PREVALENCE OF β-LACTAMASES IN ENTEROTOXIGENIC *ESCHERICHIA COLI* CLINICAL ISOLATES COLLECTED IN CAMBODIA, KENYA, NEPAL, THAILAND, UZBEKISTAN, AND VIETNAM, FROM 2001 TO 2010

Warawadee Nirdnoy, Paksathorn Kietsiri, Orntipa Sethabutr, Kamonporn Poramathikul, Supaporn Ruksasiri, Apichai Srijan, Katie R Margulieux, Ladaporn Bodhidatta, Brett E Swierczewski and Carl J Mason

Department of Enteric Diseases, Armed Forces Research Institute of Medical Sciences (AFRIMS), Bangkok, Thailand

Abstract. A collection of enterotoxigenic *Escherichia coli* (ETEC) isolates from diarrheal patients in Cambodia, Kenya, Nepal, Thailand, Uzbekistan, and Vietnam during 2001-2010 were isolated and investigated for prevalence of class A extended spectrum β-lactamases (ESBLs) or class C β-lactamases. A total of 377 ETEC strains were screened for β-lactamase activity using standard disk diffusion and confirmed with double-disk synergy and combination tests using clavulanic and boronic acids. Conjugation studies were then conducted to determine the possibility of horizontal gene transfer of the identified ESBL resistance mechanism(s) to previously antimicrobial susceptible parent strains. Six β-lactam resistant strains were identified (1.6% overall prevalence rate of tested strains), which demonstrated phenotypes that suggested the presence of class A ESBLs and/or plasmid-encoded class C β-lactamases. PCR amplification of class A and class C β-lactamase genes from the identified isolates revealed presence of Amp*C* (100%), CTX-M-I (83%), TEM-105 (67%), CTX-M-IV (17%), and CMY-2 (17%) genes. Additional conjugation studies demonstrated all ETEC strains harboring ESBLs completely transferred β-lactamase genes to previously ESBL-negative *E. coli* recipients. ETEC is a common pathogen in the geographic regions investigated, and this retrospective study reveals the early emergence and diversity of plasmid-encoded class A ESBLs and class C β-lactamases that were circulating among community-acquired, clinically relevant ETEC strains from 2001-2010.

Keywords: β-lactamases, enterotoxigenic *Escherichia coli*, prevalence

INTRODUCTION

Escherichia coli is a gram-negative bacterium commonly found in the gastrointestinal tract of humans and other warm-blooded animals. Even though *E. coli* is typically a harmless commensal bacterium, there are multiple pathogenic strains that cause illness in humans, ranging from self-limiting gastrointestinal infections to bacterial sepsis (Qadri *et al*, 2005).

Enterotoxigenic *E. coli* (ETEC) is a pathogenic strain that colonizes the gastrointestinal tract and produces intestinal cell-targeting toxins, resulting in mild

Correspondence: Katie R Margulieux, Department of Enteric Diseases, AFRIMS, 315/6 Ratchawithi Road, Bangkok 10400, Thailand. Tel: +66 (0) 2696 2700; Fax: +66 (0) 2644 4980 E-mail: katie.margulieux.ctr@afrims.org

to severe osmotic diarrhea. ETEC is an endemic pathogen found worldwide and is considered one of the leading causes of infantile diarrhea in developing countries as well as travelers' diarrhea (Adachi *et al,* 2002; Qadri *et al,* 2005; Pandey *et al,* 2011). Treatment of ETEC infections has typically been managed through β-lactam antibiotics, which are widely used as a first treatment option in human and veterinary medicine (Hornish and Kotarski, 2002). However, the past few decades have seen the development and expression of multiple extended-spectrum β-lactamases (ESBLs) in gram-negative bacteria, resulting in the global dissemination of antibiotic resistant bacterial strains and an increasingly shorter list of reliable treatment options to address severe ETEC infections (Batchelor *et al,* 2005; Oteo *et al,* 2010).

β-Lactam antibiotics are divided into six different groups consisting of carbapenems, cephalosporins, cephamycins, monobactams, β-lactamase inhibitors, and penicillins. Resistance to β-lactam antibiotics in gram-negative bacteria occurs via the synthesis of β-lactamases that act by hydrolyzing the four membered β-lactam ring, rendering the β-lactams inactive against the intended target bacterium (Bradford, 2001). Multiple β-lactamases have been described, and are separated into various classes based on their mechanism of action (Jacoby, 2006). The most prevalent classes detected globally are class A enzymes (TEM, SHV, CTX-M) and class C β-lactamases (AmpC, CMY-2) (Ambler, 1980; Jacoby and Han, 1996; Jarlier *et al,* 1998; Nordmann, 1998; Pai *et al,* 1999; Canton and Coque, 2006).

An increasing number of hospital-acquired and community-associated infections were reported to have been caused by β-lactamase producing *E. coli*, with various class A enzymes, such as CTX-M, being reported as endemic in some regions (Pitout and Laupland, 2008). Even though prevalence rates are relatively lower than plasmid-encoded ESBLs, plasmid-based class C β-lactamases are also spreading worldwide and have been detected in clinical *E. coli* isolates (Horii *et al,* 1994; Gazouli *et al,* 1998; Shaikh *et al,* 2015). Initially, the class C β-lactamase was defined as the chromosomally encoded gene, *ampC*, but several plasmid-encoded transferable class C β-lactamase genes have now been identified, including *ampC* and CMY-2 gene (Philippon *et al,* 2002; Jacoby, 2009). Notably, ETEC strains have been identified that carry multiple β-lactamase enzymes from different class characterizations, resulting in severely limited treatment options (Jacoby, 2009).

Detection of colonizing ESBL-producing enterobacteria, particularly *E. coli*, isolated from fecal samples from patients with diarrhea has increased rapidly in many areas of the world since 2000 (Hernandez *et al,* 2003; Mirelis *et al,* 2003; Munday *et al,* 2004; Valverde *et al,* 2004; Pitout *et al,* 2005). Bacterial production of ESBLs has become a major challenge in treating gram-negative enteric infections, especially pathogens such as ETEC that carry multiple β-lactamases (Bradford, 2001; Sturenburg and Mack, 2003). Clinically, ESBL-producing bacteria are not only resistant to cephalosporins and penicillin, but also frequently are resistant to fluoroquinolones and trimethoprim-sulfamethoxazole (Bradford, 2001). As a result, these bacteria may be resistant to a majority of antimicrobial agents that typically are recommended for the treatment of community-acquired infections caused by enteric bacterial species (Rodriguez-Bano and Navarro, 2008).

The inability to detect these resistance phenotypes during the early years of

emergence may have contributed to the uncontrolled spread of ESBL-producing organisms and subsequent related treatment failures. Therefore, it is necessary to utilize sensitive and reliable assays for ESBL detection to avoid the risk of reporting false-positive susceptibility to aztreonam, cephalosporins and penicillin (Spanu *et al,* 2006; CLSI, 2009c). It is also imperative that the retrospective screening of stored clinical isolates is undertaken and reported to understand the global spread of current antimicrobial mechanisms. The current study examined prevalence, phenotype and genotype of ESBL-type resistance of ETEC strains isolated from patients presenting with diarrhea in Cambodia, Kenya, Nepal, Thailand, Uzbekistan, and Vietnam from 2001 to 2010. The objective was to further understand and acquire tools necessary to combat the risk of multidrug- resistant bacteria, as well as to study the initial rates of ESBL acquisition and mechanisms during the early stages of global antimicrobial resistance development and spread.

MATERIALS AND METHODS

Bacterial strains

A total of 377 ETEC strains isolated from human stool of diarrheal patients in Cambodia, Kenya, Nepal, Thailand, Uzbekistan, and Vietnam during 2001 - 2010 were selected for this study (Table 1). ETEC identification was performed by identifying heat-stable enterotoxin (ST) or heat-labile enterotoxin (LT) using a monosialoganglioside-G_{MI} enzyme-linked immunosorbent assay (G_{MI}-ELISA) (Sjöling *et al,* 2007).

ESBL screening and antimicrobial susceptibility testing

Initial screening for ESBLs in the ETEC isolates were determined by anti-

Table 1
Number of ETEC strains from country of origin and year collected.

Country of origin (*n*)	Year of specimen collection									
	2001	2002	2003	2004	2005	2006	2007	2008	2009	2010
Cambodia (17)	0	0	0	0	33	33	1	0	0	0
Kenya (12)	0	0	0	0	0	0	0	0	12	0
Nepal (8)	5	7	3	0	0	0	14	42	6	1
Thailand (213)	10	37	28	7	28	41	7	7	28	8
Uzbekistan (1)	0	0	0	0	0	1	0	0	0	0
Vietnam (2)	2	0	0	0	0	0	0	0	0	0

microbial disk susceptibility testing according to the Clinical and Laboratory Standards Institute (CLSI, 2009b,c). Antimicrobial agents tested included aztreonam, cefpodoxime, ceftazidime, cefotaxime, and ceftriaxone. Phenotypic confirmation was performed using double - disk synergy (DDST) and combination disk tests. Selection criteria were based on ETEC isolates with zone diameters produced by 30 μg of aztreonam (ATM-30) of ≤ 27 mm, 10 μg of cefpodoxime (CPD-10) of ≤ 17 mm, 30 of μg ceftazidime (CAZ-30) of ≤ 22 mm, 30 μg of cefotaxime (CTX-30) of ≤ 27 mm, or 30 μg of ceftriaxone (CRO-30) of ≤ 25 mm.

DDST was performed using standard disk diffusion method according to the Clinical and Laboratory Standards M2-A10 (Ho *et al*, 1998; CLSI, 2009b). In brief, two separate Mueller-Hinton agar (Becton, Dickinson, Sparks, MD) plates were inoculated. Amoxicillin with clavulanic acid (20/10 μg) (AmC-30) and piperacillin/tazobactam (100/10 μg) (TZP-110) disks were placed separately at the center of each plate. Along the periphery, five third-generation β-lactams, namely, aztreonam, cefpodoxime, ceftazidime, cefotaxime and ceftriaxone were placed 30 mm (center to center) from the β-lactamase inhibitors (AmC-30 and TZP-110) prior to incubation. Positive ESBL production was inferred when at least one expanded β-lactam zone was observed by visual inspection.

Table 2
Oligonucleotide sequence of primers and probes used in the study.

Target gene	Primer/probes sequence (5′ to 3′)		
MOX, CMY(1, 8-11)	F: TCAAGGGATCCGT*CTTTGACA T*--C	R: GCGCATCTTCTCGGATGAAT	P: CTTGCCACCTACAGCGCCGGAG
LAT, CMY (2-7), BIL	F: CCTGCTGCACTTAGCCACCTA	R: AATGCGGCTTTATCCCTAACG	P: CGGCCTACCGCTGCAGATCCC
DHA1/2	F: GCTCTGCCGCAGTGGAA	R: TCCGCACGGCTTTTTACC	P: CTACCTATACCGCAGGCGGACTGCC
ACC	F: GAAGTGGGTTCGCTGAGTAAAAC	R: CACGCAACTCTGGAACGTAATG	P: TTTGCTGCCCACCTTGGCGTCC
MIR, ACT	F: CCGGATGAGGTCACGGATA	R: CTTTTTGGTGCGCTGGCGG	P: AAAACTGGCAGCCGCAGTGGAAG
FOX	F: GGAGATGCCGCGCTAAAAA	R: CCAGCCCCTGAGTCATGTCT	P: ATCGCGCTGACCCAT*ACCGGTTTCT T*--C
AMPC	F: CTATGCGGACATCGCCAAA	R: TTGCTGACCGAACCTAACTCAA	P: CAGCCCGTCACACAGCAAACGTTG
TEM	F: CTGAATGAAGCCATACCAAACG	R: ACTTTATCCGCCTCCATCCA	P: CGAGCGTGACACCACGATGCCT
SHV	F: TGGATGCCGGTGACGAA	R: CGCTGACCGGCGAGTAGT	P: CGAAAGATCCACTATCGCCAGCAGGA
CTX-MI (CTX-M1)	F: CGGTGATCTGGCCAAAAGA	R: CTTAGGTTGAGGCTGGGTGAAG	P: TGCGCCGCTGATTCTGGTCACTT
CTX-MII (CTX-M2)	F: GCGGCGGTGCTTAAACAG	R: CCAGGTCGCTCTTCTTGATTTC	P: AGCGATAAGCACCTGCTAAATCAGCGC
CTX-MIII (CTX-M8)	F: ATTGACACCGCCGATAACG	R: TGCTGCACATGGCAAAGC	P: ACAGACGCTCTACCGCGCCGA
CTX-MIV (CTX-M9)	F: CGCTGGTTCTGGTGACCTATTT	R: CCGCTGAAGCCAGCACAT	P: ACCCAGCCGCAACAGAACGCA
CTX-MV (CTX-M5)	F: ATCTGACGTTGGGCAATGC	R: GGTAGCCCTGCCTGAATGC	P: TGGCTGAAAGGCAACACCACCG

For additional confirmation of ESBL presence, CLSI standards M2-A10 and M100-S19 (CLSI, 2009b,c) recommends a combination disk test using a comparison of the zones generated by CTX-30 to 30/10 μg of cefotaxime/clavulanate and CAZ-30 to 30/10 μg of ceftazidime/clavulanate. ESBL production is inferred if the zones produced by clavulanate are ≥ 5 mm larger than those without clavulanate.

Detection of plasmid-encoded AmpC β-lactamase production in ETEC isolates by combination disk test using boronic acid

Boronic acid disk test was performed using the standard disk diffusion method (CLSI, 2009a). In short, suspensions of select ETEC isolates (0.5 McFarland unit) were inoculated onto two separate Mueller-Hinton (Becton Dickinson) agar plates. Onto the first plate, disks were placed containing CAZ-30, 30 μg of ceftazidime/10 μg of clavulanic acid (CAZ/CLA), 30 μg of ceftazidime/400 μg of 3-aminophenylboronic acid hydrochloride (CAZ and APB), and 30 μg of ceftazidime/10 μg clavulanic acid/400 μg of 3-aminophenylboronic acid hydrochloride (CAZ/CLA and APB). Onto a second plate, disks were placed containing CTX-30, 30 μg of cefotaxime/10 μg of clavulanic acid (CTX/CLA), 30 μg of cefotaxime/400 μg of 3-aminophenylboronic acid hydrochloride (CTX and APB), and 30 μg of cefotaxime/10 μg of clavulanic acid/400 μg of 3-aminophenylboronic acid hydrochloride (CTX/CLA and APB). Comparisons of combinations of CAZ and APB with CAZ-30 or CTX and APB with CTX-30 were performed. An increase of ≥ 5 mm in zone diameter for either antimicrobial agent tested in combination with boronic acid against boronic acid alone is interpreted as class C β-lactamase-positive. Similarly, comparisons of combinations of CAZ/CLA and APB with CAZ/CLA or CTX/CLA and APB with CTX/CLA were performed. An increase of ≥ 5 mm in zone diameter for either antimicrobial agent tested in combination with both CLA and boronic acid against tested antimicrobial agent in combination with CLA is interpreted as both ESBL and class C β-lactamase-positive.

Minimal inhibitory concentration (MIC) assay to confirm ESBL-producing ETEC strains using broth microdilution method

MICs of CAZ and CTX alone and in combination with CLA were determined for ETEC strains according to CLSI standard (CLSI, 2009a,b,c). Stock antimicrobial solutions were prepared according to the manufacturer's instructions and serially diluted 2-fold to the following concentrations: CAZ (0.008-128 mg/l) and CTX (0.015-8,192 mg/l). A 100 μl aliquot of diluted antimicrobial solutions was dispensed into each well of pre-prepared microdilution trays. Addition of 10 μl aliquot of 5 x 10^5 CFU/ml of each tested ETEC strain was used to inoculate each test well. Following mixing of the test drugs with CLA, fixed concentrations (4 mg/l) were prepared and dispensed into microdilution trays followed by addition of 10 μl aliquot of 5 x 10^5 CFU/ml of each test ETEC strain. A 3-fold reduction in drug MIC in the presence of CLA is interpreted as positive for ESBL production.

Quantitative (q)-PCR assay

Q-PCR assays were developed to detect β-lactamase genes. Primers and TaqMan probes for each gene were designed using Primer Express software version 2 (Applied Biosystems, Foster City, CA) utilizing well characterized β-lactamase GenBank nucleotide sequences (Table 2). TaqMan probes 5′-ends were labeled with reporter 6-carboxyfluoscein (FAM) or VIC® dye and 3′-ends with quencher 6- carboxytetramethyl-

Table 3
Screening of ESBL-producing ETEC strains employing standard disk diffusion tests.

Country of origin	Sample ID	Toxin type	ESBL-screening from inhibition zone results (ATM[a]≤ 27 mm, or CPD ≤ 17 mm, or CAZ ≤ 22 mm, or CTX ≤ 27 mm, or CRO ≤ 25 mm)	Phenotypic confirmatory test by double disk synergy test (positive = expanded zone of test antimicrobial agents)
Thailand	CH04-0493	LT[b]	Pos[c]	Pos[d]
Nepal	BH07-0209	LT	Pos	Pos
Nepal	SH08-0511	LT, STI[b]	Pos	Pos
Nepal	KH08-0783	LT	Pos	Pos
Nepal	BH08-0929	STI[b]	Pos	Neg[e]
Nepal	BH08-1025	LT	Pos	Pos

[a]ATM, aztreonam; CPD, cefpodoxime; CAZ, ceftazidime; CTX, cefotaxime.
[b]LT, heat-labile toxin; STI, heat-stable toxin I.
[c]Pos, positive (suspected ESBL production); [d]Pos, positive ESBL production; [e]Neg, negative ESBL production.

rhodamine dye. Q-PCRs were performed in a 25-µl final volume containing 1X TaqMan buffer, 100 µM dNTPs, 2.0 mM $MgCl_2$, 200 nM forward and reverse primers, 40 nM probe, and 0.025 U *Taq* polymerase (Thermo Fisher, Waltham, MA). All reactions were performed on a Sequence Detector ABI Prism 7900 (Applied Biosystems) in duplicate using the following cycling conditions: 95°C for 5 minutes; followed by 40 cycles of 94°C for 15 seconds and 60°C for 1 minute.

Transconjugation of ESBL assay

Conjugation between the six identified individual ESBL-producing ETEC isolates, either CAZ- or CTX-resistant, and a recipient DH5α *E. coli* strain (F^-, lac^-, Nal^R) was performed to determine if the ESBL resistance gene was transferable by a plasmid. Cultures of the donor and recipient *E. coli* strains were incubated in 5 ml of Luria-Bertani (LB) broth (tryptone and yeast extract from Becton, Dickinson, Sparkers, MD, and sodium chloride from Sigma-Aldrich, St Louis, MO) for 16 hours at 37°C with shaking in a ratio of 1:1 followed by plating onto tryptic soy agar (TSA) (Becton, Dickinson) plates for 6 hours at 37°C. Bacterial colonies were resuspended in 2 ml of MH broth and 0.1 ml aliquot was inoculated onto TSA plates supplemented with 15 mg/l nalidixic acid and either 20 mg/l CTX or 8 mg/l CAZ. Transconjugant colonies were selected at random and screened for transferred ESBL resistance genes and MIC values as described above.

RESULTS

Phenotypic detection of class A ESBLs and class C β-lactamases

A total of six β-lactamase-producing ETEC isolates were detected during the initial antibiotic resistance screen for an overall prevalence of 1.6%, comprising of 5/78 (6%) ETEC strains from Nepal and 1/213 (0.5%) ETEC strains from Thailand (Table 3). No ESBL-producing ETEC strains were detected in the 86 remaining isolates collected from Camboida, Kenya,

Uzbekistan, or Vietnam. The putative ESBL-positive ETEC strains were confirmed phenotypically for ESBL production using DDST and combination disk assays. Of the tested strains, 5/6 were phenotypically confirmed for ESBL production through these methods.

Presence of class C β-lactamases was assayed using a combination disk test with boronic acid (Table 4). Only one strain, Nepal BH08-0929, was positive for production of class C β-lactamases. Nepal BH08-0929 was also the only strain that tested negative in the DDST and combination disk assays, indicating that the mechanism of β-lactam resistance is mediated through a class C enzyme instead of class A ESBL as with the other five strains.

Antimicrobial susceptibility of ESBL-producing ETEC isolates

All five class A ESBL-producing ETEC isolates showed resistance to CTX with MICs in the range of 256-1,024 mg/l and were effectively inhibited by CLA (Table 5). One ESBL-producing ETEC strain, SH08-0511, demonstrated intermediate resistance (16 mg/l) to CAZ, while all other strains were resistant. A single ESBL-producing ETEC strain, CH04-0493, was sensitive to CAZ (MIC = 2 mg/l), but was resistant to CTX (MIC = 256 mg/l). However, inhibition by CLA when combined with either CAZ or CTX was still observed. The ETEC strain harboring plasmid-encoded class C β-lactamase, BH08-0929, had intermediate resistance against both CAZ and CTX (MIC = 16 and 8 mg/l, respectively) (Table 5). Addition

Table 4
Detection of plasmid-encoded class C β-lactamases production by combination disk test with boronic acid.

Country of origin	Sample ID	Toxin type	Inhibition zone diameter difference in combination with boronic acid (< 5 or ≥ 5 mm)					Interpretation	
			CAZ/CLA[a] *vs* CAZ	CAZ/Bor[b] *vs* CAZ	CAZ/CLA /Bor[c] *vs* CAZ/CLA	CTX/CLA[d] *vs* CTX	CTX/Bor[e] *vs* CTX	CTX/CLA/Bor[f] *vs* CTX/CLA	Class A ESBL production	Class C β-lactamase production
Thailand	CH04-0493	LT[g]	≥ 5	< 5	< 5	≥ 5	< 5	< 5	Pos	Neg
Nepal	BH07-0209	LT	≥ 5	< 5	< 5	≥ 5	< 5	< 5	Pos	Neg
Nepal	SH08-0511	LT, STI	≥ 5	< 5	< 5	≥ 5	< 5	< 5	Pos	Neg
Nepal	KH08-0783	LT	≥ 5	< 5	< 5	≥ 5	< 5	< 5	Pos	Neg
Nepal	BH08-0929	STI	< 5	≥ 5	< 5	< 5	≥ 5	< 5	Neg	Pos
Nepal	BH08-1025	LT	≥ 5	< 5	< 5	≥ 5	< 5	< 5	Pos	Neg

Neg, negative result; Pos, positive result; *vs*, versus. [a]CAZ/CLA, ceftazidime-clavulanic acid; [b]CAZ/Bor, ceftazidime-boronic acid; [c]CAZ/CLA/Bor, ceftazidime-clavulanic acid-boronic acid; [d]CTX/CLA, cefotaxime-clavulanic acid; [e]CTX/Bor, cefotaxime-boronic acid; [f]CTX/CLA/Bor, cefotaxime-clavulanic acid-boronic acid; [g]LT: heat-labile toxin; STI, heat-stable toxin I.

Table 5
Minimum inhibitory concentrations (MICs) of ESBL-producing ETEC against ceftazidime (CAZ) and cefotaxime (CTX) with and without β-lactamase inhibitors.

Sample ID	Toxin type	MIC (mg/l)			
		CAZ	CAZ+CLA	CTX	CTX+CLA
CH04-0493	LT[a]	2	0.25	256	0.125
BH07-0209	LT	32	0.25	1,024	0.125
SH08-0511	LT, STI	16	0.25	256	0.06
KH08-0783	LT	64	0.5	512	0.125
BH08-0929	STI	16	8	8	8
BH08-1025	LT	32	0.25	512	0.06

[a]LT, Heat-labile toxin; STI, Heat-stable toxin I.

Table 6
β-Lactamase genes in ETEC clinical and transconjugant isolates.

ETEC isolate	Detection by PCR	Transconjugant (trc) isolate	Detection by PCR
CH04-0493	*ampC*, $bla_{CTX\text{-}M\text{-}IV}$, bla_{TEM}	Trc isolate 2	*ampC*, $bla_{CTX\text{-}M\text{-}IV}$, bla_{TEM}
BH07-0209	*ampC*, $bla_{CTX\text{-}M\text{-}I}$	Trc isolate 4	*ampC*, $bla_{CTX\text{-}M\text{-}I}$
SH08-0511	*ampC*, $bla_{CTX\text{-}M\text{-}I}$	Trc isolate 1	*ampC*, $bla_{CTX\text{-}M\text{-}I}$
KH08-0783	*ampC*, $bla_{CTX\text{-}M\text{-}I}$, bla_{TEM}	Trc isolate 1	*ampC*, $bla_{CTX\text{-}M\text{-}I}$, bla_{TEM}
BH08-0929	*ampC*, $bla_{CMY\text{-}2}$	Trc isolate 5	*ampC*, $bla_{CMY\text{-}2}$
BH08-1025	*ampC*, $bla_{CTX\text{-}M\text{-}I}$, bla_{TEM}	Trc isolate 1	*ampC*, $bla_{CTX\text{-}M\text{-}I}$, bla_{TEM}

of CLA resulted in no change of MIC value when combined with CAZ or CTX and the level of resistance remained at the intermediate level.

ESBL and AmpC-β-lactamase genotypes

The six ESBL identified ETEC strains were examined for genes encoding ESBL β-lactamase production through q-PCR identification. All six ETEC strains carried class C β-lactamase gene, *ampC* (Table 6). Notably, there is no evidence of functional AmpC enzyme production in any of the phenotypic assays, a finding that has been noted in other studies (Jacoby, 2009; Zeng and Lin, 2013). Four ETEC strains carried a class A ESBL gene encoding CTX-M-I and one strain harbored the gene encoding CTX-M-IV. TEM gene was also identified in two strains encoding CTX-M-I gene and in the single strain carrying CTX-M-IV gene, showing that multiple ESBL genes were present in a single isolate. BH08-0929, phenotypically positive for a class C enzyme, contained CMY-2 gene. All strains carried at least two β-lactamase producing genes, including *ampC*, but there is no phenotypic evidence that multiple enzymes were expressed and active.

Conjugative transfer of ESBLs and AmpC-β-lactamases

ESBLs, both class A and class C, from

Table 7
Minimum inhibitory concentrations of transconjugants, donors and recipients against selected β-lactam antibiotics.

Sample ID	Organism	Recipient	Minimal inhibitory concentration (mg/l)							
			Donor/ recipient CAZ[b]	Trans-conjugant CAZ	Donor/ recipient CAZ/CLA[c]	Trans-conjugant CAZ/CLA	Donor/ recipient CTX[d]	Trans-conjugant CTX	Donor/ recipient CTX/CLA	Trans-conjugant CTX/CLA
CH04-0493	LT[a]	Top 10	4	8	0.25/4	0.5/4	256	512	≤ 0.03/4	0.12/4
BH07-0209	LT	Top 10	32	64	0.5/4	1/4	1,024	512	≤ 0.03/4	0.06/4
KH08-0783	LT	DH5α	128	32	1/4	0.5/4	512	512	0.06/4	≤ 0.03/4
BH08-0929	STI	Top 10	16	64	16/4	32/4	16	8	4/4	8/4
BH08-1025	LT	Top 10	64	128	0.5/4	2/4	512	1,024	0.03/4	0.06/4
		DH5α	0.12		0.12/4		0.03		0.008/4	
		Top 10	0.5		0.5/4		0.06		0.12/4	

[a]LT, heat-labile toxin; STI: heat-stable toxin I.
[b]CAZ, ceftazidime; [c]CLA, clavulanic acid; [d]CTX, cefotaxime.

all identified ETEC strains were capable of being conjugatively transferred to the DH5α *E. coli* strain (Table 7). All transconjugant β-lactamase genes from each ETEC donor strain showed 100% sequence homology to donor strains. The MIC of the transconjugants to CAZ was 8-128 mg/l, representing a 6- to 11- fold increase relative to that of the recipient parent strains, *E. coli* DH5α (0.12 mg/l) and Top10 (0.5 mg/l) (Table 7). Similarly, the MIC of the transconjugants toward cefotaxime was 8-1,024 mg/l, representing a 8- and 15-fold increase relative to the recipient parent strain, *E. coli*; DH5α (0.03 mg/l) and Top10 (0.06 mg/l), respectively.

DISCUSSION

Of the 377 ETEC isolates investigated in this study, 6 strains were identified as producing class A ESBLs or class C β-lactamases. The prevalence of ESBL-producing ETEC strains found in the current study was relatively low when compared to previously reported studies that identified in Asia 5-24% of *E. coli* isolates as ESBL-positive (Paterson and Bonomo, 2005). This is most likely due to the study site locations and the community-acquired nature of the isolates compared to hospital-acquired or hospital-associated infections (Horcajada *et al*, 2013; Kassakian and Mermel, 2014). Among the ESBL (AmpC, CMY-2, CTX-M, SHV, and TEM) genes examined, AmpC was the most frequently detected resistance gene, followed by CTX-M, TEM and the plasmid-encoded class C β-lactamase. CTX-M-I group was observed only in those strains isolated in Nepal, and CTX-M-IV was detected

from a single diarrhea patient in Thailand.

In this study, three unique ETEC strains from two different geographic locations, namely, KH08-0783 and BH08-1025 from Nepal and CH04-0493 from Thailand, were found harboring TEM β-lactamases. In addition to carrying a TEM β-lactamase, these three ETEC strains also harbored a CTX-M-β-lactamase that was detected in two other strains. Predominant types of CTX-M vary in different geographic locales: CTX-M ESBL genes are believed to be the predominant type in Asia and responsible for outbreaks in several countries including China (Chanawong *et al*, 2002; Xiong *et al*, 2002; Wang *et al*, 2003), India (Karim *et al*, 2001; Poirel *et al*, 2002), Japan (Komatsu *et al*, 2001; Ma *et al*, 2002), Korea (Pai *et al*, 2001), and Taiwan (Yu *et al*, 2002).

In Thailand, detection of CTX-M-type was first documented in a study of 48 Enterobacteriaceae isolates recovered during 1998 to 1999 from the same hospital where prevalence of 52% of a single CTX-M-type were found to carry β-lactamase gene encoding CTX-M-IV type ESBL (Chanawong *et al*, 2007). Among 52 isolates collected in 2003, prevalence of CTX-M-type increased to 65% since 1998 and comprised of three groups, namely, 44% CTX-M-15, 11% CTX-M-14 and 10% other ESBLs in CTX-M-IV cluster (Chanawong *et al*, 2007). Kiratisin *et al* (2008) demonstrated that CTX-M-type ESBL is highly endemic with 99% of ESBL-producing *E. coli* strains isolated during 2004 to 2005 carrying the β-lactamase gene encoding CTX-M-type. In this study, a single ETEC strain from Thailand, CH04-0493, harbored a CTX-M-IV β-lactamase gene, in addition to those of AmpC and TEM β-lactamases. All other CTX-M-positive strains carried CTX-M-I gene.

Plasmid-encoded class C or transferable class C β-lactamases are found worldwide in many gram-negative bacterial species (Jacoby, 2009; Zeng and Lin, 2013). In the current study, a single ETEC strain from Nepal, BH08-0929, harbored a plasmid-encoded class C β-lactamase CMY-2 in addition to AmpC β-lactamase gene. β-Lactamase CMY-2 is the most prevalent of the plasmid-encoded class C β-lactamases, with the widest geographical distribution, being reported in Algeria, France, Germany, Greece, India, Pakistan, Spain, Taiwan, UK, and USA (Bauernfeind *et al*, 1998; Navarro *et al*, 2001; Philippon *et al*, 2002). There is no evidence in our study that *ampC* was over-expressed to produce an active AmpC-β-lactamase. It has been observed by other groups that *E. coli* does not over-express *ampC* in response to β-lactamase like other bacterial species (Jacoby, 2009; Zeng and Lin, 2013). This makes *ampC* unlikely to play a role in the observed resistance.

Genotypic results of the current study demonstrated that 3/6 ETEC isolates harbored at least 3 ESBL genes. An increasing number of studies have shown more than one ESBL within the same strain (Paterson and Bonomo, 2005). For example, a study from China demonstrated that some ESBL-producing *E. coli* and most ESBL-producing *K. pneumoniae* strains produce more than one type of β-lactamase (Xiong *et al*, 2002). A study from Thailand revealed almost 90% of ESBL-producing *E. coli* strains carry multiple β-lactamase genes with the combination of CTX-M and TEM-type ESBLs being the most prevalent (Kiratisin *et al*, 2008). In this study, ETEC strain CH04-0493 isolated in 2004 from Thailand harbored at least three β-lactamase genes (encoding AmpC, CTX-M and TEM), confirming that isolates carrying multiple ESBL genes are com-

monly detected in Asia.

It has been reported that β-lactamase genes coding for multiple β-lactamases may be found on different plasmids; however, increasing evidence suggests that multiple β-lactamase genes may actually be present on the same plasmid (Philippon *et al*, 2002). In the current study, transmissibility of ESBL-encoding genes was demonstrated via conjugation experiments as all β-lactamase genes present in each ETEC donor were completely transferred to the recipient strain. This observation is consistent with reports of CTX-M genes being present on plasmids that confer multi-drug resistance (Rossolini *et al*, 2008; Karczmarczyk *et al*, 2011). Additionally, the presence of transferred ESBL genes in transconjugants suggests that β-lactamase genes may spread horizontally. Therefore, these data suggest ESBL encoding plasmids may facilitate spread of β-lactam antibiotic resistance among *E. coli* as the current data are consistent with previous reports, indicating plasmids as a factor in spreading β-lactam antibiotic resistance from ETEC to other *E. coli* strains.

In conclusion, this study demonstrates the emerging presence of extended spectrum β-lactamases in enterotoxigenic *E. coli* isolated from diarrhea patients during 2001 to 2010 from Nepal and Thailand, and reveals the important role that horizontal transfer may have played in the early spread of β-lactam antibiotic resistance from ETEC to other enteric pathogens. Continued retrospective and current surveillance along with detailed studies on the identification of resistance mechanisms are necessary to combat the spread of pathogenic isolates as well as to provide essential information for proper treatment regiments.

ACKNOWLEDGEMENTS

The authors thank Dr Trent J Peacock for his critical review and organization for completion of the manuscript, Ms Pattrapan Jullasing for kind assistance in manuscript preparation, and staff in Molecular Laboratory Sciences Section, Department of Enteric Diseases, AFRIMS for identification of ETEC using G_{MI}-ELISA. The work was supported by AFHSC-GEIS.

REFERENCES

Adachi JA, Ericsson CD, Jiang ZD, Dupont MW, Pallegar SR, Dupont HL. Natural history of enteroaggregative and enterotoxigenic *Escherichia coli* infection among US travelers to Guadalajara Mexico. *J Infect Dis* 2002 185: 1681-3.

Ambler RP. The structure of beta-lactamases. *Philos Trans R Soc Lond B Biol Sci* 1980; 289: 321-31.

Batchelor M, Threlfall EJ, Liebana E. Cephalosporin resistance among animal-associated *Enterobacteria*: a current perspective. *Expert Rev Anti Infect Ther* 2005; 3: 403-17.

Bauernfeind A, Chong Y, Lee K. Plasmid-encoded AmpC beta-lactamases: how far have we gone 10 years after the discovery? *Yonsei Med J* 1998; 39: 520-5.

Bradford PA. Extended-spectrum beta-lactamases in the 21st century: characterization epidemiology and detection of this important resistance threat. *Clin Microbiol Rev* 2001; 14: 933-51.

Canton R, Coque TM. The CTX-M beta-lactamase pandemic *Curr Opin Microbiol* 2006; 9: 466-75.

Chanawong A, Lulitanond A, Kaewkes W, Lulitanond V, Srigulbutr S, Homchampa P. CTX-M extended-spectrum beta-lactamases among clinical isolates of Enterobacteriaceae in a Thai university hospital. *Southeast Asian J Trop Med Public Health* 2007; 38: 493-500.

Chanawong A, M'zali FH, Heritage J, Xiong JH, Hawkey PM. Three cefotaximases CTX-M-9 CTX-M-13 and CTX-M-14 among *Enterobacteriaceae* in the People's Republic of China. *Antimicrob Agents Chemother* 2002; 46: 630-7.

Clinical and Laboratory Standards Institute (CLSI). Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria That Grow Aerobically; Approved Standard M07-A8. Wayne: CLSI, 2009a.

Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial disk susceptibility tests; Approved Standard- Tenth Edition M2-A10. Wayne: CLSI, 2009b.

Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; Nineteeth Informational Supplement M100-S19. Wayne: CLSI, 2009c.

Gazouli M, Tzouvelekis LS, Vatopoulos AC, Tzelepi E. Transferable class C beta-lactamases in *Escherichia coli* strains isolated in Greek hospitals and characterization of two enzyme variants (LAT-3 and LAT-4) closely related to *Citrobacter freundii* AmpC beta-lactamase. *J Antimicrob Chemother* 1998; 42: 419-25.

Hernandez JR, Pascual A, Canton R, Martinez-Martinez L. Grupo De Estudio De Infeccion Hospitalaria G. Extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in Spanish hospitals (GEIH-BLEE Project 2002). *Enferm Infecc Microbiol Clin* 2003; 21: 77-82.

Ho PL, Chow KH, Yuen KY, Ng WS, Chau PY. Comparison of a novel inhibitor-potentiated disc-diffusion test with other methods for the detection of extended-spectrum beta-lactamases in *Escherichia coli* and *Klebsiella pneumoniae. J Antimicrob Chemother* 1998; 42: 49-54.

Horcajada JP, Shaw E, Padilla B, *et al.* Healthcare-associated community-acquired and hospital-acquired bacteraemic urinary tract infections in hospitalized patients: a prospective multicentre cohort study in the era of antimicrobial resistance. *Clin Microbiol Infect* 2013; 19: 962-8.

Horii T, Arakawa Y, Ohta M, *et al.* Characterization of a plasmid-borne and constitutively expressed blaMOX-1 gene encoding AmpC-type beta-lactamase. *Gene* 1994; 139: 93-8.

Hornish RE, Kotarski SF. Cephalosporins in veterinary medicine - ceftiofur use in food animals. *Curr Top Med Chem* 2002; 2: 717-31.

Jacoby GA. Beta-lactamase nomenclature. *Antimicrob Agents Chemother* 2006; 50: 1123-9.

Jacoby GA. AmpC β-Lactamases. *Clin Microbiol Rev* 2009; 22: 161-82.

Jacoby GA, Han P. Detection of extended-spectrum beta-lactamases in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli. J Clin Microbiol* 1996; 34: 908-11.

Jarlier V, Nicolas MH, Fournier G, Philippon A. Extended broad-spectrum beta-lactamases conferring transferable resistance to newer beta-lactam agents in *Enterobacteriaceae*: hospital prevalence and susceptibility patterns. *Rev Infect Dis* 1998; 10: 867-78.

Karczmarczyk M, Abbott Y, Walsh C, Leonard N, Fanning S. Characterization of multidrug-resistant *Escherichia coli* isolates from animals presenting at a university veterinary hospital. *Appl Environ Microbiol* 2011; 77: 7104-12.

Karim A, Poirel L, Nagarajan S. Nordmann P. Plasmid-mediated extended-spectrum beta-lactamase (CTX-M-3 like) from India and gene association with insertion sequence ISEcp1. *FEMS Microbiol Lett* 2001; 201: 237-41.

Kassakian SZ, Mermel LA. Changing epidemiology of infections due to extended spectrum beta-lactamase producing bacteria. *Antimicrobial Resist Infect Control* 2014 Mar 25; 3: 9.

Kiratisin P, Apisarnthanarak A, Laesripa C, Saifon P. Molecular characterization and epidemiology of extended-spectrum-beta-lactamase-producing *Escherichia coli*

and *Klebsiella pneumoniae* isolates causing health care-associated infection in Thailand where the CTX-M family is endemic. *Antimicrob Agents Chemother* 2008; 52: 2818-24.

Komatsu M, Ikeda N, Aihara M, *et al*. Hospital outbreak of MEN-1-derived extended spectrum beta-lactamase-producing *Klebsiella pneumoniae. J Infect Chemother* 2001; 7: 94-101.

Ma L, Ishii Y, Chang FY, Yamaguchi K, Ho M, Siu LK. CTX-M-14 a plasmid-mediated CTX-M type extended-spectrum beta-lactamase isolated from *Escherichia coli. Antimicrob Agents Chemother* 2002; 46: 1985-8.

Mirelis B, Navarro F, Miro E, Mesa RJ, Coll P, Prats G. Community transmission of extended-spectrum beta-lactamase. *Emerg Infect Dis* 2003; 9: 1024-5.

Munday CJ, Whitehead GM, Todd NJ, Campbell M, Hawkey PM. Predominance and genetic diversity of community- and hospital-acquired CTX-M extended-spectrum beta-lactamases in York UK. *J Antimicrob Chemother* 2004; 54: 628-33.

Navarro F, Perez-Trallero E, Marimon JM, Aliaga R, Gomariz M, Mirelis B. CMY-2-producing *Salmonella enterica, Klebsiella pneumoniae, Klebsiella oxytoca, Proteus mirabilis* and *Escherichia coli* strains isolated in Spain (October 1999-December 2000). *J Antimicrob Chemother* 2001; 48: 383-9.

Nordmann P. Trends in beta-lactam resistance among Enterobacteriaceae. *Clin Infect Dis* 1998; 27 (suppl 1): S100-6.

Oteo J, Perez-Vazquez M, Campos J. Extended-spectrum beta-lactamase producing *Escherichia coli:* changing epidemiology and clinical impact. *Curr Opin Infect Dis* 2010; 23: 320-6.

Pai H, Choi EH, Lee HJ, Hong JY, Jacoby GA. Identification of CTX-M-14 extended-spectrum beta-lactamase in clinical isolates of *Shigella sonnei, Escherichia coli* and *Klebsiella pneumoniae* in Korea. *J Clin Microbiol* 2001; 39: 3747-9.

Pai H, Lyu S, Lee JH, *et al*. Survey of extended-spectrum beta-lactamases in clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*: prevalence of TEM-52 in Korea. *J Clin Microbiol* 1999; 37: 1758-63.

Pandey P, Bodhidatta L, Lewis M, *et al.*Travelers' diarrhea in Nepal: an update on the pathogens and antibiotic resistance. *J Travel Med* 2011; 18: 102-8.

Paterson DL, Bonomo RA. Extended-spectrum beta-lactamases: a clinical update. *Clin Microbiol Rev* 2005; 18: 657-86.

Philippon A, Arlet G, Jacoby GA. Plasmid-determined AmpC-type beta-lactamases *Antimicrob Agents Chemother* 2002; 46: 1-11.

Pitout JD, Laupland KB. Extended-spectrum beta-lactamase-producing Enterobacteriaceae: an emerging public-health concern *Lancet Infect Dis* 2008; 8: 159-66.

Pitout JD, Nordmann P, Laupland KB, Poirel L. Emergence of Enterobacteriaceae producing extended-spectrum beta-lactamases (ESBLs) in the community. *J Antimicrob Chemother* 2005; 56: 52-9.

Poirel L, Gniadkowski M, Nordmann P. Biochemical analysis of the ceftazidime-hydrolysing extended-spectrum beta-lactamase CTX-M-15 and of its structurally related beta-lactamase CTX-M-3. *J Antimicrob Chemother* 2002; 50: 1031-4.

Qadri F, Svennerholm A-M, Faruque ASG, Sack RB. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment and prevention. *Clin Microbiol Rev* 2005; 18: 465-83.

Rodriguez-Bano J, Navarro MD. Extended-spectrum beta-lactamases in ambulatory care: a clinical perspective. *Clin Microbiol Infect* 2008; 14 (suppl 1): 104-10.

Rossolini GM, D'andrea MM, Mugnaioli C. The spread of CTX-M-type extended-spectrum beta-lactamases. *Clin Microbiol Infect* 2008; 14 (suppl 1): 33-41.

Shaikh S, Fatima J, Shakil S, Rizvi SMD, Kamal MA. Antibiotic resistance and extended spectrum beta-lactamases: types, epide-

miology and treatment. *Saudi J Biol Sci* 2015: 90-101.

Sjöling Å, Wiklund G, Savarino SJ, Cohen DI, Svennerholm AM. Comparative analyses of phenotypic and genotypic methods for detection of enterotoxigenic *Escherichia coli* toxins and colonization factors. *J Clin Microbiol* 2007; 45: 3295-301.

Spanu T, Sanguinetti M, Tumbarello M, *et al*. Evaluation of the new VITEK 2 extended-spectrum beta-lactamase (ESBL) test for rapid detection of ESBL production in *Enterobacteriaceae* isolates. *J Clin Microbiol* 2006; 44: 3257-62.

Sturenburg E, Mack D. Extended-spectrum beta-lactamases: implications for the clinical microbiology laboratory therapy and infection control. *J Infect* 2003; 47: 273-95.

Valverde A, Coque TM, Sanchez-Moreno MP, Rollan A, Baquero F, Canton R. Dramatic increase in prevalence of fecal carriage of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* during nonoutbreak situations in Spain. *J Clin Microbiol* 2004; 42: 4769-75.

Wang H, Kelkar S, Wu W, Chen M, Quinn JP. Clinical isolates of *Enterobacteriaceae* producing extended-spectrum beta-lactamases: prevalence of CTX-M-3 at a hospital in China. *Antimicrob Agents Chemother* 2003; 47: 790-3.

Xiong Z, Zhu D, Wang F, Zhang Y, Okamoto R, Inoue M. Investigation of extended-spectrum beta-lactamase in *Klebsiella pneumoniae* and *Escherichia coli* from China. *Diagn Microbiol Infect Dis* 2002; 44: 195-200.

Yu WL, Pfaller MA, Winokur PL, Jones RN. Cefepime MIC as a predictor of the extended-spectrum beta-lactamase type in *Klebsiella pneumoniae*, Taiwan. *Emerg Infect Dis* 2002; 8: 522-4.

Zeng X, Lin J. Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria. *Front Microbiol* 2013; 4: 128.