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

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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 10^2-10^3 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. col*. 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

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epidemic infection. In recent years the incidence of *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 diarrhegenic *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 37ºC 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 37ºC. After incubation, sorbitol-non-fermenting colonies were picked and streaked onto CHROMagar O157 (CHROMagar, Paris, France) and incubated for 24 hours at 37ºC. 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 37ºC and 42ºC 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 -10^8 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, EIEC 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 $MgCl_2$ (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 37ºC 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.

Table 1
Control strains used in the study.

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

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 (37ºC *vs* 42ºC) and washing buffer (PBS-Tween with and without 0.04% casein) using spiked stool samples. We found enrichment in TSB at 37ºC 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.

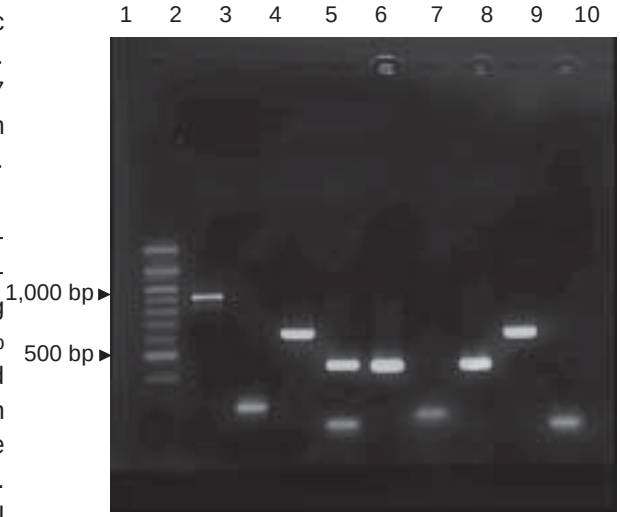

Fig 1–PCR detection of gene specific to diarrheagenic *E. coli.* Lane 1: molecular size markers; lanes 2-7: positive control strains including *eae* gene of EPEC (lane 2), *bfp* gene of EPEC (lane 3), *ipaH* gene of EIEC (lane 4), *elt* and *est* genes of ETEC (lane 5), *aggR* gene of EAggEC (lane 6), and *stx* gene of EHEC (lane 7); lanes 8-10: strains isolated from patients in this study including EAggEC (lane 8), EIEC (lane 9), and *est*-positive ETEC (lane 10).

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 EAggEC). 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 (EAggEC and

Table 2
Primer and amplicon size.

Primer	Sequence (5'... 3')	Target gene	Product size (bp)	Reference
MK-1	TTT ACG ATA GAC TTC TCG AC	*stx*	224 or 227	(Karch and Meyer, 1989)
MK-2	CAC ATA TAA ATT ATT TCG CT			
AggR-1	CAG AAT ACA TCA GTA CAC TG	*aggR*	433	(Tsukamoto, 1996)
AggR-2	GAA GCT TAC AGC CGA TAT AT			
EaeP1	CTG AAC GGC GAT TAC GCG AA	*eae*	917	(Reid *et al*, 1999)
Eae P2	CCA GAC GAT ACG ATC CAG			
Ecoeaeα	CTG GAG TTG TCG ATG TT	*bfp*	265	(Reid *et al*, 1999)
Ecoeaeβ	GTA ATT GTG GCA CTC C			
Ecoeaeγ	GCC TCT GAC ATT GTT AC			
BFP-1	CGT CTG ATT CCA ATA AGT CGC			
BFP-2	GCC GCT TTA TCC AAC CTG GTA			
TW 20	GGC GAC AGA TTA TAC CGT GC	*elt*	450	(Stacy-Phipps *et al*, 1995)
JW 11	CGG TCT CTA TAT TCC CTG TT			
JW 14	ATT TTT ACT TTC TGT ATT AGT CTT	*est*	190	(Stacy-Phipps *et al*, 1995)
JW 7	CAC CCG GTA CAA GGC AGG ATT			
ipaIII	GTT CCT TGA CCG CCT TTC CGA TAC CGT C	*ipaH*	603, 619	(Sethabutr *et al*, 1993)
ipa IV	GCC GGT CAG CCA CCC TCT GAG AGT AC			

EPEC in two samples and EAggEC and ETEC in one sample). The results are summarized in Table 4. EAggEC-positive strain was the most frequent (5.8% of the samples), followed by ETEC-positive (2.5%), EPEC-positive (2.5%), and EIEC-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.

EAggEC 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 EAggEC, EPEC, ETEC and EIEC, respectively. Watery stool (14 patients, 58%) and fever (12 patients, 50%) were common symptoms in EAggEC-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. EAggEC, ETEC and EPEC was detected in two, two and one of these patients, respectively.

Table 3
PCR conditions.

Step of PCR	Type of *E. coli*		
	EHEC, EAggEC, EPEC	ETEC	ETEC
First denaturation	94ºC, 5 minutes	95ºC, 5 minutes	94ºC, 5 minutes
Denaturation	94ºC, 1 minute	95ºC, 4 seconds	94ºC, 1 minute
Extension	72ºC, 3 minutes	72ºC, 5 minutes	72ºC, 1 minute
Cycle	30	40	35

Table 4
Diarrheagenic *Escherichia coli* isolated from 530 stool samples collected at Songklanagarind Hospital.

Diarrheagenic *E. coli*	No. of strains isolated (%)
EAggEC	31 (5.8)
ETEC, total	13 (2.5)
est-positive ETEC	7
elt-positive ETEC	5
el-t and *est*-positive ETEC	1
EPEC	13 (2.5)
EIEC	1 (0.2)

Table 5
Age distribution of patients infected with diarrheagenic *Escherichia coli*.

Age	No. of patients	No. of patients positive for diarrheagenic *E. coli* (%)
Children		
0-2 years	159	24 (15.1)
3-14 years	73	8 (11)
Adult (>15 years)	298	23 (7.7)
Total	530	55 (10.5)

Antibiotic susceptibility test

Fifty-two strains of diarrheagenic *E. coli* were available for antibiotic susceptibility tests (Table 7). These diarrheagenic *E. coli* showed high resistance to commonly used antibiotics 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.

Clinical symptom	No. (%) patients infected with			
	EAggEC (n = 24)	EPEC (n = 13)	ETEC (n = 11)	EIEC (n = 1)
Stool characteristics				
Watery	14 (58.3)	8 (61.5)	5 (45.5)	1
Mucous	6 (25)	4 (30.8)	1 (9.1)	
Mucous and bloody	2 (8.3)	1 (7.7)	2 (18.2)	
Loose	2 (8.3)	-	3 (27.3)	
Fever	12 (50)	6 (46.2)	4 (36.4)	1
Vomiting	7 (29.2)	3 (23.1)	4 (36.4)	

Table 7
Antibiotic susceptibility of diarrheagenic *Escherichia coli.*

Antibiotic	No. susceptible (%)		
	EAggEC (n = 30)	ETEC (n = 9)	EPEC (n = 13)
Ampicillin	18 (60)	7 (78)	6 (46)
Chloramphenicol	20 (67)	9 (100)	10 (77)
Ciprofloxacin	30 (100)	9 (100)	11 (85)
Cefotaxime	30 (100)	9 (100)	12 (92)
Fosfomycin	30 (100)	9 (100)	13 (100)
Gentamicin	30 (100)	9 (100)	11 (85)
Nalidixic acid	27 (90)	7 (78)	8 (62)
Norfloxacin	30 (100)	9 (100)	10 (77)
Cotrimoxazole	7 (23)	7 (78)	4 (31)
Tetracycline	15 (50)	7 (78)	6 (46)

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 (stx_1, stx_2, 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 (Ratchtrachenchai *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; Ratchtrachenchai *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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