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ORIGINAL RESEARCH

Phenotypic and genotypic characterizations of extended-spectrum beta-lactamase-producing *Escherichia coli* in Thailand

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Purpose: Extended-spectrum β-lactamases (ESBLs) have become an issue in community worldwide due to an increase in antibiotic resistance over the past decade. This study was aimed to investigate the phenotypic and genotypic characteristics of ESBL-producing *Escherichia coli* in Thailand.

Materials and methods: In this study, all clinical isolates collected from tertiary hospitals in Thailand were identified as *E. coli* by biochemical tests and MALDI-TOF mass spectrometry. ESBL-producing *E. coli* was preliminary screened with disk diffusion method by cephalosporin disks and confirmed by the method of combination disk diffusion. Antimicrobial susceptibility test was used to determine MIC values of all ESBL-producing *E. coli*. For genotypic detection, a variety of ESBL genes were determined by PCR. Moreover, multilocus sequence typing (MLST) analysis was performed on internal portions of seven housekeeping genes for the diversity and phylogenetic relatedness of *E. coli* clonal group.

Results: Of the 285 ESBL-producing *E. coli*, most were susceptible to carbapenems. These strains showed a high resistance rate to ciprofloxacin (85.26%). The most frequently detected gene was $bla_{\text{CTX-M1}}$ group at about 71.23% followed by $bla_{\text{CTX-M9}}$ group (38.95%). The bla_{TEM}, bla_{PER}, bla_{GES}, bla_{VEB}, and bla_{SHV} genes were identified in 31.93%, 5.96%, 4.56%, 3.51%, and 0.70% of ESBL-producing isolates, respectively. The $bla_{\text{OXA-10}}$ gene was detected in only one strain. ESBL-producing *E. coli* isolates with high antimicrobial resistance were further investigated. Among those, *E. coli* sequence type ST38 was mostly found, followed by ST405, ST410, and ST131. It is noteworthy that the $bla_{\text{CTX-M}}$ gene was mainly detected in all four ST-type *E. coli* clones (ST38, ST405, ST410, and ST131).

Conclusion: This study provided a recent evidence of the genetic diversity of ESBL-producing *E. coli* in Thailand. In addition, the profile related to antimicrobial resistance pattern in this region was also demonstrated.

Keywords: epidemiology, prevalence, antimicrobial resistance, MLST, ESBLs, antibiotic resistance genes

Introduction

The rapid emergence of antibiotic resistance threatens effective prevention and treatment of an increasing range of infections. Some bacteria are naturally resistant to certain antibiotics and others can acquire resistance through mutations in some of their genes when they are exposed to an antibiotic. This acquired resistance can spread to other bacterial species. The main mechanism of antibiotic resistance mostly found is enzyme production such as β-lactamase enzymes. The β-lactamase enzymes produced by some bacteria provide resistance to β-lactam antibiotics by hydrolyzing β-lactam rings.[1]

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Among antimicrobial resistant bacteria, *Escherichia coli* is one of highly concerned bacteria in the family Enterobacteriaceae. *E. coli* is a common cause of urinary tract infection and intra-abdominal infection in humans and is the second most common Gram-negative bacteria causing community-acquired bloodstream infection, accounting for 7.3% of all bloodstream infection isolates.[2] ESBL-producing *E. coli* isolates have become an importance in community-onset infections, as well as nosocomial infections. The prevalence of resistance to fluoroquinolones and extended-spectrum cephalosporins in *E. coli* had highly increased over the past decade rendering severely limited therapeutic options for these infections.[3]

Extended-spectrum β-lactamases (ESBLs) are extremely broad spectrum β-lactamase enzymes, which can be produced by Gram-negative bacteria. They are mainly found in a family of Enterobacteriaceae. ESBLs are produced by the mutation of the TEM-1, TEM-2, and SHV-1 β-lactamases, which have been discovered since 1980–1990 and first detected in Western Europe.[4] To date, more than 350 different natural ESBL variants are known, which have been classified into nine distinct structural and evolutionary families based on amino acid sequence comparisons such as TEM, SHV, CTX-M, PER, VEB, GES, BES, TLA, and OXA.[5–7] The main types of ESBL variants include TEM, SHV, CTX-M, and OXA. Interestingly, bla_{CTX-M} has rapidly increased and is now widely found in clinically isolated *E. coli* across the world.[8] ESBLs, especially of the CTX-M type, are strongly associated with specific clonal *E. coli* strains.[9] To date, little is known about the epidemiology of ESBL variants in Thailand. Moreover, it is critical to provide up-to-date resistance pattern which affect the treatment decision in this region. Therefore, this study was aimed to investigate the phenotypic characteristic and variation of genetically related ESBL-producing *E. coli* in Thailand.

Material and methods

Bacterial isolates

ESBL-producing *E. coli* isolates were collected from tertiary care hospitals located in various regions in Thailand from 2014 to 2015. Tertiary care hospitals were defined over 500-bed hospitals that usually provided a full complement of services. These hospitals were regional hospitals which were generally referral for patients with serious conditions. A total of 285 *E. coli* isolates were obtained from clinical specimens, including urine, sputum, pus, blood, and feces, which were a part of routine hospital procedures. Confirmation and identification of *E. coli* strains were performed by biochemical tests and MALDI-TOF mass spectrometry. This study was approved by Mahidol University Institutional Review Board (MU-IRB) [Approval No. MU-IRB 2014/019.0705].

Determination of ESBL-producing *E. coli* isolates

ESBL-producing *E. coli* was screened with disk diffusion method by cephalosporin disks as recommended by the CLSI guideline.[10] To confirm ESBL production in *E. coli*, the method of combination disk diffusion technique was performed. Briefly, disks of ceftazidime (30 μg), ceftazidime/clavulanate (30/10 μg), cefotaxime (30 μg), and cefotaxime/clavulanate (30/10 μg) were placed on the Mueller–Hinton agar plate (Difco) with 30 mm distance from the center of each and were incubated at 37°C for 18 hours. The test result is considered as positive if the inhibition zone diameter is 5 mm or larger with clavulanate than without. The strain of *Klebsiella pneumoniae* ATCC700603 (carrying bla_{SHV-18} gene) was used for the positive control and *E. coli* ATCC25922 was used as the negative control in this study.

Antimicrobial susceptibility testing

The determination of MIC values was performed by broth microdilution method with nine antimicrobial agents, namely, ciprofloxacin, prulifloxacin, ceftazidime, fosfomycin, imipenem, meropenem, doripenem, biapenem, and piperacillin/tazobactam. *E. coli* isolates were grown in Mueller Hinton broth (MH broth), then *E. coli* isolates were diluted with MH broth to 0.5 McFarland (cell approximately 10^8 CFU/mL), and diluted with normal saline to adjust the cells to 10^6 CFU/mL before adding into 96-well plates containing antibiotics in triplicates. The working stock concentrations of antibiotics were based on the CLSI guideline.[10] Finally, the plate was kept at 37°C for 18 hours. The results were evaluated by the MIC values from the minimum concentration of the drugs that gave no visible growth.

Characterization of ESBL genotypes

The standard PCR was performed to screen for the presence of ESBL genes: bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{OXA}, bla_{GES}, bla_{VEB}, and bla_{PER} using specific primers described in Table 1. PCR reactions contained 1× buffer, 1.5 mM of $MgCl_2$, 400 μM of dNTPs, 0.5 μM of forward and reverse primers each, 1 U *Taq* polymerase, and the concentration of DNA template depending on specific primers. For PCR cycling condition, the denaturation step was achieved at 96°C for 30 seconds, followed by the annealing step, the temperature of which depended upon the specific primers, and the final step of

Table 1 Specific primers for ESBL genes detection

Genes	Primer names	Primer sequences (5′–3″)	Product size (bp)	Temperatures of annealing step (°C)	References
bla_{TEM}	TEM-F	ATG AGT ATT CAA CAT TTC CGT	861	56	Ryoo et al 2005[12]
	TEM-R	TTA CCA ATG CTT AAT CAG TGA			
bla_{SHV}	SHV-F	CGC CTG TGT ATT ATC TCC CTG	849	64	In this study
	SHV-R	TTA GCG TTG CCA GTG CTC GAT			
bla_{CTX-M}	CTX-M 1 group-F	AGT TCA CGC TGA TGG CGA CG	839	64	In this study
	CTX-M 1 group-R	GAC GAT TTT AGC CGC CGA CG			
	CTX-M 9 group-F	GCG TGC ATT CCG CTG CTG C	832	65	In this study
	CTX-M 9 group-R	ACA GCC CTT CGG CGA TGA TTC			
bla_{OXA}	OXA2 group-F	ATG GCA ATC CGA ATC TTC GC	760	60	In this study
	OXA2 group-R	GCA CGA TTG CCT CCC TCT T			
	OXA10 group-F	ATG AAA ACA TTT GCC GCA TAT G	801	60	In this study
	OXA10 group-R	TTA GCC ACC AAT GAT GCC CT			
bla_{GES}	GES-F	TAC TGG CAG SGA TCG CTC AC	838	62	In this study
	GES-R	TTG TCC GTG CTC AGG ATG AG			
bla_{VEB}	VEB-F	GCC AGA ATA GGA GTA GCA AT	703	58	In this study
	VEB-R	TGG ACT CTG CAA CAA ATA CG			
bla_{PER}	PER-F	CTC AGC GCA ATC CCC ACT GT	851	62	In this study
	PER-R	TTG GGC TTA GGG CAG AAA GCT			

Abbreviation: ESBL, extended-spectrum β-lactamase.

extension at 72°C for 30 seconds. All steps were repetitively performed for 30 cycles. Then, the PCR products were analyzed by agarose gel electrophoresis.

Molecular typing by MLST

Forty-eight strains of ESBL-producing *E. coli* were selected and determined sequence types by MLST. The criteria for *E. coli* strain selection were based on ESBL gene pattern. PCR method was used to amplify internal portions of seven housekeeping genes of *E. coli* (*adk, fumC, gyrB, icd, mdh, purA,* and *recA)* with specific primers. Seven-locus MLST was performed using published criteria and primers.[11] Amplification products were submitted to the commercial sequencing service (Macrogen Sequencing, Korea). DNA sequences of each genes were identified using the website: https://pubmlst.org/bigsdb?db=pubmlst_mlst_seqdef&page=sequenceQuery. The allelic profile for each isolate was determined using the BioNumerics MLST Plug-in in accordance with the Achtman scheme available at http://enterobase.warwick.ac.uk/species/ecoli/allele_st_search.

Results

Phenotypic detection of ESBL-producing *E. coli*

All 285 *E. coli* collected from tertiary care hospitals located in various regions in Thailand were determined by combination disk diffusion. Among these positive ESBL-producing *E. coli*, 55 isolates were from the northeast region, 61 isolates from the north region, 45 isolates from the east region, 60 from the central region, and 64 isolates from the south region of Thailand. Antibiotic resistance patterns of isolated ESBL-producing *E. coli* were further examined according to the CLSI guideline. The results demonstrated that all of ESBL-producing *E. coli* were sensitive to carbapenems with 100% susceptibility to meropenem and doripenem followed by biapenem (99.65%) and imipenem (98.86%). High susceptibility was also observed with piperacillin and tazobactam (86.32%). Nevertheless, most of them showed a high resistance rate to ciprofloxacin at 85.26% (243/285 isolates), ceftazidime at 80% (228/285 isolates), prulifloxacin at 79.30% (226/285 isolates), and fosfomycin at 10.88% (31/285 isolates) (Figure 1). It was of interest that MIC_{90} of meropenem and doripenem were about ≤0.125 μg/mL followed by those of biapenem (MIC_{90}=0.25 μg/mL) and doripenem (MIC_{90}=0.5 μg/mL), respectively (Table 2).

Molecular detection of ESBL gene variants

The presence of ESBL genes including bla_{TEM}, bla_{SHV}, bla_{CTX-M} (CTX-M1 and CTX-M9 group), bla_{OXA} (OXA-2 and OXA-10 group), bla_{GES}, bla_{VEB}, and bla_{PER} genes in all clinical isolates were investigated by PCR method (Figure 2). The results showed that bla_{CTX-M1} group genes were predominantly presented in Thailand at about 71.23% (203/285 isolates) followed by 38.95% (111/285 isolates) bla_{CTX-M9} group, 31.93% (91/285 isolates) bla_{TEM}, 5.96% (17/285 isolates) bla_{PER},

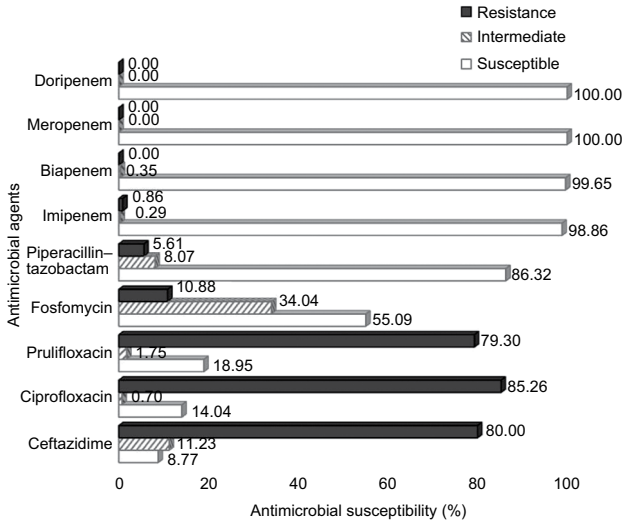

Figure 1 Antimicrobial susceptibility test of antimicrobial agents against ESBL-producing *E. coli* performed by broth dilution method.
Abbreviation: ESBL, extended-spectrum β-lactamase.

Table 2 Antimicrobial susceptibility of nine antimicrobial agents against ESBL-producing *E. coli*

Antimicrobial agents	MIC range (μg/ml)	MIC_{50} (μg/ml)	MIC_{90} (μg/ml)
Imipenem	0–8	≤0.125	0.5
Meropenem	0–0.5	≤0.125	≤0.125
Doripenem	0–0.5	≤0.125	≤0.125
Biapenem	0–0.5	≤0.125	0.25
Piperacillin/tazobactam	2–128/4	4	32
Fosfomycin	4–256	64	256
Prulifloxacin	0.125–8	8	>8
Ciprofloxacin	0.125–8	>8	>8
Ceftazidime	0.5–32	32	>32

Abbreviation: ESBL, extended-spectrum β-lactamase.

4.56% (13/285 isolates) bla_{GES}, 3.51% (10/285 isolates) bla_{VEB}, 0.70% (2/285 isolates) bla_{SHV}, and the least common 0.35% (1/285 isolate) bla_{OXA}. It was noteworthy that the bla_{OXA} gene was not detected in the chromosome but found on the plasmid. Interestingly, 52.63% (150/285 strains) of ESBL-producing *E. coli* carried only one ESBL gene. Among these, 33.33% (95/285 strains) of these contained two ESBL genes and 12.63% (36/285 strains) carried three ESBL genes. In addition, the coexistence of all three genes mostly was $bla_{CTX\text{-}M1}$ group, $bla_{CTX\text{-}M9}$ group, and bla_{TEM}.

Forty-seven strains of ESBL-producing *E. coli* with high antimicrobial resistance were selected to determine 23 STs using the Achtman MLST scheme. The majority of ST-type high antimicrobial resistant strains was ST38 (10/47 isolates), followed by ST405 (6/47 isolates), ST410 (5/47 isolates), and ST131 (4/47 isolates) (Table 3). Among *E. coli* ST38 isolates,

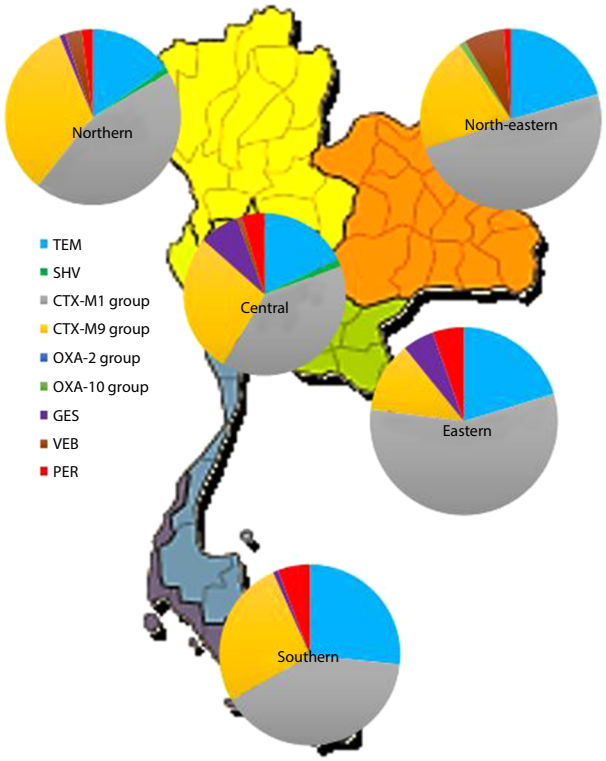

Figure 2 Epidemiology of genotypic variants of ESBL-producing *E. coli* in all regions of Thailand.
Abbreviation: ESBL, extended-spectrum β-lactamase.

eight strains carried bla_{PER} genes, four strains contained bla_{GES} genes, and two strains possessed bla_{TEM} genes. Interestingly, all of these strains carried $bla_{CTX\text{-}M}$ genes and were sensitive to carbapenems, prulifloxacin, and ciprofloxacin. However, 8 out of 10 *E. coli* ST38 isolates were resistant to ceftazidime and two strains were intermediate sensitive. Most *E. coli* ST38 strains were sensitive to piperacillin/tazobactam. All of *E. coli* ST405 contained $bla_{CTX\text{-}M}$ genes, while bla_{VEB} could be detected in only two strains and only one strain contained bla_{TEM} gene. *E. coli* ST405 were sensitive to carbapenems and fosfomycin but were resistant to ceftazidime, prulifloxacin, and ciprofloxacin. All *E. coli* ST410 carried $bla_{CTX\text{-}M}$ and two of them carried bla_{TEM}. Interestingly, only one strain possessed bla_{OXA} and bla_{VEB} genes. This ST type was sensitive to carbapenems and fosfomycin but was resistant to prulifloxacin, ciprofloxacin, ceftazidime, and piperacillin/tazobactam. For *E. coli* ST131, all of them contained $bla_{CTX\text{-}M}$ genes, one strain carried bla_{TEM}, and one strain carried bla_{VEB}. All of these were sensitive to meropenem, doripenem, and fosfomycin. They were resistant to ciprofloxacin, prulifloxacin, and ceftazidime. Two strains of *E. coli* ST131 were resistant to imipenem and one strain showed intermediate sensitivity to biapenem.

Table 3 STs of ESBL-producing *E. coli*

Strains	ESBL genes								STs
	bla_{TEM}	bla_{SHV}	$bla_{CTX\text{-}M\ group\ 1}$	$bla_{CTX\text{-}M\ group\ 9}$	bla_{OXA}	bla_{GES}	bla_{VEB}	bla_{PER}	
MTC33011	−	−	+	−	−	−	−	−	131
MTC33019	+	−	+	−	−	+	−	−	3,171
MTC33025	−	−	−	+	−	+	−	+	38
MTC33031	+	−	−	+	−	−	−	+	3,028
MTC33035	+	−	+	−	−	−	−	−	131
MTC33036	+	−	+	+	−	−	−	−	5,520
MTC33057	−	−	+	+	−	−	+	−	405
MTC33058	−	−	+	−	−	−	+	−	457
MTC33075	−	−	+	−	−	−	−	+	2003
MTC33077	−	+	+	+	−	−	−	−	12
MTC33082	−	−	+	−	−	−	−	−	131
MTC33090	+	−	+	−	−	+	−	−	7,096
MTC33129	−	−	+	−	−	−	−	−	410
MTC33137	−	−	−	−	−	−	+	+	127
MTC33140	+	−	+	−	−	−	+	−	212
MTC33144	−	−	+	−	−	−	+	−	131
MTC33145	+	−	−	+	−	−	−	−	405
MTC33148	+	−	+	−	−	−	+	−	648
MTC33149	−	−	+	−	−	−	+	−	405
MTC33152	−	−	+	−	+	−	+	−	410
MTC33169	−	−	+	+	−	−	−	−	405
MTC33178	−	−	+	−	−	−	−	−	410
MTC33180	−	−	+	−	−	−	−	+	2003
MTC33185	−	−	+	+	−	−	−	+	38
MTC33191	+	−	+	−	−	+	−	−	38
MTC33192	−	−	+	+	−	−	−	−	38
MTC33193	−	−	−	+	−	+	−	−	1,543
MTC33194	+	−	−	−	−	+	+	−	5,026
MTC33197	−	+	−	−	−	+	−	−	2,473
MTC33198	−	−	+	−	−	+	−	+	2,659
MTC33201	−	−	+	−	−	+	−	−	155
MTC33215	+	−	−	+	−	−	−	+	38
MTC33218	+	−	−	+	−	−	−	−	617
MTC33247	+	−	+	−	−	+	−	−	7,228
MTC33252	+	−	+	−	−	+	−	−	624
MTC33261	+	−	+	+	−	−	−	−	405
MTC33266	−	−	+	+	−	−	−	−	405
MTC33270	−	−	−	+	−	+	−	+	38
MTC33274	−	−	+	−	−	−	−	+	38
MTC33275	−	−	+	−	−	−	−	+	38
MTC33277	−	−	+	−	−	+	−	+	38
MTC33281	+	−	+	−	−	−	−	−	410
MTC33302	−	−	+	−	−	−	−	+	2003
MTC33326	−	−	−	+	−	−	−	+	38
MTC33337	+	−	+	−	−	−	−	+	1,193
MTC33353	+	−	+	−	−	−	−	−	410
MTC33354	+	−	+	+	−	−	−	−	648

Abbreviations: ESBL, extended-spectrum β-lactamase; STs, Sequence Types.

Discussion

ESBLs are bacterial enzymes that mediate resistance to the third-generation cephalosporins and monobactams. The spreading and outbreaks of ESBLs are often found in the family of Enterobacteriaceae especially *E. coli*.[13] Our findings were based on the evaluation of *E. coli* strains isolated from clinical specimens obtained from tertiary care hospitals in Thailand. This study clearly showed high resistance rates of ESBL-producing *E. coli* to ciprofloxacin (85.26%), ceftazidime (80%), and prulifloxacin (79.30%), which raised serious concern and became a challenge for clinicians. In 2013, researchers reported high resistance to cefotaxime, ceftriaxone, and ceftazidime, and only some isolates were resistant to ciprofloxacin while most strains were susceptible to carbapenems in Thailand.[14] In Korea, the study of Kang et al determined antimicrobial susceptibility of ESBL-producing *E. coli* against imipenem and meropenem and the resistance rates were only 1.5% (1/68) and 0% (0/50), respectively.[15] The previous study in Thailand showed that CTX-M family had the highest prevalence of ESBL-related *bla* genes (87.3%) similar to our findings in which CTX-M type was still predominant.[12] This study reported the presence of 31.93% bla_{TEM} genes, while in 2011, bla_{TEM} genes were found at 42% and the previous study in 2007 demonstrated 67% bla_{TEM} genes. This indicated that the spreading of bla_{TEM} ESBL-producing *E. coli* strains were decreased.[16,17] In addition, only few bla_{SHV} carrying strains were detected by the study of Kiratisin et al in 2007 and our study found only two strains of bla_{SHV} ESBL-producing strains.[16] In India, the prevalence of bla_{TEM} genes was reported at about 48.7% (38/78 strains), followed by bla_{CTX-M} at 7.6% (6/78 strains) and bla_{SHV} at 5.1% (4/78 strains) which were different from our findings in Thailand.[7,18] This study revealed that the majority of ST type with high antimicrobial resistant rate was ST38 which showed high sensitivity to piperacillin/tazobactam. According to the MLST results described by previous study, ST38 *E. coli* isolates were identified in China (CTX-M-14 producer),[19] Japan (CTX-M-9 and CTX-M-14 producers),[20] and France (OXA-48 producer).[21] Additionally, Rodríguez et al investigated MLST in bla_{CTX-M} ESBL-producing *E. coli* from Germany, Netherlands, and UK. The study showed high prevalence of ST38.[22] Moreover, Alghoribi et al found CTX-M-positive ST38, ST131, and ST405 in Saudi Arabia.[23]

Conclusion

This study demonstrated that the CTX-M type remained the most common spreading in Thailand. Although carbapenems remain drugs of choice to treat ESBL-producing bacterial infections, we reported that ESBL-producing *E. coli* have increased the severity of resistance to antibiotics. It was clearly shown that ESBL outbreaks have been a problem worldwide and we hope to raise the awareness on proper antibiotics use to control spreading of these strains in both hospitals and community.

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Disclosure

The authors report no conflicts of interest in this work.

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