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

This article was published in the following Dove Press journal: Infection and Drug Resistance

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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 blaCTX-M group at about 71.23% followed by blaCTX-APP group (38.95%). The blaTEM, blaPER, blaGES, blaVEA, and blaSHV genes were identified in 31.93%, 5.96%, 4.56%, 3.51%, and 0.70% of ESBL-producing isolates, respectively. The blaOXA-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 blaCTX-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.
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. 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.

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. 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. The main types of ESBL variants include TEM, SHV, CTX-M, and OXA. Interestingly, the *bla*<sub>CTX-M</sub> has rapidly increased and is now widely found in clinically isolated *E. coli* across the world. ESBLs, especially of the CTX-M type, are strongly associated with specific clonal *E. coli* strains. 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. 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*<sub>SHV-15</sub> 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<sup>6</sup> CFU/mL), and diluted with normal saline to adjust the cells to 10<sup>6</sup> CFU/mL before adding into 96-well plates containing antibiotics in triplicates. The working stock concentrations of antibiotics were based on the CLSI guideline. 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*<sub>TEM</sub>, *bla*<sub>SHV</sub>, *bla*<sub>CTX-M</sub>, *bla*<sub>OXA</sub>, *bla*<sub>GES</sub>, *bla*<sub>PER</sub>, and *bla*<sub>BES</sub> using specific primers described in Table 1. PCR reactions contained 1× buffer, 1.5 mM of MgCl<sub>2</sub>, 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
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. 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 $\leq$ 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*$_{CTX-M}$ group genes were predominantly presented in Thailand at about 71.23% (203/285 isolates) followed by 38.95% (111/285 isolates) *bla*$_{CTX-M8}$ group, 31.93% (91/285 isolates) *bla*$_{TEM}$, 5.96% (17/285 isolates) *bla*$_{PER}$.

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**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 |                   | 64                                  |                                   |
| *bla*$_{SHV}$| SHV-F        | CGC CTG TGT ATT ATC TCC CGT | 849               | 64                                  | In this study                     |
|              | SHV-R        | TTA CGG TTG CCA GTG CTC GAT |                   | 65                                  | In this study                     |
| *bla*$_{CTX-M}$| CTX-M 1 group-F | AGT TCA CGG TGA TGG CGA CG | 839               | 64                                  | In this study                     |
|              | CTX-M 1 group-R | GAC GAT TTT AGC CCG CCA CG |                   | 65                                  | In this study                     |
|              | 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 CCG CGA TGA TTC |                   | 65                                  | In this study                     |
| *bla*$_{OXA}$| OXA2 group-F | ATG GCA ATC CGA ATC TCC GC | 760               | 60                                  |                                   |
|              | OXA2 group-R | GCA CGA TTT CCT CCC TCT T |                   | 60                                  | In this study                     |
|              | 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 |                   | 62                                  | In this study                     |
| *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 |                   | 62                                  | In this study                     |
| *bla*$_{VEB}$| VEB-F        | GCC AGA ATA GGA GTA GCA AT | 703               | 58                                  | In this study                     |
|              | VEB-R        | TGG ACT CTG CAA AAA ATG CG |                   | 58                                  | In this study                     |
| *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 |                   | 62                                  | In this study                     |

Abbreviation: ESBL, extended-spectrum β-lactamase.
4.56% (13/285 isolates) bla\textsubscript{GES}, 3.51% (10/285 isolates) bla\textsubscript{SHV}, 0.70% (2/285 isolates) bla\textsubscript{PER}, and the least common 0.35% (1/285 isolate) bla\textsubscript{OXA}. It was noteworthy that the bla\textsubscript{OXA} gene was not detected in the chromosome but found on the plasmid. Interestingly, 52.63% (150/285 strains) of these strains carried bla\textsubscript{TEM} genes, and two strains possessed bla\textsubscript{PER} genes. This ST type was sensitive to carbapenems, fosfomycin, 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\textsubscript{CTX-M} genes, while bla\textsubscript{PER} could be detected in only two strains and only one strain contained bla\textsubscript{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\textsubscript{CTX-M} and two of them carried bla\textsubscript{TEM}. Interestingly, only one strain possessed bla\textsubscript{OXA} and bla\textsubscript{PER} genes. This ST type was sensitive to carbapenems and fosfomycin but was resistant to cipfofaxcin, ciprofloxacin, and ceftazidime. For E. coli ST131, all of them contained bla\textsubscript{CTX-M} genes, one strain carried bla\textsubscript{TEM}, and one strain carried bla\textsubscript{PER}. All of these were sensitive to meropenem, doripenem, and fosfomycin. They were resistant to ciprofloxacin, prulifoxacin, 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  |
|--------------|------------|------|
|              | \(\text{bla}_{\text{TEM}}\) | \(\text{bla}_{\text{SHV}}\) | \(\text{bla}_{\text{CTX-M }\text{group I}}\) | \(\text{bla}_{\text{CTX-M }\text{group }\#}\) | \(\text{bla}_{\text{OXA}}\) | \(\text{bla}_{\text{GES}}\) | \(\text{bla}_{\text{VEB}}\) | \(\text{bla}_{\text{PER}}\) |
| MTC33011     | –          | –    | +    | –    | –          | –          | –          | –          | –          | 131        |
| MTC33019     | +          | –    | +    | –    | –          | –          | –          | +          | –          | 3,171      |
| MTC33025     | –          | –    | –    | +    | –          | +          | +          | +          | 38         |
| MTC33031     | +          | –    | –    | +    | –          | –          | –          | –          | 3028       |
| 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.11 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 ceftotaxime, 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% blaTEM genes, while in 2011, blaTEM genes were found at 42% and the previous study in 2007 demonstrated 67% blaTEM genes. This indicated that the spreading of blaTEM ESBL-producing E. coli strains were decreased.16,17 In addition, only few blaSHV carrying strains were detected by the study of Kiratisin et al in 2007 and our study found only two strains of blaSHV ESBL-producing strains.16 In India, the prevalence of blaTEM genes was reported at about 48.7% (38/78 strains), followed by blaCTX-M at 7.6% (6/78 strains) and blaSHV at 5.1% (4/78 strains) which were different from our findings in Thailand.7,11 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, Rodriguez et al investigated MLST in blaCTX-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.21

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.

Acknowledgments

The authors wish to thank all staffs in the Department of Microbiology for technical support and suggestion on this work. The authors also thank all staffs at the hospital sites for their help and collaboration.

Disclosure

The authors report no conflicts of interest in this work.

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