# SHV-12 EXTENDED SPECTRUM β-LACTAMASE ASSOCIATED WITH HIGH-LEVEL CEFTAZIDIME RESISTANCE IN *ENTEROBACTER CLOACAE* ISOLATED FROM THAILAND

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**Abstract.** Four *Enterobacter cloacae* clinical isolates with reduced susceptibility to ceftazidime from two hospitals in Thailand were studied. Production of extended-spectrum β-lactamase was confirmed by double disk synergy test and combination disk method. All isolates were highly resistant to ceftazidime but retained susceptibility to imipenem. One isolate was able to hydrolyze cefotaxime, ceftazidime and cefepime, the latter being one of the treatment choices for infection by *Enterobacter* spp. PCR analysis demonstrated the presence of $bla_{SHV-12}$ in addition to $bla_{TEM-1}$ in all isolates suggesting that SHV-12 was associated with high-level resistance to ceftazidime in the *E. cloacae* isolates.

## INTRODUCTION

Extended spectrum β-lactamases (ESBLs) are an increasing cause of resistance to third generation cephalosporins and aztreonam and are present most commonly in *Klebsiella pneumoniae* and *Escherichia coli*. ESBL genes are usually carried by plasmids, some of which are located within transposable elements, thereby facilitating their spread between bacteria (Paterson and Bonomo, 2005). Previous reports revealed that patients infected with ESBL-producing bacteria have a higher mortality rate than with non-ESBL producers (Chayakulkeeree *et al*, 2005; Henshke-Bar-Meir *et al*, 2006). Most ESBLs are mutants of classical plasmid-mediated TEM and SHV β-lactamases. There are also new families of ESBLs, including the CTX-M type enzymes (Paterson and Bonomo, 2005). In recent years, ESBLs have also become more prevalent among species with an inducible AmpC type β-lactamases such as *Enterobacter* spp, which is a leading cause of nosocomial infections (Sanders and Sanders, 1997). *Enterobacte*r spp has been associated with several outbreaks generally involving mutants overproducing their chromosomal β-lactamase or, more infrequently, expressing ESBL.

Resistance to broad spectrum β-lactam antimicrobial agents in *Enterobacter* spp is a significant problem in Thailand. From a study in 1999 resistance to ceftazidime and ceftriaxone in *Enterobacter* spp is 42.3% and 28.2%, respectively (Biedenbach *et al*, 1999). Study in a teaching hospital in southern Thailand revealed that ESBL production in *E. cloacae* is 15.4% (Jitsurong and Yodsuwat, 2006). To

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date, there is relatively little information on ESBL-producing *E. cloacae* although it has been suggested that *Enterobacter* spp may represent the main reservoir of ESBL-producing enteric isolates in Thailand (Girlich *et al*, 2001). Thus this study was undertaken to investigate resistant determinants of the ceftazidime-resistant *E. cloacae* isolated from Thailand.

## MATERIALS AND METHODS

### Bacterial strains and assay of ESBL production

Four different clinical isolates with reduced susceptibility to ceftazidime were studied. Isolates were collected during the period of July 2004-January 2005 from Hospital A (northeast of Thailand) and Hospital B (Bangkok). Species identification was performed by standard biochemical tests. *E. coli* ATCC 25922 (susceptible isolate) and *K. pneumoniae* ATCC 700603 (ESBL-producing isolate) were used as control isolates. All bacterial isolates were supplied by National Antimicrobial Resistance Surveillance of Thailand.

Examination of ESBL phenotypes were performed by double disk synergy test (DDST) and combination disk method. For DDST, an amoxicillin-clavulanate disk was placed in the center of a plate and ceftazidime, cefotaxime, ceftriaxone and aztreonam disks were placed 25 mm (center to center) from amoxicillin-clavulanate disk. Extension of the zone of inhibition towards the disk containing clavulanate indicates the presence of an ESBL (Jarlier *et al*, 1988). Combination disk method employed the use of disks containing cefotaxime and cefotaxime-clavulanate. ESBL production was noted if the zone diameter given by a disk with clavulanate is ≥ 5 mm than that without inhibitor (CLSI, 2008).

### Susceptibility testing and β-lactamase activity assay

Minimum inhibitory concentrations (MICs) of ceftazidime, cefotaxime, ceftriaxone and aztreonam were determined by a standard agar dilution method according to CLSI guidelines (CLSI, 2008). Determination of imipenem susceptibility was performed by E-test method as described in the manufacturer's instructions (AB Biodisk, Solna, Sweden).

β-Lactamase activity was determined spectrophotometrically by monitoring the hydrolysis of β-lactam (Waley, 1974). β-Lactam substrates used were cefotaxime, ceftazidime and cefepime. For inhibition study, clavulanic acid was added to the cell extract at a final concentration of 4 μg/ml and the solution was left at room temperature for 10 minutes before β-lactamase activity was determined. Protein concentration was measured using *DC*™ Protein assay kit (Bio-Rad Laboratories, Hercules, CA). Specific activity is expressed as μmole of ß-lactam hydrolyzed per minute per mg of protein.

### Amplification and sequence analysis of ESBL-encoding genes

Detection of genes coding for TEM-, SHV- and CTX-M- related ESBLs was performed by polymerase chain reaction (PCR). Plasmid DNA was isolated from ESBL producers using alkaline lysis method and used as template (Sambrook and Russell, 2001). The primers for detection of ESBL genes were designed to amplify the entire region of the structural genes as previously described (Coque *et al*, 2002; Ma *et al*, 2002) and were purchased from Invitrogen (Carlsbad, CA). PCR was performed in 50 μl volume mixture containing 100 ng of template, 0.25 μM of each oligonucleotide primer, 200 μM of dNTPs and 1 unit of Platinum *Taq* polymerase (Invitrogen, arlsbad, CA) together with its reaction buffer. The conditions comprised 1 cycle at 94ºC for 5 minutes, followed by 30 cycles at 94ºC for 30 seconds, 55ºC (42ºC for $bla_{TEM}$) for 45 seconds and 72ºC for 1 minute with a final step at 72ºC for

10 minutes. The amplicons were analyzed by 1% agarose gel-electrophoresis, purified using QIAquick PCR purification kit (Qiagen, Valencia, CA) and sequenced on both strands using an automated DNA sequencer (Applied Biosystem, Foster city, CA). The nucleotide and deduced amino acid sequences were analyzed with software available over the internet at the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

**Pulse field gel electrophoresis (PFGE)**

PFGE was performed as described by Xiong *et al* (2002). Chromosomal DNA was digested with *Xba*I (New England Biolabs, Beverly, MA) at 37ºC for 16 hours. DNA was electrophoresed in 1% Pulse Field Certified agarose using a CHEF Mapper® XA System (Bio-Rad Laboratories, Hercules, CA). The gel was stained for 30 minutes with 1 μg/ml of ethidium bromide and photographed under UV light (GelDoc 2000, Bio-Rad Laboratories, Hercules, CA).

## RESULTS

Four *E. cloacae* isolates with reduced susceptibility to ceftazidime were studied. The characteristics of *E. cloacae* isolates, including source and type of specimen are shown in Table 1. Three isolates were obtained from Hospital A and the remaining one isolate from Hospital B. All *E. cloacae* isolates were positive by DDST (results not shown). Furthermore, all isolates demonstrated reduced inhibition zone diameters for cefotaxime in combination with clavulanic acid versus that for cefotaxime when tested alone (Table 1).

Using CLSI interpretive criteria for Enterobacteriaceae, resistance to cefotaxime, ceftazidime, ceftriaxone and aztreonam was defined as MIC ≥ 64, 32, 64 and 32 μg/ml, respectively (CLSI, 2008). All isolates demonstrated resistance to one or more β-lactams tested (Table 1). High level resistance to

Table 1
Bacterial isolates, ESBL test, minimum inhibitory concentration (MIC) and presence of β-lactamase genes.

| Isolate | Source | Type of specimen | ESBL confirmatory test (inhibition zone, mm) | | MIC (μg/ml) | | | | | β-lactamase gene |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Ctx | Ctx+Clav | Ctx | Caz | Cro | Azt | Imp | |
| *E. coli* ATCC 25922 | | | 34 | 1 | <0.125 | <0.125 | <0.125 | <0.125 | <0.125 | - |
| *K. pneumoniae* ATCC 700603 | | | 26 | 5 | 2 | 32 | 4 | 32 | 0.25 | - |
| *E. cloacae* 19527 | Hospital A | Blood | 22 | 9 | 32 | 512 | 32 | >64 | 0.25 | TEM-1, SHV-12 |
| *E. cloacae* 19610 | Hospital A | Blood | 27 | 9 | <0.125 | 128 | 0.25 | <0.125 | 0.38 | TEM-1, SHV-12 |
| *E. cloacae* 19612 | Hospital A | Blood | 23 | 10 | 16 | 128 | 16 | >64 | 0.25 | TEM-1, SHV-12 |
| *E. cloacae* 21522 | Hospital B | Urine | 11 | 5 | 4 | 128 | <0.125 | 0.25 | 0.5 | TEM-1, SHV-12 |

Caz, ceftazidime; Ctx, cefotaxime; Cro, ceftriaxone; Azt, aztreonam; Imp, imipenem; Clav, clavulanic acid.

Table 2
β-lactamase activity of *E. cloacae*.

| Bacterial isolate | β-lactamase activity[a] | | |
|---|---|---|---|
| | Caz | Ctx | Fep |
| *E. coli* ATCC 25922 | 12.8 | ND | ND |
| *K. pneumoniae* ATCC 700603 | 9.7 | 37.6 | ND |
| *E. cloacae* 21522 | 48.1 | 2,953.5 | 567.3 |
| *E. cloacae* 21522 + 4 μg/ml clavulanic acid | ND | 35.5 | 40.8 |

[a]μmole substrate hydrolyzed $min^{-1}$ mg $protein^{-1}$.
Caz, ceftazidime; Ctx, cefotaxime; Fep, cefepime; ND, no detectable activity.

ceftazidime (MIC = 128-512 μg/ml) was observed in all isolates. Isolates 19527 and 19612 showed cross-resistance to aztreonam (MIC > 64 μg/ml) and demonstrated intermediate resistance to cefotaxime and ceftriaxone. All isolates were susceptible to imipenem.

To assess the ability of *E. cloacae* to hydrolyse β-lactams, we investigated the β-lactamase activities in these isolates. Three bloodstream infection isolates (19527, 19610 and 19612) from Hospital A showed no detectable activity against all β-lactams tested (results not shown). However, isolate 21522 from Hospital B was able to hydrolyze cefotaxime, ceftazidime and cefepime, which was inhibited by 4 μg/ml of clavulanic acid (Table 2).

PCR amplification and sequence analysis of ESBL encoding genes were performed. Amplification of $bla_{CTX-M}$ was negative. All isolates carried the ESBL gene $bla_{SHV-12}$ in addition to the non ESBL $bla_{TEM-1}$ (Table 1).

The relationship between the three *E. cloacae* isolates obtained from Hospital A was studied by PFGE. Isolates 19610 and 19612 demonstrated identical PFGE pattern, whereas isolate 19527 had a unique PFGE pattern (Fig 1).

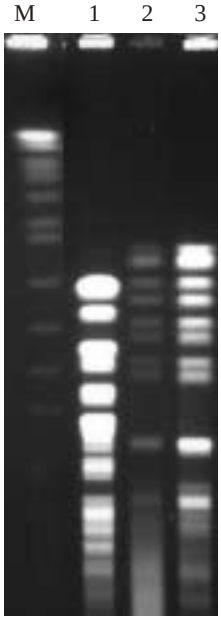


Fig 1–PFGE profile of three SHV-12-producing *E. cloacae* from Hospital A. For PFGE analysis, the chromosomal DNA was digested with *Xba*I and electrophoresed in 1% agarose. Lane M: *S. cerevisiae* chromosomal DNA (Bio-Rad Laboratories). Lanes 1-3: *E. cloacae* isolate 19527, 19610 and 19612, respectively.

## DISCUSSION

Resistance to expanded-spectrum cephalosporins in most *Enterobacter* species as well

as in other natural AmpC β-lactamase-producing members of the family Enterobacteriaceae is mainly produced by a constitutive overexpression of their chromosomal β-lactamase (Ehrhardt and Sanders, 1993). Plasmid-mediated ESBLs have also been described in these species and have been responsible for several outbreaks (Cantón *et al*, 2002; Manzur *et al*, 2007). Although less common than AmpC overexpression, ESBL production is also known to be another important mechanism for extended-spectrum cephalosporins resistance in *Enterobacter* spp (Sanders and Sanders, 1997). ESBL-producing *Enterobacter* spp, particularly *E. cloacae*, has been identified in many Asian countries including Thailand (Chanawong *et al*, 2001). In this study, four isolates of *E. cloacae* with reduced susceptibility to ceftazidime were positive by DDST and combination disk methods, suggesting the production of an ESBL by every isolate. All isolates demonstrated resistance to one or more high efficacy β-lactams. High resistance to ceftazidime was observed in all isolates. However, all isolates were susceptible to imipenem, which is the drug of choice for treatment of patients infected with ESBL-producing Enterobacteriaceae (Paterson, 2006). One *E. cloacae* isolate was detected that effectively hydrolyzed broad spectrum cephalosporins, such as cefotaxime, ceftazidime as well as cefepime. Hydrolysis of cefepime is of concern because cefepime is considered one of the treatment choices for infections by *Enterobacter* spp due to its activity against *Enterobacter* spp even against derepressed mutants (Palmer *et al*, 1995; Sanders *et al*, 1996). Although it is infrequently reported, studies have shown that some *Enterobacter* spp isolates are able to hydrolyze cefepime (Barnaud *et al*, 2004; Fernández-Cuenca *et al*, 2006). Addition of clavulanic acid inhibited the β-lactamase activity in this isolate, suggesting that class A β-lactamase (TEM or SHV type enzyme) may be responsible for this characteristic.

SHV-12 β-lactamase was first described in 1997 for *E. coli* and *K. pneumoniae* isolates of human clinical origin in Switzerland (Nüesch-Inderbinen *et al*, 1997). SHV-12 ESBL is also present in *Enterobacter* spp (Szabó *et al*, 2005; Wu *et al*, 2006; Yu *et al*, 2006). In Thailand, *E. cloacae* producing SHV-12 has been reported in Srinagarind Hospital, Khon Kaen (Chanawong *et al*, 2001). In the present study, we detected SHV-12 in four ceftazidime-resistant isolates, suggesting that SHV-12 was responsible for resistance to ceftazidime in the *E. cloacae* isolates tested.

The spread of ESBL-producing *K. pneumoniae* within the hospital in the Northeast of Thailand has been reported (Chanawong *et al*, 2001). We investigated the genetic relationship by PFGE among the three *E. cloacae* isolates obtained from Hospital A. Two SHV-12 producing isolates (19610 and 19612) were clonally related suggesting an intra-hospital spread. Isolate 19610 was resistant only to ceftazidime whereas isolate 19612 exhibited intermediate resistance or resistance to cefotaxime, ceftazidime, ceftriaxone and aztreonam. The reason for this is not known. It is possible that the level of resistance in different isolates harboring identical ESBL may vary depending on their hosts (Nüesch-inderbinen *et al*, 1997). Another explanation for the same genotype exhibiting different resistant patterns is that other mechanisms (outer membrane permeability and efflux pump) may be involved in resistance phenotype. In addition, we found that the isolate 19527 from the Hospital A showed different PFGE pattern. The plasmid profile of isolate 19527 was indistinguishable from that found in isolate 19610 and 19612 (results not shown), suggesting that the spread of ceftazidime resistance mediated by SHV-12 in *E. cloacae* may also result from a horizontal transfer of the gene.

In summary, this work shows that SHV-12 β-lactamase is associated with high-level ceftazidime resistance in *E. cloacae* isolates from two hospitals in Thailand. Possible intra-hospital dissemination of resistant isolates was observed. Despite the limited numbers of isolates tested, the presence of cefepime hydrolysis in *E. cloacae* is of concern.

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