

Prevalence of extended-spectrum β -lactamases in *Enterobacter cloacae* in Taiwan and comparison of 3 phenotypic confirmatory methods for detecting extended-spectrum β -lactamase production

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Background and purpose: The prevalence of extended-spectrum β -lactamases (ESBLs) in *Enterobacter cloacae* remains unclear in Taiwan. This study was conducted to investigate the prevalence of ESBL-producing *E. cloacae* in Taiwan.

Methods: 116 clinical isolates of *E. cloacae* were tested for cefepime susceptibility. Isolates with a minimal inhibitory concentration (MIC) of ≥ 0.25 $\mu\text{g/mL}$ for cefepime were tested for the ESBL phenotype by the combination-disk synergy tests (CDSTs) using cefotaxime and ceftazidime alone or in combination with clavulanic acid, the ESBL Etest using cefepime with or without clavulanic acid, and the double-disk synergy test (DDST) using cefepime and amoxicillin-clavulanate.

Results: Thirty eight isolates had an MIC of ≥ 0.25 $\mu\text{g/mL}$ for cefepime. Of these, 27 had an ESBL phenotype; 24 were determined by DDST, 25 by CDST, and 21 by ESBL Etest. ESBL genes were identified in 24 isolates. One isolate without an ESBL phenotype but with an MIC of ≥ 0.25 $\mu\text{g/mL}$ for cefepime was confirmed to have the ESBL gene. DDST, CDSTs, and ESBL Etest detected 24, 22, and 21 of 25 isolates with the ESBL genotype, respectively. DDST had the highest sensitivity of 96.0% for detecting ESBLs among isolates and a specificity of 69.2%. CDSTs and ESBL Etest had sensitivities of 88.0% and 84.0%, respectively, and specificities of 69.2% and 100%, respectively. The overall prevalence of ESBL-producing *E. cloacae* was 21.6%.

Conclusion: A combination of DDST using cefepime and amoxicillin-clavulanate and CDSTs using cefotaxime and ceftazidime alone or in combination with clavulanic acid enhance the detection of ESBL production in Taiwan.

Key words: beta-Lactamases; *Enterobacter cloacae*; Prevalence; Taiwan

Introduction

Acquisition and expression of the genes encoding extended-spectrum β -lactamases (ESBLs) have resulted in resistance to a broad range of extended-spectrum β -lactam antibiotics among *Enterobacteriaceae*, of which *Escherichia coli* and *Klebsiella pneumoniae* are the most frequently reported pathogens [1]. Among AmpC β -lactamase-producing organisms such as *Enterobacter* spp., *Serratia marcescens*, and *Citrobacter*

spp., ESBL producers are less common [2,3]. However, the prevalence has increased worldwide, and is high in some regions of South and East Asia, for example, China, Korea, The Philippines, Singapore, and Taiwan [1,4]. Although there has been a regional report of ESBL-producing *Enterobacter cloacae* [5], the exact prevalence in Taiwan remains unclear.

In addition, there is no recommendation from the Clinical and Laboratory Standards Institute (CLSI) for the phenotypic detection of ESBL among these AmpC β -lactamase-producing *Enterobacteriaceae*. One reason is that clavulanic acid may induce high-level expression of chromosomal AmpC enzyme and may then antagonize rather than protect the antibacterial

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activity of the partner β -lactam, masking the synergy required to detect ESBL production [6]. To overcome this problem, fourth-generation cephalosporins, which are stable to AmpC β -lactamases, have been used as the indicator drug in various phenotypic detection methods, including the double-disk synergy test (DDST) [7] and ESBL Etest [8]. The third method of combination-disk synergy tests (CDSTs), which uses cefotaxime or ceftazidime, either alone or combined with clavulanic acid, have been reported previously to be simple and reliable for ESBL detection among *E. cloacae* in Taiwan [5]. A screening criterion of a minimal inhibitory concentration (MIC) of cefepime of $>0.25 \mu\text{g/mL}$ was reported to have a sensitivity of 100% and a specificity of 74% for predicting the presence of ESBLs among *E. cloacae* in the Asia-Pacific region, with higher specificity than using the MIC of third-generation cephalosporins or aztreonam [4].

The aims of this study were to investigate the prevalence of ESBL-producing *E. cloacae* in Taiwan, to evaluate the performance of 3 different phenotypic methods for detection of ESBL production, and to determine the MICs of various antimicrobials for evaluation of their possible clinical application. The above-mentioned screening criterion was modified as cefepime MIC $\geq 0.25 \mu\text{g/mL}$ to screen the isolates.

Methods

Bacterial isolates

116 non-duplicate consecutive *E. cloacae* isolates collected from the Taiwan Surveillance of Antimicrobial Resistance (TSAR) IV program were used in this study. The hospitals in the TSAR IV program include medical centers and regional hospitals located throughout the 4 geographic regions (North, Central, South, and East) of Taiwan. The collection process and identification of each isolate have been described previously [9]. Of the 116 clinical isolates of *E. cloacae*, 24.1% were collected from specimens of respiratory tract secretions, 22.4% from wound discharge, wound pus, or pus, 20.7% from blood, 18.1% from urine, 4.3% from catheter tips, 3.4% from bile, 1.7% from abdominal fluids, 0.9% from cerebrospinal fluid, and 4.3% from other body sites.

Minimal inhibitory concentrations of antimicrobial agents

The MIC levels for cefepime were determined by the agar dilution method described by the CLSI [10]. In

brief, inocula of 10^4 colony-forming units (CFUs) of aerobic bacteria were inoculated onto Mueller-Hinton agar plates containing a series of 2-fold dilutions of tested antimicrobial agents with Steers' replicator. Following inoculation, the agar plates were incubated at 35°C in 5% carbon dioxide for 18 to 20 h; the MIC that was read as the lowest concentration of antimicrobial agents tested for all bacteria ranged from $0.03 \mu\text{g/mL}$ to $256 \mu\text{g/mL}$. As for the isolates with the ESBL phenotype, the MICs of ampicillin, amoxicillin-clavulanate, cefazolin, cefuroxime, cefmetazole, flomoxef, moxifloxacin, levofloxacin, ciprofloxacin, ertapenem, imipenem, meropenem, gentamicin, amikacin, and tigecycline were determined further by the agar dilution method.

Phenotypic methods for detection of extended-spectrum β -lactamase production

All of the 116 isolates were tested for ESBL production using the following 3 phenotypic confirmatory methods. The first method was CDSTs using cefotaxime and ceftazidime as the indicator drugs. The inhibitory zone diameters of disks containing $30 \mu\text{g}$ of cefotaxime or ceftazidime, either alone or in combination with $10 \mu\text{g}$ of clavulanate, were compared. An increase of $\geq 5 \text{ mm}$ in the zone diameter for at least 1 of the combination disks relative to its corresponding single antibiotic disk was considered as a positive result [10]. The second method was the ESBL Etest using Etest strip (AB Biodisk, Solna, Sweden) containing cefepime (MIC test range, $0.25\text{--}16 \text{ mg/L}$) and cefepime (MIC test range, $0.064\text{--}4 \text{ mg/L}$) plus 4 mg/L clavulanic acid. According to the manufacturer's instruction, a reduction of MIC of 3 or more 2-fold dilutions in the presence of clavulanic acid was indicative of ESBL production, as well as deformation of ellipses or the presence of a 'phantom' zone, even if the MIC ratio was <8 or could not be read [8]. The third method was DDST using a $30\text{-}\mu\text{g}$ disk of cefepime and a disk of amoxicillin-clavulanate ($20 \mu\text{g}$ to $10 \mu\text{g}$) positioned at a distance of 20 mm (center to center). The presence of keyhole phenomenon was regarded as positive [7]. *E. coli* American Type Culture Collection (ATCC) 25922 and *K. pneumoniae* ATCC 700603 were used as negative and positive controls, respectively, for ESBL production. The sensitivity and specificity of each phenotypic confirmatory method for detecting the ESBLs among isolates with a MIC of $\geq 0.25 \mu\text{g/mL}$ were evaluated based on carriage of ESBL genes as the gold standard for being an ESBL producer.

Detection of genes encoding for extended-spectrum β -lactamases

All isolates with an ESBL phenotype, as well as those without an ESBL phenotype but with an MIC of ≥ 0.25 $\mu\text{g/mL}$ for cefepime, were further analyzed for the presence of ESBL genes. The ESBL genes representative of the SHV and TEM families were detected by polymerase chain reaction (PCR) with fragment length polymorphism analysis [11], and the genes encoding CTX-M-type ESBLs were detected by multiplex PCR [12].

Results

Detection of extended-spectrum β -lactamase production by 3 phenotypic methods

Of 116 *E. cloacae* isolates, 38 had an MIC of ≥ 0.25 $\mu\text{g/mL}$ for cefepime. Twenty seven of the 38 isolates had an ESBL phenotype, and the test results of 3 phenotypic confirmatory methods are listed in Table 1. DDST using cefepime and amoxicillin-clavulanate, CDSTs using cefotaxime and ceftazidime, either alone or in combination with clavulanic acid, and cefepime ESBL Etest revealed 24, 25, and 21 isolates with an ESBL phenotype, respectively. Discrepant results among the 3 phenotypic methods were noted in 6 isolates (Table 2). One isolate (isolate 11) had a positive CDST result and a borderline DDST result, while 2 other isolates (isolates 85 and 92) had positive CDST but negative DDST results. Two isolates (isolates 59 and 72) had positive DDST results and were negative for the other 2 methods. Three isolates (isolates 59, 63, and 72) had negative CDST results, probably due to the inducing effect of clavulanic acid for the AmpC enzyme, because a decrease in inhibitory zone diameter of cefotaxime-clavulanic acid disks compared with cefotaxime disks alone was observed. Meanwhile, the inhibitory zone diameter of the ceftazidime plus clavulanic acid disks in these isolates did not decrease concordantly but remained stationary (isolate 59) or even increased slightly (isolate 72), suggesting that the ESBL of these isolates belonged to the SHV family [13]. Three isolates that had positive CDST results but negative DDST results (isolates 11, 85, and 92) did not have ESBL genes detected. Of the 6 ESBL Etest-negative but CDST- and/or DDST-positive strains, reduction in MICs in the presence of clavulanate ranged from no change in 1 isolate (isolate 59) to less than three 2-fold dilutions in the other 5 isolates (isolates 72, 77, 11, 85, and 92). All isolates with an MIC of < 0.25 $\mu\text{g/mL}$ for

cefepime were confirmed to be negative for the ESBL phenotype. Among the 38 isolates that had an MIC of ≥ 0.25 $\mu\text{g/mL}$ for cefepime, DDST detected 24 of 25 isolates with an ESBL genotype, and had a sensitivity of 96%. Three isolates (isolates 6, 11, and 29) had borderline DDST results. If these 3 isolates were excluded as a negative result, DDST identified only 9 isolates of 13 non-ESBL producers, and had a specificity of 69.2%. CDSTs detected 22 of 25 ESBL producers and 9 of 13 non-ESBL producers, with a sensitivity of 88.0% and specificity of 69.2%. ESBL Etest had the lowest sensitivity, at 84%, detecting 21 of 25 ESBL producers, but a good specificity of 100%.

Detection of extended-spectrum β -lactamase genes

ESBL genes were detected in 24 of the 27 isolates with the ESBL phenotype. Three isolates (isolates 11, 85, and 92) that gave positive CDST results but negative or borderline DDST results were negative for ESBL genes by the PCR methods. ESBL genes were not detected in another 2 isolates (isolates 6 and 29), also with borderline DDST results. PCR detection for ESBL genes was also undertaken for another 11 isolates without an ESBL phenotype but with MICs of ≥ 0.25 $\mu\text{g/mL}$ for cefepime. One of these isolates (isolate 63) was found to be carrying bla_{SHV} , which was confirmed by DNA sequencing as an ESBL gene, SHV-12. Therefore, 25 isolates were found to carry ESBL genes, and all of them had an MIC of > 0.25 $\mu\text{g/mL}$ for cefepime. None of them had an MIC of 0.25 $\mu\text{g/mL}$ for cefepime. Sixteen of the 25 isolates harbored both bla_{SHV} and $bla_{\text{CTX-M}}$, and all 25 isolates carried bla_{SHV} . The overall prevalence of ESBL-producing *E. cloacae* based on the genotypic results was 21.6% (25/116).

Antimicrobial susceptibilities

The susceptibilities of the 25 ESBL-producing isolates to various antimicrobial agents are listed in Table 3. By CLSI breakpoint criteria, 16 of the 25 ESBL-producing isolates (64.0%) were not susceptible to cefotaxime, including 9 (36.0%) that were intermediately resistant to cefotaxime and 7 (28.0%) that were resistant. The rate of non-susceptibility to cefepime among the 25 ESBL producers was 16% (4/25), with an intermediate resistance rate of 8% (2/25) and a resistance rate of 8% (2/25). Among the 38 isolates with an MIC of ≥ 0.25 $\mu\text{g/mL}$ for cefepime, the 25 ESBL producers had a higher rate of non-susceptibility to cefepime compared with the 13 non-ESBL producers.

Table 1. Concordance of test results of different phenotypic methods for extended-spectrum β -lactamase detection in 25 *Enterobacter cloacae* isolates.

Isolate no.	Combination-disk synergy tests				Extended-spectrum β -lactamase Etest		Double-disk synergy test	Extended-spectrum β -lactamase genes
	Cefotaxime/cefotaxime and clavulanic acid		Ceftazidime/ceftazidime and clavulanic acid		Cefepime/cefepime and clavulanic acid		Cefepime and amoxicillin-clavulanate disks	
	Result	Finding (zone diameter [mm])	Result	Finding (zone diameter [mm])	Result	Finding	Result	
Strains with concordant test results for phenotypic methods								
1	+	15/31	+	12/25	+	Deformation of ellipse	+	SHV, CTX-M
7	-	9/12	+	8/13	+	12/0.094	+	SHV, CTX-M
8	+	10/24	+	No zone/20	+	Phantom zone	+	SHV
17	+	18/30	+	10/25	+	Deformation of ellipse	+	SHV, CTX-M
20	+	18/28	+	15/23	+	Deformation of ellipse	+	SHV, CTX-M
22	+	17/30	+	17/25	+	Deformation of ellipse	+	SHV, CTX-M
23	+	13/20	-	12/9	+	Deformation of ellipse	+	SHV, CTX-M
25	+	20/30	+	15/25	+	Deformation of ellipse	+	SHV, CTX-M
35	+	21/30	+	18/27	+	Phantom zone	+	SHV
39	+	20/30	+	15/27	+	Deformation of ellipse	+	SHV, CTX-M
41	+	17/29	+	18/25	+	Deformation of ellipse	+	SHV
46	+	18/30	+	16/25	+	Deformation of ellipse	+	SHV
50	+	20/30	+	16/27	+	Deformation of ellipse	+	SHV
56	+	12/18	+	11/16	+	Deformation of ellipse	+	SHV, CTX-M
61	+	No zone/12	-	No zone/9	+	3/<0.064	+	SHV, CTX-M
71	+	16/28	+	12/24	+	Deformation of ellipse	+	SHV, CTX-M
76	+	8/13	-	8/11	+	1.5/<0.064	+	SHV, CTX-M
78	+	16/28	+	12/26	+	Deformation of ellipse	+	SHV, CTX-M
87	+	16/29	+	7/24	+	Deformation of ellipse	+	SHV, CTX-M
94	+	16/29	+	13/24	+	Deformation of ellipse	+	SHV, CTX-M
113	-	16/18	+	14/22	+	Deformation of ellipse	+	SHV, CTX-M
Isolates with discrepant test results for phenotypic methods								
59	-	11/9	-	11/11	-	2/2	+	SHV
72	-	12/10	-	10/14	-	1/0.25	+	SHV
77	-	14/11	+	No zone/15	-	2/0.75	+	SHV
Isolates with negative results for phenotypic methods but with positive genotypes								
63	-	13/13	-	10/11	-	8/1.5	-	SHV
Isolates with discrepant test results or borderline test results but without genotype								
6	-	No zone/9	-	No zone/11	-	3/0.75	+/-	-
11	+	No zone/13	-	10/14	-	1.0/0.25	+/-	-
29	-	No zone/10	-	No zone/10	-	2/1	+/-	-
85	-	No zone/9	+	No zone/12	-	0.5/0.094	-	-
92	-	No zone/8	+	No zone/12	-	0.75/0.25	-	-

Abbreviations: + = positive; - = negative.

Regarding the susceptibility rate to carbapenems, all 25 strains with ESBLs were susceptible to either meropenem or imipenem, and 88% were susceptible to ertapenem. Most of the *E. cloacae* isolates with ESBLs (96%) were susceptible to tigecycline, with an MIC range from 0.5 to 8.0 $\mu\text{g/mL}$. The MIC₉₀ of tigecycline was 4 $\mu\text{g/mL}$ and the MIC₅₀ was 1 $\mu\text{g/mL}$.

Discussion

This study found that the prevalence of ESBL producers among *E. cloacae* collected from the TSAR IV program was 21.6%, which was higher than that reported from a single teaching hospital in Taiwan (15.0%) [5] and in Korea (12.8%) [14]. However, a

Table 2. Discrepant results for the phenotypic confirmatory methods for extended-spectrum β -lactamases.

Double-disk synergy test	Combination-disk synergy tests	Cefepime extended-spectrum β -lactamase Etest	Genotype	No. of isolates	Isolate no.
+	+	-	+	1	77
+	-	-	+	2	59, 72
-	-	-	+	1	63
-	+	-	-	3	11, 85, 92

Abbreviations: + = positive; - = negative.

Table 3. Antimicrobial susceptibility testing for 25 *Enterobacter cloacae* isolates.

Antimicrobial	Range	Minimal inhibitory concentration ₅₀ (μ g/mL)	Minimal inhibitory concentration ₉₀ (μ g/mL)	Susceptibility (%)
Ampicillin	>128.00	>128.00	>128.00	0
Amoxicillin-clavulanate	32.00-128.00	64.00	128.00	0
Cefazolin	64.00->128.00	>128.00	>128.00	0
Cefuroxime	32.00->128.00	128.00	>128.00	0
Cefotaxime	4.00->128.00	16.00	>128.00	36
Cefepime	0.50-64.00	1.00	32.00	84
Cefmetazole	32.00->128.00	128.00	>128.00	0
Flomoxef	0.13->128.00	16.00	128.00	40
Ertapenem	<0.03-8.00	0.13	4.00	88
Imipenem	0.13-2.00	0.25	0.50	100
Meropenem	<0.03-1.00	0.06	0.25	100
Ciprofloxacin	<0.03->128.00	0.50	16.00	76
Levofloxacin	<0.03-64.00	0.50	8.00	76
Moxifloxacin	0.06-128.00	2.00	16.00	- ^a
Gentamicin	0.50->128.00	128.00	>128.00	12
Amikacin	1.00->128.00	8.00	32.00	88
Tigecycline	0.50-8.00	1.00	4.00	96

^aNo interpretive breakpoints are provided by the Clinical and Laboratory Standards Institute.

much higher proportion of *E. cloacae* with ESBL production (28%) was reported from a single tertiary hospital in Taiwan during 2000 to 2001 [15]. *E. cloacae* with ESBL production has been reported to exceed 35% in some other countries such as China, The Philippines, and Singapore [4].

Phenotypic confirmatory methods for ESBL production among AmpC-hyperproducing organisms remains a problem. According to a previous report [5], CDSTs using both cefotaxime and ceftazidime, either alone or in combination with clavulanic acid, is considered to be simple and reliable for ESBL detection among *E. cloacae* in Taiwan compared with the MIC-based method or DDST using cefepime and amoxicillin-clavulanate disks. As genotypic detection of ESBLs among all 116 *E. cloacae* isolates was not performed, the exact sensitivity and specificity of each phenotypic confirmatory method could not be determined. Nevertheless, it seemed that DDST using cefepime and amoxicillin/clavulanate disks had a

higher sensitivity than CDSTs using cefotaxime and ceftazidime as indicator drugs for the detection of ESBLs among *E. cloacae* isolates with an MIC of ≥ 0.25 μ g/mL. However, there are still potential limitations [11,12] of the genotypic methods used in this study, which may give a false-negative genotype result and alter the sensitivity of each of the confirmatory methods. For example, there were 3 isolates with positive CDSTs results but negative DDST results (isolates 11, 85, and 92). If these 3 isolates had been ESBL producers that could not be detected by the PCR methods used in this study, CDSTs would have been more sensitive than DDST to detect ESBL producers. In addition, the results of DDST are sometimes difficult to interpret. Yu et al demonstrated that ESBL producers have negligible increases in cefepime zone diameter in the presence of clavulanic acid in DDST, other than the typical keyhole phenomenon [5]. Although both CDSTs and DDST have limitations, a combination of both DDST using cefepime and amoxicillin-clavulanate

and CDSTs using cefotaxime and ceftazidime as indicator drugs seems to be able to enhance the sensitivity for detecting ESBLs. These tests are also simple to perform in a clinical laboratory.

According to previous studies in Taiwan [5,16], the predominant ESBL type in *E. cloacae* is the SHV-type but not CTX-M. In this study, all 25 isolates harbored *bla*_{SHV} and 16 isolates also carried *bla*_{CTX-M}, which is not consistent with previous data. Therefore, DNA sequencing of the 16 isolates was performed and this method confirmed the result. This suggests that there might be changes in the epidemiology of the ESBL type of *E. cloacae* within a few years, and further continuous investigation is warranted. In addition, the CTX-M-type ESBL usually confers resistance to cefepime, which is not consistent with the findings of this study. This might possibly be explained by the inoculum effect on the MIC of cefepime. As Varela et al have shown, *E. cloacae* isolates harboring only *bla*_{CTX-M} had markedly increased MICs for cefepime if using a high inoculum (10^7 CFUs/mL) compared with a low inoculum (10^3 CFUs/mL) or a 10^5 CFUs/mL inoculum [17]. The inoculum size used in this study was 10^4 CFUs/mL, which might explain the relatively low cefepime resistance rate. However, isolates in this study also carried *bla*_{SHV}. A special study design may be needed to examine whether the inoculum effect on the MIC of cefepime exists among these isolates after excluding for the effect of SHV-type ESBL.

As a significant proportion of clinical isolates of *E. cloacae* were positive for ESBL, the selection of antibiotics for the treatment of infections caused by ESBL-producing organisms is an important issue for clinicians. Fourth-generation cephalosporins such as cefepime and ceftazidime could be a first-line treatment for infections caused by *E. cloacae* [18], but there are controversies over their use in the treatment of infection caused by ESBL-producing organisms [19]. Carbapenems are considered to be the drugs of choice against ESBL-producing organisms [20]. In this study, all ESBL-producing *E. cloacae* isolates were susceptible to imipenem and meropenem. However, only 88% of isolates were susceptible to ertapenem, with an MIC₅₀ of 0.12 µg/mL and an MIC₉₀ of 4 µg/mL. It has been reported that loss of porins in the outer membranes of some bacteria may result in reduced susceptibility to ertapenem but good susceptibility to imipenem and meropenem [21]. Whether the isolates with reduced susceptibility to ertapenem in this study is due to the same mechanism needs to be explored further.

Tigecycline, an antimicrobial with good in vitro activity against ESBL-producing organisms, may be another drug of choice for treatment of ESBL-producing bacterial infections. According to the Tigecycline Evaluation and Surveillance Trial program, tigecycline was active against ESBL-producing *E. coli* and *K. pneumoniae* in the Asia-Pacific region from 2004 to 2007 [22]. Ninety six percent of isolates of *Enterobacter* spp. (including ESBL-producing and –non-producing isolates) were susceptible to tigecycline. This study demonstrated a similar result, with 96% of the ESBL-producing *E. cloacae* being susceptible to tigecycline. Further investigation is warranted to evaluate the effectiveness of tigecycline in clinical practice.

In conclusion, the prevalence of ESBL-producing *E. cloacae* was 21.6% in Taiwan. A combination of phenotypic methods of DDST using cefepime and amoxicillin-clavulanate and CDSTs using cefotaxime and ceftazidime, either alone or in combination with clavulanic acid will increase the sensitivity for detecting ESBL production. As a significant proportion of *E. cloacae* clinical isolates were positive for ESBLs, the choice of empirical antibiotics to treat *E. cloacae* infection needs further evaluation.

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