Yoshitsugu Nakaguchi² and Mitsuaki Nishibuchi²

¹Department of Pathology, Faculty of Medicine, Prince of Songkla University, Hat Yai, Thailand; ²Center for Southeast Asian Studies, Kyoto University, Yoshida, Kyoto, Japan

Abstract. A total of 493 stool samples from diarrheal patients in Songklanagarind Hospital, in southern Thailand, were examined for Escherichia coli O157 by the culture method combined with an immunomagnetic separation (IMS) technique. E. coli O157 was not found, although the IMS-based method could detect 10²-10³ CFU of artificially inoculated O157/g of stool samples. Polymerase chain reaction was also used for the detection and identification of diarrheagenic E. coli from 530 stool samples. The target genes were eae for enteropathogenic E. coli (EPEC), stx for enterohemorrhagic E. coli (EHEC), elt and est for enterotoxigenic E. coli (ETEC), ipaH for enteroinvasive E. coli (EIEC), and aggR for enteroaggregative E. coli (EAggEC). Fifty-eight diarrheagenic E. coli strains were detected in 55 stool samples (10%) from 32 children and 23 adults. These included 31 EAggEC strains (5.8%), 13 ETEC strains (2.5%), 13 EPEC strains (2.5%), and one EIEC strain (0.2%). EHEC was not detected. The diarrheagenic E. coli strains were found mainly in children under 2 years of age (24 of 32 children). EAggEC strains and ETEC strains were susceptible to several antibiotics whereas the EPEC strains exhibited resistance to these antibiotics.

INTRODUCTION

Enteropathogenic bacteria causing diarrhea is still an important problem in Thailand. Stool samples from diarrheal patients are routinely cultured and bacteria belonging to the genera Salmonella, Shigella, Vibrio, Aeromonas and Plesiomonas are often isolated as the causative enteropathogens. These pathogens can be identified by standard biochemical tests. Escherichia coli is also one of the most common etiologic agents of diarrhea. There are diarrheagenic and non-diarrheagenic E. coli among E. coli isolates from stool, and they cannot be distinguished by colony morphology or biochemical tests, nor is serogrouping of the O antigen sufficient to identify the isolated E. coli as being diarrheagenic E. coli. Five major diarrheagenic E. coli have been identified: enteropathogenic E. coli (EPEC), enteroinvasive E. coli (EIEC), enterotoxigenic E. coli (ETEC), enterohemorrhagic E. coli (EHEC), and enteroaggregative E. coli (EAggEC). The O antigen may be used in typing diarrheagenic E. coli in diarrheal stool, but it is not perfectly correlated with the presence of specific virulence factors (Sunabe and Honma, 1998). Thus, diagnostic laboratory can not easily isolate and detect diarrheagenic E. coli from fecal samples.

E. coli O157:H7, a causative agent of bloody diarrhea (hemorrhagic colitis), belongs to the EHEC type and causes sporadic and
E. coli O157:H7 infection has increased in Europe, USA and Asia (Reida et al., 1994; Su and Brandt, 1995; Watanabe et al., 1996; Hilborn et al., 1999; Fey et al., 2000; Olorunshola et al., 2000). In Asia, the largest outbreak of E. coli O157:H7 occurred in Japan in 1996, and involved more than 6,000 cases (Watanabe et al., 1996; Yukioka et al., 1997). However, information on E. coli O157:H7 infection is scarce in Southeast Asia. This is partly because specific and sensitive methods are needed to isolate E. coli O157:H7 from stool samples. There has as yet been no report of any diarrhea case caused by E. coli O157:H7 in Thailand to our knowledge, although E. coli O157:H7 has been found in retail beef and bovine feces in Hat Yai City in southern Thailand (Vuddhakul et al., 2000).

Thus, the first purpose of this study was to determine the presence of E. coli O157:H7 in stool samples of diarrheal patients in Hat Yai City employing a combination of an immunomagnetic separation (IMS) method, sorbitol MacConkey (SMac) agar and CHROMagar that allows detection of E. coli O157:H7 with high specificity and sensitivity. The second purpose was to examine distribution of the five major diarrheagenic E. coli in stool samples of diarrheal patients in Hat Yai City employing PCR-based molecular genetic methods that are very specific and sensitive for the detection of diarrheagenic E. coli (Schmidt et al., 1995; Stacy-Phipps et al., 1995; da Silva Duque et al., 2002). A number of papers have reported detection of diarrheagenic E. coli using PCR methods from patients in Bangkok and the surrounding areas (Echeverria et al., 1992; Sethabutr et al., 1994; Leelaporn et al., 2003; Ratchtrachenchai et al., 2004).

MATERIALS AND METHODS

Detection of E. coli O157

Stool samples from diarrheal patients were collected at Songklanagarind Hospital in Hat Yai City between September 1999 to October 2000. Samples were examined for E. coli O157 by culture on SMac agar, and by IMS followed by isolation on SMac agar and CHROMagar.

(i) Culture on SMac agar. Samples were collected in Stuart’s transport medium and cultured on SMac agar at 37°C overnight. All sorbitol-non-fermenting (colorless and translucent) colonies on SMac agar were examined by standard biochemical tests. Colonies identified as E. coli were then examined by a slide agglutination method with anti-E. coli O157 antiserum (E. coli O antiserum O157, Denka Seiken, Tokyo, Japan).

(ii) IMS followed by isolation on SMac agar and CHROMagar. IMS using magnetic beads coated with the anti-O157 antibody (Dynabeads anti-E.coli O157, Dynal A.S, Oslo, Norway) was performed according to the manufacturer’s instructions. In brief, the stool sample was incubated in tryptic soy broth (TSB) for 5 hours for enrichment, and then one-ml aliquots were taken for IMS. Twenty µl aliquot of the immunomagnetic beads suspension was incubated with 1 ml of the enriched sample at room temperature for 30 minutes with continuous mixing. The bead-bacteria complexes were separated using a magnetic concentrator (Dynal MPC-M; Dynal A.S, Oslo, Norway). After discarding the supernatant, beads with attached bacterial cells were washed three times in 1 ml of washing buffer. The beads were resuspended in 20 µl of phosphate-buffered saline (PBS), cultured on SMac agar overnight at 37°C. After incubation, sorbitol-non-fermenting colonies were picked and streaked onto CHROMagar O157 (CHROMagar, Paris, France) and incubated for 24 hours at 37°C. All mauve (pink to purple) colonies on CHROMagar were used for identification of E. coli by biochemical tests and for the O157 antigen by agglutination test with anti-E. coli O157 antiserum.
Prior to testing the clinical samples, the IMS procedure was optimized and the sensitivity of the IMS was evaluated. To optimize IMS, incubation temperatures of 37°C and 42°C were compared for enrichment in TSB. Also, PBS containing 0.05% Tween 20 (PBS-Tween) with and without 0.04% casein were compared to select a suitable washing buffer (Islam et al, 1992). For evaluation of the sensitivity of the IMS, known numbers of E. coli O157 EDL933 (10^{-10} to 10^8 CFU/ml as determined by the standard viable plate count technique) were added to 0.2 g of normal stool samples from healthy volunteers. Then, E. coli O157-spiked samples were processed by the IMS as described above.

Detection of diarrheagenic E. coli

Stool samples and bacteriology. Stool samples and rectal swabs were collected from patients diagnosed with diarrhea at Songklanagarind Hospital from April 2001 to December 2002. Samples collected in the transport medium were inoculated onto MacConkey agar and incubated at 37°C overnight. Sixteen E. coli-like colonies (pink to red colonies) were selected from one plate and pooled for PCR assays.

PCR assay. PCR was used to detect the presence of the genes specific to EHEC, EPEC, ETEC, EIIEC and EAggEC. A small amount of the growth was picked from the pooled sixteen E. coli-like colonies and suspended in 500 µl of distilled water. DNA was extracted from the suspension by boiling for 10 minutes, then a 2 µl aliquot was used as template. The PCR mixture contained 1x PCR buffer, 2 mM MgCl₂ (Promega, Madison WI, USA) 200 µM of each deoxy nucleoside triphosphate (dATP, dCTP, dGTP and dTTP; Promega, Madison WI, USA), 300 µM of each primer, 1 to 2 U of Taq DNA polymerase (Promega, Madison WI, USA) and 2 µl of template DNA. The final volume of the reaction was adjusted with sterile distilled water to 50 µl. Then the PCR tube was placed in the block of a DNA thermal cycler (Model 2400, Perkin-Elmer, Norwalk, USA). Positive control strains (Table1) were included in each PCR assay. A buffer control with no template DNA was included in each PCR assay as a negative control to monitor interior contamination. The primers and amplification conditions used are shown in Tables 2 and 3, respectively. Ten µl of each PCR product was analyzed in 1.5% agarose gel by electrophoresis in Tris-acetate-EDTA buffer at a constant voltage of 100 V for 40 minutes. A 100-bp DNA ladder (Invitrogen, USA) was used as markers for determining the sizes of the amplicons. The gel was stained with 4 µg/ml of ethidium bromide and visualized under UV illumination (Sambrook et al, 1989).

When the DNA template from pooled colonies gave positive results, individual isolates were examined by the above PCR protocol to identify the PCR-positive isolate.

Antibiotic susceptibility test

The antibiotic susceptibility test was performed using the Bauer-Kirby standard disk diffusion method according to protocols of the National Committee for Clinical Laboratory Standards (Wayne, PA, USA). The diarrheagenic E. coli isolates were tested with the following antibiotics: ampicilin (AM, 30 µg), chloramphenecol (C, 30 µg), cefotaxime (CTX, 30 µg), ciprofloxacin (CIP, 5 µg), fosfomycin (FO, 50 µg), gentamicin (GM, 10 µg), nalidixic acid (NA, 30 µg), norfloxacin (NX, 10 µg), cotrimoxazole (SXT: sulfamethoxazole, 23.75 µg; and trimethoprim, 1.25 µg), and tetracycline (Te, 30µg). E. coli ATCC 25922 was used as the standard strain. The inoculated agar plates were incubated overnight at 37°C and the diameters of inhibition zones around bacterial growth were measured.

RESULTS

Detection of E. coli O157

A total of 493 stool samples from diarrheal patients were examined for E. coli O157.
Sorbitol-non-fermenting colonies on SMac agar that were subsequently identified as E. coli were tested for the presence of the O157 antigen by a slide agglutination method with anti-E. coli O157 antiserum. None of the E. coli isolates agglutinated with the antiserum.

To optimize the IMS method, we compared the incubation temperature during enrichment in TSB (37°C vs 42°C) and washing buffer (PBS-Tween with and without 0.04% casein) using spiked stool samples. We found enrichment in TSB at 37°C and washing with PBS-Tween added with 0.04% casein improve the recovery of E. coli O157 (data not shown). This IMS method could detect 10^2 to 10^3 CFU of spiked E.coli O157 EDL933/g of stool.

When we examined the clinical stool samples using this IMS method followed by isolation on SMac agar, 653 sorbitol-non-fermenting colonies were isolated from 39 stool samples. When these isolates were grown on CHROMagar, 78 isolates formed mauve colonies and were identified as E. coli. However, these isolates did not agglutinate with the anti-E.coli O157 antiserum and thus were judged to belong to O serogroups other than O157.

Detection of diarrheagenic E. coli

Stool and rectal swab samples that gave predominately E. coli-like colonies on MacConkey agar totaled 530 samples. The E. coli-like colonies were examined by PCR to detect strains belonging to diarrheagenic E. coli (EHEC, ETEC, EIEC, EPEC or EAEGEc).

Diarrheagenic E. coli were detected in 55 stool samples (10.3%), 32 samples from children and 23 samples from adults. Two types of diarrheagenic E. coli were detected simultaneously in three of the samples (EAEGEc and

Table 1
Control strains used in the study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>E. coli type</th>
<th>Target gene(s)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDL933</td>
<td>EHEC</td>
<td>stx (stx(_1) and stx(_2))</td>
<td>(Karch and Meyer, 1989)</td>
</tr>
<tr>
<td>PE27</td>
<td>EPEC</td>
<td>eae, bfp</td>
<td>(Reid et al, 1999)</td>
</tr>
<tr>
<td>PE7</td>
<td>EAEGEc</td>
<td>aggR</td>
<td>(Tsukamoto, 1996)</td>
</tr>
<tr>
<td>H10407</td>
<td>ETEC (LT)</td>
<td>elt</td>
<td>(Stacy-Phipps et al, 1995)</td>
</tr>
<tr>
<td>KETE</td>
<td>ETEC (LT+ST)</td>
<td>est (STh and STp)</td>
<td>(Stacy-Phipps et al, 1995)</td>
</tr>
<tr>
<td>KEIE</td>
<td>EIEC</td>
<td>ipaH</td>
<td>(Sethabutr et al, 1993)</td>
</tr>
</tbody>
</table>

Fig 1–PCR detection of gene specific to diarrheagenic E. coli. Lane 1: molecular size markers; lanes 2-7: positive control strains including eae gene of EPEC (lane 2), bfp gene of EPEC (lane 3), ipaH gene of EIEC (lane 4), elt and est genes of ETEC (lane 5), aggR gene of EAEGEc (lane 6), and stx gene of EHEC (lane 7); lanes 8-10: strains isolated from patients in this study including EAEGEc (lane 8), EIEC (lane 9), and est-positive ETEC (lane 10).
EPEC in two samples and EAEGEC and ETEC in one sample. The results are summarized in Table 4. EAEGEC-positive strain was the most frequent (5.8% of the samples), followed by ETEC-positive (2.5%), EPEC-positive (2.5%), and EIIEC-positive (0.2%) strains. No EHEC strain was detected. Of the ETEC strains isolated, those carrying the heat-stable enterotoxin gene (est) were dominant (53.8% of ETEC strains). Examples of the amplified DNA bands detected by agarose gel electrophoresis are shown in Fig 1.

EAEGEC was isolated more frequently from children (22 of 31, 71%) than from adults (9 of 31, 29%) whereas EPEC and ETEC were commonly found in both children and adults (data not shown). Age distribution of diarrheagenic E. coli-positive individuals is summarized in Table 5. In children, diarrheagenic E. coli strains were isolated mainly in the children less than 2 years of age (24 of 32, 75%).

In this study, clinical symptoms of 49 patients were available (Table 6). They included 24, 13, 11 and one patients infected with EAEGEC, EPEC, ETEC and EIIEC, respectively. Watery stool (14 patients, 58%) and fever (12 patients, 50%) were common symptoms in EAEGEC-infected patients. Watery diarrhea (8 patients, 62%) was the dominant symptom recorded for EPEC-infected patients. Watery stool (5 patients (45%)) were found from ETEC-infected patients. Five patients had mucous bloody stool. EAEGEC, ETEC and EPEC was detected in two, two and one of these patients, respectively.
such as ampicillin, cotrimoxazole, and tetracycline. All of the EAggEC and ETEC strains tested were susceptible to ciprofloxacin, cefotaxime, fosfomycin, gentamicin and norfloxacin, whereas all strains of EPEC exhibited susceptibility only to fosfomycin.

**DISCUSSION**

We examined if E. coli O157 was present in 493 stools of diarrheal patients in Hat Yai area using two isolation methods. E. coli O157 was not detected by the isolation method using only SMac agar. Most E. coli O157 strains do not ferment sorbitol while most non-O157 E. coli strains can ferment sorbitol. Based on this characteristic, many laboratories use SMac agar for initial screening of E. coli O157 from stool samples, because it is inexpensive and easy to use (March and Ratnam, 1986; Ritchie et al, 1992). However, many bacteria form colorless and translucent colonies similar to E. coli O157 on SMac agar. Therefore, the isolation method using only SMac agar is not specific for E. coli O157 and thus the sensitivity is low when other sorbitol-non-fermenting bacteria are present in the sample. The detection limit of the spiked E. coli O157 in stool samples by direct detection on SMac agar was about 10^8 CFU/g in our experiment (data not shown). The infectious dose of E. coli O157 is estimated to be very low (Willshaw et al, 1994), and thus a more sensitive detection technique is required.
Table 6
Clinical symptoms of patients infected with diarrheagenic Escherichia coli at Songklanagarind Hospital from April 2001 to December 2002.

<table>
<thead>
<tr>
<th>Clinical symptom</th>
<th>EAggEC (n = 24)</th>
<th>EPEC (n = 13)</th>
<th>ETEC (n = 11)</th>
<th>EIEC (n = 1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stool characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Watery</td>
<td>14 (58.3)</td>
<td>8 (61.5)</td>
<td>5 (45.5)</td>
<td>1</td>
</tr>
<tr>
<td>Mucous</td>
<td>6 (25)</td>
<td>4 (30.8)</td>
<td>1 (9.1)</td>
<td></td>
</tr>
<tr>
<td>Mucous and bloody</td>
<td>2 (8.3)</td>
<td>1 (7.7)</td>
<td>2 (18.2)</td>
<td></td>
</tr>
<tr>
<td>Loose</td>
<td>2 (8.3)</td>
<td>-</td>
<td>3 (27.3)</td>
<td></td>
</tr>
<tr>
<td>Fever</td>
<td>12 (50)</td>
<td>6 (46.2)</td>
<td>4 (36.4)</td>
<td>1</td>
</tr>
<tr>
<td>Vomiting</td>
<td>7 (29.2)</td>
<td>3 (23.1)</td>
<td>4 (36.4)</td>
<td></td>
</tr>
</tbody>
</table>

Table 7
Antibiotic susceptibility of diarrheagenic Escherichia coli.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>EAggEC (n = 30)</th>
<th>ETEC (n = 9)</th>
<th>EPEC (n = 13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>18 (60)</td>
<td>7 (78)</td>
<td>6 (46)</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>20 (67)</td>
<td>9 (100)</td>
<td>10 (77)</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>30 (100)</td>
<td>9 (100)</td>
<td>11 (85)</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>30 (100)</td>
<td>9 (100)</td>
<td>12 (92)</td>
</tr>
<tr>
<td>Fosfomycin</td>
<td>30 (100)</td>
<td>9 (100)</td>
<td>13 (100)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>30 (100)</td>
<td>9 (100)</td>
<td>11 (85)</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>27 (90)</td>
<td>7 (78)</td>
<td>8 (62)</td>
</tr>
<tr>
<td>Norfloxacin</td>
<td>30 (100)</td>
<td>9 (100)</td>
<td>10 (77)</td>
</tr>
<tr>
<td>Cotrimoxazole</td>
<td>7 (23)</td>
<td>7 (78)</td>
<td>4 (31)</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>15 (50)</td>
<td>7 (78)</td>
<td>6 (46)</td>
</tr>
</tbody>
</table>

We included IMS and CHROMagar O157 isolation step before and after the SMac agar isolation step, respectively. The IMS method is rapid and facilitates selective isolation of pathogens from samples containing microflora and growth inhibitors. It is often combined with enrichment culture techniques and is widely used for detection of pathogens from food and other specimens (Cudjoe et al, 1995; Ogden et al, 2001). Several investigators applied IMS to detect E. coli O157 from food and stool samples (Wright et al, 1994; Tomoyasu, 1998; Islam et al, 2006; Lejeune et al, 2006). E. coli O157 had been found in 4 of 95 retail beef samples and one of 55 bovine feces samples collected in Hat Yai City by using IMS (Vuddhakul et al, 2000). CHROMagar O157 is a very selective medium for E. coli O157 (Bettelheim, 1998). The method using IMS followed by isolation on SMac agar and CHROMagar allowed detection of $10^2$ to $10^3$ CFU of spiked E. coli O157/g of stool. Nevertheless, E. coli O157 was not detected by this method from the 493 diarrheal stool samples. This result indicates that E. coli O157 is an uncommon diarrhea-caus-
ing pathogen at least in Hat Yai City, Thailand. This is also supported by the result of PCR assay examining 530 samples of stools and rectal swabs from patients in this study. Clinical strains of E. coli O157 belong to EHEC and they are expected to carry stx gene (stx₁, stx₂, or both). The stx gene was not detected in all 530 samples. Absence of the infection by E. coli O157 in this area may be partly due to immunity to the O157 antigen (Tsai et al, 2003).

Although EHEC including E. coli O157 was not detected, examination of the 530 samples by PCRs demonstrated that infections by other types of diarrheagenic E. coli are prevalent in Hat Yai City. PCR is a powerful molecular biological technique that has been applied to the detection of many pathogens including diarrheagenic E. coli (Keskimaki et al, 2001; da Silva Duque et al, 2002; Toma et al, 2003; Vidal et al, 2004). However, its direct application to clinical specimens is difficult due to the fact that fecal specimens often contain substance(s) that may interfere with the PCR assay, leading to false-negative results (Wilde et al, 1990; Persing, 1991). For this reason, DNA purification from stool samples or isolated colonies from stool culture are required for PCR (Leelaporn et al, 2003). In our study we pooled 16 E. coli-like colonies from a single MacConkey agar plate and the DNA template prepared from the pooled colonies were subjected to PCR examination. We believe this approach is an effective screening method for detection of diarrheagenic E. coli. Diarrheagenic E. coli was detected in 11% of the samples from the diarrheal patients. EAggEC was detected most frequently (5.8 %), followed by ETEC, EPEC and EIEC. A similar tendency was observed among the diarrheagenic E. coli detected in stool samples of children with diarrhea in the Bangkok area (Ratchrachenchai et al, 2004). Other studies from India, Japan, Taiwan, Mongolia and Thailand have also reported high incidences of diarrhea caused by EAggEC (Dutta et al, 1999; Nishikawa et al, 2002; Tsai et al, 2003; Kahali et al, 2004; Ratchrachenchai et al, 2004; Sarantuya et al, 2004) and epidemiological studies suggest that EAggEC is a significant cause of acute and persistent diarrhea in children and adults (Sullivan et al, 1994; Schultsz et al, 2000). Our study showed that EAggEC was more frequently isolated from children than from adults.

In summary, the present study shows, for the first time, that EAggEC, EPEC, and ETEC, but not EHEC, are the etiologies of diarrhea in Hat Yai City, and that EAggEC is especially an important pathogen causing diarrhea among children in this area. Further studies on the epidemiology of diarrheagenic E. coli in southern Thailand including outer urban and rural areas, where hygienic and social conditions are different from those of the city area, is needed.

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