J Microbiol Immunol Infect 2010;43(1):26–34

Contents lists available at ScienceDirect

Journal of Microbiology, Immunology and Infection

Journal homepage: http://www.e-jmii.com

Original Article

Screening Extended-spectrum β-Lactamase Production in *Enterobacter cloacae* and *Serratia marcescens* Using Antibiogram-based Methods

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BACKGROUND/PURPOSE: In *Enterobacter cloacae* and *Serratia marcescens*, AmpC β-lactamases can confer resistance to the third-generation cephalosporins and oxacephems, but not to the fourth-generation cephalosporins. Extended-spectrum β-lactamases (ESBL) may confer resistance to all extended-spectrum cephalosporins but not flomoxef. As difficult to detect, the ESBL phenotype of the intrinsically AmpC-producing *E. cloacae* and *S. marcescens* is not routinely screened in the clinical microbiology laboratories. The distinct antibiotic resistance phenotype between ESBL- and AmpC-producers may assist to differentiate the type of secreted β-lactamases. Therefore, we attested the validity of an antibiogram-based method to predict the presence of ESBLs in both species.

METHODS: Polymerase chain reaction-based methods and antibiogram-based methods were compared for their detection of ESBL in 74 *E. cloacae* and 69 *S. marcescens* isolates recovered from patients hospitalized at two medical centers in Taiwan. Three major types of antibiogram were defined: type I (3s4s), susceptible to cefotaxime, ceftazidime, and cefepime; type II (3r4s), resistant to cefotaxime or ceftazidime, but susceptible to cefepime; and type III (3r4r), resistant to cefepime plus cefotaxime and/or ceftazidime. Furthermore, subtype-a and subtype-b were defined as being resistant and susceptible to flomoxef, respectively.

RESULTS: Overall, ESBL producers were identified in 20 (27.0%) of *Enterobacter* and 11 (15.9%) of *Serratia* isolates by polymerase chain reaction-based methods. All type I isolates of both species ($n=49$) were non-ESBL

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Article History:
Received: Nov 18, 2008
Revised: Jan 20, 2009
Accepted: Feb 17, 2009

producers. In *E. cloacae*, all subtype IIb ($n=6$) and type III ($n=6$) isolates produced ESBLs, but only 8 of 17 IIa isolates produced ESBLs. The IIb and III types had the highest positive predictive value (100%) and specificity (100%) for ESBL detection. In *S. marcescens*, type II isolates rarely produced ESBLs (4/57 isolates), while seven of type III ($n = 8$) isolates produced ESBLs. Type III antibiogram had the highest positive predictive value (87.5%) and specificity (98.3%) for ESBL detection.

CONCLUSION: The antibiograms of subtype IIb and type III are highly predictive for ESBL detection in *E. cloacae*, while type III is highly predictive for ESBL detection in *S. marcescens*. It is imperative to further examine ESBLs, focusing on the *E. cloacae* isolates with antibiogram subtype IIa.

KEYWORDS: antibiogram, *Enterobacter cloacae*, extended spectrum β-lactamases, resistance, *Serratia marcescens*

Introduction

Plasmid-mediated extended-spectrum β-lactamases (ESBL), derived mainly from Temoniera (TEM)-type and sulfhydryl variable (SHV)-type β-lactamases, are able to confer resistance to all extended-spectrum oxyiminocephalosporins (e.g. cefotaxime, ceftazidime, and cefepime), and monobactams (e.g. aztreonam).[1] ESBLs do not hydrolyze 7-α-methoxy β-lactams, including cephamycins (e.g. cefoxitin and cefotetan) or oxacephems (e.g. moxalactam and flomoxef).[2]

Since the first report of *Klebsiella pneumoniae* expressing an ESBL was published in 1983,[3] the frequency of ESBL-producing *Enterobacteriaceae* has increased, and encompasses *Escherichia coli* and other organisms with chromosomal Bush group 1 (AmpC) β-lactamase, such as *Enterobacter* spp., *Citrobacter* spp., and *Serratia* spp.[4–9] Production of small amounts of inducible AmpC β-lactamases causes intrinsic resistance of the AmpC producers to ampicillin and first-generation cephalosporins. Antibiotic induction of high AmpC β-lactamase leads to expression or selection of derepressed mutants, which produce high levels of the AmpC β-lactamase,[10–12] has conferred resistance to 7-α-methoxy β-lactams and to all cephalosporins except the fourth-generation agents (e.g. cefepime and cefpirome).[2] The distinct antibiotic resistance phenotypes (antibiogram) between ESBL- and AmpC-producers may assist to differentiate the type of secreted β-lactamase.

Gene or protein sequencing is used for accurate identification of the *Enterobacteriaceae* β-lactamases. ESBL production can be screened by both cefotaxime and ceftazidime, alone and in combination with clavulanate using phenotypic disks.[13–15] The Clinical and Laboratory Standards Institute has issued recommendations for ESBL screening and confirmation for isolates of *E. coli*, *K. pneumoniae*, *K. oxytoca*, and *Proteus mirabilis*, but not for AmpC-producing organisms.[16,17] Although phenotypic disk methods can be applied to AmpC producers, the major concern is that high-level expression of AmpC may prevent recognition of an ESBL in an AmpC-inducible species.[7]

ESBL-producing isolates of *Enterobacter cloacae* and *Serratia marcescens* have been identified in several hospitals in Taiwan.[18–25] However, most clinical microbiology laboratories do not currently screen for ESBL in *E. cloacae* and *S. marcescens* on a routine basis. Therefore, clinical physicians need alternative ways to recognize these ESBL-producing bacteria, such as an antibiogram-based method. Accordingly, the present study assesses the reliability of the antibiogram-based method using a panel containing cefotaxime, ceftazidime, cefepime, and flomoxef to predict the presence or absence of ESBLs in a consecutive collection of clinical isolates of *E. cloacae* and *S. marcescens*. We initially hypothesized that ESBLs occurred in isolates with an antibiogram of resistance to ceftazidime, cefotaxime, and/or cefepime but susceptibility to flomoxef; whereas isolates with resistance to ceftazidime, cefotaxime and flomoxef, but susceptibility to cefepime, implied a predominant expression of AmpC β-lactamase.

Methods

Bacterial strains

Non-repetitive *E. cloacae* clinical isolates ($n=74$) were obtained from patients hospitalized at a university affiliated medical center in Central Taiwan from January 1 to June 30,

2002. Non-repetitive *S. marcescens* bloodstream isolates ($n = 69$) were recovered from a 1,300-bed medical center in Southern Taiwan from August 1999 to July 2003. The isolates were identified on the basis of routine microbiologic methods and species identification was confirmed using the VITEK system (BioMerieux Vitek Inc, Hazelwood, MD, USA).

Antibiogram

Antimicrobial susceptibility testing was performed using the disk diffusion method.[16] The antibiogram in this study included the susceptibility profiles of cefepime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), and flomoxef (30 μg). The breakpoints for susceptible and resistant categories of flomoxef were > 18 mm and < 12 mm respectively, according to the manufacturer's instructions (BD Biosciences, USA). *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as control strains.

Confirmation test of ESBL phenotype

The combination-disk synergistic test (CDST) using cefotaxime, ceftazidime, and cefepime plus clavulanate (10 μg) was performed to detect ESBL production for all isolates. An ESBL phenotype was confirmed by a ≥ 5 mm increase in zone diameter for cefotaxime, ceftazidime or cefepime in combination with clavulanate versus its zone when tested alone. The combination disks included cefotaxime/clavulanate and ceftazidime/clavulanate (BBL Sensi-Disc, Becton Dickinson, Cockeysville, MD, USA) and cefepime/clavulanate (Neo-Sensitabs, Rosco Diagnostica, Taastrup, Denmark). The minimum inhibitory concentration (MIC) of antimicrobial agents were determined by the agar dilution method.[17] The ESBL phenotype was confirmed by a reduction of ≥ 3 $\log_2$ dilutions for the MIC of cefotaxime, ceftazidime or cefepime in the presence of clavulanic acid (4 μg/mL). Control experiments were assured by testing *E. coli* ATCC 25922 and *K. pneumoniae* 700603.

Detection of bla_{SHV}, bla_{TEM}, bla_{CTX-M} and AmpC genes

Polymerase chain reaction (PCR) was used to amplify the entire sequences of the bla_{SHV-12}, $bla_{CTX-M-3}$ and $bla_{CTX-M-14}$, as well as the partial sequences of bla_{TEM}.[22,24,26] Specific primers were used to *AmpC* gene and bla_{SRT} gene in the genomes of *E. cloacae* and *S. marcescens* isolates, respectively.[24,27] Primer sequences were listed in Table 1. Amplicons were purified with PCR clean up kits (Roche Diagnostics, GmbH, Penzberg, Germany) and sequenced on an ABI PRISM 3730 sequencer analyzer (Applied Biosystems, Foster City, CA, USA). Sequence analyses were performed online at the National Center for Biotechnology Information website.[28]

Statistical analysis

The PCR-based method was used as the standard detection method with the accuracy of ESBL detection results by other methods evaluated by sensitivity, specificity, positive predictive value, and negative predictive value.

Table 1. Primer sequences used in this study

Gene	Primer	Sequences	Reference
bla_{SHV-12}	forward	5′-ATG CGT TAT ATT CGC CTG TG-3′	22,24,26
	reverse	5′-TTA GCG TTG CCA GTG CTC G-3′	
$bla_{CTX-M-3}$	forward	5′-GGA TCC ATG GTT AAA AAA TCA CTG CG-3′	
	reverse	5′-AAG CTT TTA CAA ACC GTC GGT GAC-3′	
$bla_{CTX-M-14}$	forward	5′-AAA AAT GAT TGA AAG GTG GTT GT-3′	
	reverse	5′-TTA CAG CCC TTC GGC GAT GA-3′	
bla_{TEM}	forward	5′-ATA AAA TTC TTG AAG ACG AAA-3′	
	reverse	5′-GAC AGT TAC CAA TGC TTA ATC-3′	
AmpC	forward	5′-TCG GAA TTC CGG AGG ATT ACT GAT GAT GA-3	27
	reverse	5′-TTA GTC GAC AAT GTT TTA CTG TAG CGC CTC G-3′	
bla_{SRT}	forward	5′-GCC GAT ACC CTG CAA CCT AAG-3′	24
	reverse	5′-CGC CTG GAC GAT GTG GTA AG-3′	

Results

Bacterial strains, sources, and ESBL survey

Among the 74 non-repetitive *E. cloacae* isolates, 32 (43.2%) recovered from wound pus, 21 (28.4%) from urine, six (8.1%) from blood, six (8.1%) from central venous catheter tip, three (4.1%) from ascites, two (2.7%) from sputum, and four (5.4%) from other sources (Table 2). The CDST or the MIC-based methods were able to detect 16 ESBL producers (Table 3). The 74 isolates were surveyed for ESBL genes using PCR, which detected 4 additional ESBL producers (strains E11, E24, E34, and E35). Therefore, the sensitivity, specificity, positive predictive value, and negative predictive value of the CDST or the MIC-based method for ESBL detection in *E. cloacae* was 80.0%, 100%, 100%, and 93.1%, respectively. Twenty (27.0%) isolates carried alleles for the $bla_{\text{SHV-12}}$ gene (Table 4). Subsequent DNA sequencing of all PCR products confirmed 100% homology to the $bla_{\text{SHV-12}}$ gene. The E95 and E96 isolates also contained $bla_{\text{CTX-M-14}}$ and $bla_{\text{CTX-M-3}}$, respectively. TEM-1 β-lactamase was confirmed in 10 non-ESBL isolates. The ESBL producers were most commonly isolated from central venous catheter-tip, urine, ascites, and pus specimens (Table 2).

From 69 bacteremic isolates of *S. marcescens*, the CDST or MIC-based methods were able to detect nine ESBL producers (Table 5) while a total of 11 ESBL producers were identified by PCR, including 10 isolates producing CTX-M-3 and one producing SHV-12 (Table 6). The sensitivity,

Table 2. Sources and extended-spectrum β-lactamases prevalances among the non-repetitive *Enterobacter cloacae* isolates[a]

Specimen	No of isolates	ESBL Prevalence[c]
Pus	32 (43.2)	6 (18.8)
Urine	21 (28.4)	9 (42.9)
Blood	6 (8.1)	0 (0)
Central venous catheter tip	6 (8.1)	4 (66.7)
Ascites	3 (4.1)	1 (33.3)
Sputum	2 (2.7)	0 (0)
Others[b]	4 (5.4)	0 (0)
Total	74 (100)	20 (27.0)

[a]Data presented as *n* (%); [b]one each for bile, pleural fluid, cerebrospinal fluid and bronchoalveolar lavage; [c]among the positive isolates from each specimen group.

Table 3. Antibiogram and positive ESBL tests of 74 Enterobacter cloacae isolates

Antibiogram				Positive ESBL tests by		Predictive values of various antibiogram			
Type	CAZ/CTX	FFP	FLO	CDST or MIC-based methods (*n*=16)	PCR-based methods (*n*=20)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)
Ia	S[a]	S	R	0/9	0/9	0	31.0	–	–
Ib	S[a]	S	S	0/36	0/36				
IIa	R[b]	S	R	4/17	8[c]/17	47.1	78.9	–	–
IIb	R[b]	S	S	6/6	6/6	100	87.1	60.0	100
IIIa	R[b]	R	R	5/5	5/5				
IIIb	R[b]	R	S	1/1	1/1				
Sensitivity (%)		–		80.0	Standard			–	
Specificity (%)		–		100	Standard			–	
PPV (%)		–		100	Standard			–	
NPV (%)		–		93.1	Standard			–	

[a]Susceptible to both CAZ and CTX; [b]resistant to either CAZ and/or CTX; [c]four isolates (E11, E24, E34, and E35) containing *bla*SHV-12 gave a false negative result when phenotypic disk and MIC-based extended-spectrum β-lactamases detection methods were employed (see Table 5). ESBL=extended-spectrum β-lactamases; CAZ=ceftazidime; CTX=cefotaxime; FEP=cefepime; FLO=flomoxef; CDST=combination-disk synergistic test; MIC=minimum inhibitory concentration; PCR=polymerase chain reaction ; PPV=positive predictive value; NPV=negative predictive value; S=susceptible; R=resistant.

Table 4. Minimum inhibitory concentrations, antibiogram, and genotypes of 20 strains of ESBL-producing *Enterobacter cloacae*

Strains	MIC (μg/mL)						Antibiogram	ESBL	
	CTX	CTX/CLA	CAZ	CAZ/CLA	FEP	FEP/CLA		SHV	CTX-M
E2	16	0.50	64	4	2	0.12	IIa	SHV-12	–
E6	16	0.25	64	0.50	2	0.06	IIb	SHV-12	–
E9	16	0.25	128	0.50	2	0.06	IIb	SHV-12	–
E11[a]	64	128	128	64	2	1	IIa	SHV-12	–
E13	128	4	>512	16	32	0.25	IIIa	SHV-12	–
E19	128	2	>512	8	16	0.12	IIIa	SHV-12	–
E24[a]	64	128	256	128	4	2	IIa	SHV-12	–
E25	16	1	32	0.50	1	0.12	IIa	SHV-12	–
E34[a]	32	128	64	64	2	0.50	IIa	SHV-12	–
E35[a]	16	16	32	32	0.50	0.12	IIa	SHV-12	–
E43	8	0.25	64	1	1	0.12	IIIa	SHV-12	–
E47	8	0.50	128	8	2	0.06	IIa	SHV-12	–
E68	16	0.50	128	1	2	0.06	IIb	SHV-12	–
E71	64	1	512	4	16	0.12	IIIa	SHV-12	–
E73	32	0.50	128	1	4	0.06	IIb	SHV-12	–
E75	32	0.25	256	1	4	0.06	IIb	SHV-12	–
E89	32	1	64	32	2	0.25	IIa	SHV-12	–
E95	128	0.50	8	0.50	32	0.12	IIIb	SHV-12	CTX-M-14
E96	512	16	16	32	32	0.25	IIIa	SHV-12	CTX-M-3
E98	128	64	512	32	32	1	IIIa	SHV-12	–

[a]false negative ESBL result by phenotypic methods. MIC=minimum inhibitory concentration; ESBL=extended-spectrum β-lactamases; CTX=cefotaxime; CLA=clavulanic acid; CAZ=ceftazidime; FEP=cefepime; SHV=sulfhydryl variable.

specificity, positive predictive value, and negative predictive value of the CDST or MIC-based methods for ESBL detection in *S. marcescens* were 81.8%, 100%, 100%, and 96.7%, respectively (Table 5).

Antibiogram

Antibiograms of the 74 *E. cloacae* isolates revealed susceptibility profiles to cefotaxime, ceftazidime, and cefepime, and were classified into three major types (Types I–III). Each type could be further classified into subtype-a (resistant to flomoxef) or subtype-b (susceptible to flomoxef) (Table 3). Type I (3s4s) isolates ($n=45$) were susceptible to cefotaxime, ceftazidime, and cefepime, and were all non-ESBL producers. All ESBL producers were distributed among the type II (3r4s) and III (3r4r) *E. cloacae* isolates. The occurrence of ESBL among subtype IIa (3r4sFr) isolates was 47.1% (8/17). However, four ESBL producers were not detected by phenotypic ESBL methods. The subtype IIb (3r4sFs) and type III (3r4r) isolates were confirmed as ESBL producers. Overall, the prevalence of ESBL in *E. cloacae* isolates with various antibiograms were type Ia, 0%; Ib, 0%; IIa, 47.1% (8/17 isolates); IIb, 100% (6/6 isolates); IIIa, 100% (5/5 isolates); and IIIb, 100% (1/1 isolates) (Table 3). The sensitivity, specificity, positive predictive value, and negative predictive value of the antibiograms including subtype IIb and type III for ESBL detection in *E. cloacae* was 60.0%, 100%, 100%, and 87.1%, respectively.

Similarly, the prevalence of ESBL for 69 *S. marcescens* isolates with various antibiograms were type I, 0%; IIa, 0%; IIb, 8.9% (4/45 isolates); IIIa, 66.7% (2/3 isolates); and IIIb, 100% (5/5 isolates) (Table 5). Only two ESBL-producing *S. marcescens* isolates (strains no. 109 and 122) were not detected by phenotypic methods (Table 6). The sensitivity, specificity, positive predictive value, and negative predictive values of the type III antibiogram ESBL

Table 5. The antibiogram and positive ESBL tests of 69 *Serratia marcescens* isolates

Antibiogram				Positive ESBL tests by		Predictive values of various antibiogram			
Type	CAZ/CTX	FFP	FLO	CDST or MIC-based methods (n=9)	PCR-based methods (n=11)	PPV (%)	NPV (%)	Sensitivity (%)	Specificity (%)
Ia	S[a]	S	R	0/0	0/0				
Ib	S[a]	S	S	0/4	0/4	0	79.2	–	–
IIa	R[b]	S	R	0/12	0/12				
IIb	R[b]	S	S	2/45	4[c]/45	8.9	70.8	–	–
IIIa	R[b]	R	R	2/3	2/3	87.5	9.4	63.6	98.3
IIIb	R[b]	R	S	5/5	5/5				
Sensitivity (%)		–		81.8	Standard			–	
Specificity (%)		–		100	Standard			–	
PPV (%)		–		100	Standard			–	
NPV (%)		–		96.7	Standard			–	

[a]Susceptible to both CAZ and CTX; [b]resistant to either CAZ and/or CTX; [c]two isolates (strains no. 109 and 122) containing *bla*CTX-M-3 gave false negative results by phenotypic disk and MIC-based extended-spectrum β-lactamases detection methods (see Table 6). ESBL=extended-spectrum β-lactamases; CAZ=ceftazidime; CTX=cefotaxime; FEP=cefepime; FLO=flomoxef; CDST=combination-disk synergistic test; MIC=minimum inhibitory concentration; PCR=polymerase chain reaction; PPC=positive predictive value; NPV=negative predictive value; S=susceptible; R=resistant.

Table 6. MICs, antibiogram, and genotypes of 11 strains of ESBL-producing *serratia marcescens*

Strains	MIC (μg/mL)						Antibiogram	ESBL	
	CAZ	CAZ/CLA	CTX	CTX/CLA	FEP	FEP/CLA		CTX-M	SHV
5	2	1	64	8	16	0.50	IIIb	CTX-M-3	–
101	128	1	8	4	1	0.25	IIb	–	SHV-12
109[a]	0.50	0.50	16	8	0.25	0.25	IIb	CTX-M-3	–
122[a]	0.50	0.50	16	8	0.25	0.25	IIb	CTX-M-3	–
125	16	2	>128	16	>128	2	IIIa	CTX-M-3	–
127	2	1	>128	32	64	2	IIIb	CTX-M-3	–
172	2	0.12	128	2	32	0.12	IIIb	CTX-M-3	–
174	2	0.5	64	8	16	0.50	IIIb	CTX-M-3	–
192	1	0.12	64	2	4	0.12	IIb	CTX-M-3	–
199	4	0.12	>128	0.50	64	0.06	IIIb	CTX-M-3	–
200	2	1	>128	32	64	2	IIIa	CTX-M-3	–

[a]false negative ESBL test results by phenotypic methods. MIC=minimum inhibitory concentration; ESBL=extended-spectrum β-lactamases; CAZ=ceftazidime; CLA=clavulanic acid; CTX=cefotaxime; FEP=cefepime; SHV=sulfhydryl variable.

detection in *S. marcescens* were 63.6%, 98.3%, 87.5%, and 93.4%, respectively.

Survey for chromosomal AmpC gene

The alleles of *AmpC* gene in the 29 *E. cloacae* isolates with antibiogram types II and III were characterized by PCR. Each isolate produced a 1,165-bp PCR fragment containing the *AmpC* gene. Subsequent DNA sequencing of the PCR products from strains E71 and E11 revealed 99% and 97% identity to *bla*(EcloK995120.1) and *bla* (EcloK9973), respectively, which were the chromosomal *AmpC* gene of *E. cloacae* found in Korea.[27] Furthermore,

these *AmpC*-like genes were detected in type Ia (6/9) and type Ib (7/36) *E. cloacae* isolates.

With respect to *S. marcescens*, only strain no. 125 did not harbor a chromosomal *SRT*-like AmpC enzyme. The remaining 58 non-ESBL producers were confirmed as positive AmpC producers by PCR.

MIC determination

Detailed MIC values and ESBL genotypes of the ESBL-producing *E. cloacae* isolates ($n=20$) and *S. marcescens* isolates ($n=11$) can be found in Tables 4 and 6, respectively. The MICs of cefotaxime, ceftazidime, and cefepime for the 20 *Enterobacter* ESBL producers ranged from 8 to 512 μg/mL, 8 to >512 μg/mL, and 0.5 to 32 μg/mL, respectively. Of the 20 *Enterobacter* ESBL producers, 70.0% (14/20) were susceptible to cefepime (MIC<8 μg/mL). A significant reduction of MICs for cefotaxime, ceftazidime, and cefepime in the presence of clavulanate was not achieved for four *Enterobacter* ESBL producers (strains E11, E24, E34, and E35) and two *Serratia* ESBL producers (strains no. 109 and 122).

Discussion

This analysis determined that the antibiogram-based method might be used as an initial screening method for ESBL detection in *E. cloacae* and *S. marcescens*. The antibiograms of subtype IIb and type III are highly predictive of ESBL detection in *E. cloacae*, whilst the antibiogram of type III is highly predictive of ESBL detection in *S. marcescens*. The type I antibiogram is highly predictive of non-ESBL producers for both species.

Many studies have reported a diverse prevalence of resistant strains of *Enterobacter* spp. and *S. marcescens* that produce plasmid-mediated ESBLs.[4,5,7–9,18–25,29,30] The increasing reports of ESBL-producing *Enterobacter* and *Serratia* isolates in Taiwan emphasizes the need for a better understanding of ESBL production in clinical isolates. However, ESBL detection can be challenging, because the inhibitor-based method can be confounded by the high level production of AmpC enzymes which are resistant to clavulanate. Some investigators suggested that an elevated MIC of cefepime >0.25 μg/mL,[8] or ≥1 μg/mL,[9] has the highest sensitivity (100% and 95.3%, respectively) and specificity (74% and 82.7%, respectively) for the presence of an ESBL. Similarly, no non-ESBL producing *E. cloacae* strains had MICs of cefepime ≥2 μg/mL.[31] However, most clinical microbiology laboratories perform the disk diffusion method to measure antibiotic susceptibility on a routine basis and do not use MIC-based techniques in Taiwan. Thus the disk antibiogram-based method may be an acceptable strategy for ESBL screening.

Isolates of *Enterobacter* and *Serratia* with concurrent susceptibilities to cefotaxime, ceftazidime, and cefepime (type I antibiogram), implying minimal or no induction of the chromosomal AmpC enzymes, have a low likelihood of ESBL production. Similar to our results, were those from a report in Korea stating the susceptibility of the ESBL producers *E. cloacae*, *C. freundii,* and *S. marcescens* to ceftazidime, cefotaxime, and aztreonam (MICs<8 μg/mL each) was very low (0.04–0.27%).[9] These facts suggest that these ESBL-producing organisms rarely maintain simultaneous susceptibility to all extended-spectrum cephalosporins. Thus it seems unnecessary to perform an ESBL test for isolates with a type I antibiogram, because the likelihood of a false negative result that might lead to inadequate treatment is negligible.

The mechanisms conferring resistance to the cephalosporins tested in our study are multifactorial. Isolates of subtype Ia with resistance to flomoxef likely exhibited an outer membrane protein defect.[32] The isolates of subtype IIa with resistance to flomoxef plus ceftazidime and/or cefotaxime but susceptibility to cefepime had a characteristic AmpC phenotype, confirmed by the *AmpC* PCR. Nevertheless, 47% of subtype IIa *Enterobacter* isolates were ESBL producers. These findings differ from our initial hypothesis in which antibiogram pattern would be used to predict AmpC producers. Four of the eight subtype IIa ESBL producers exhibited a positive ESBL phenotype, implying a greater activity of ESBL than that of AmpC β-lactamase.[1,2] These data refute the interpretation of all subtype IIa *E. cloacae* isolates as non-ESBL producers and support mandatory testing for ESBL production.

The subtype IIb or IIIb isolates which exhibit resistance to ceftazidime, cefotaxime and/or cefepime but susceptibility to flomoxef are characteristic of ESBL producers.[1,2] However, subtype IIb significantly correlated with ESBL production in *E. cloacae* but not *S. marcescens* isolates, as ESBLs occurred in only 8.9% of subtype IIb *S. marcescens*. The antibiogram-based method correctly

predicted ESBL production in isolates with cefepime resistance (type III) regardless of flomoxef susceptibility. Once again, these findings contrast from our initial hypothesis of using an antibiogram pattern to predict ESBL producers. A cost–benefit evaluation of antibiograms for the detection of ESBL-producing subtype IIb *S. marcescens* may be warranted in hospitals. We suggest that there is no need to test for ESBL from *S. marcescens* with a subtype IIb antibiogram. In summary, these results indicated that only type III *S. marcescens* and type IIb and III *E. cloacae* should be regarded as ESBL producers.

The mortality of patients from *E. cloacae* bacteremia is significantly higher in ESBL carriers with most harboring SHV-12 when compared with non-ESBL carriers.[19] Additionally, reduced susceptibility to cefepime among ESBL producers in the members of *Enterobacteriaceae* has become a major reason for potential therapeutic failure.[29,31,33] The possibly high rates of susceptibility to cefepime by ESBL producers re-emphasizes the importance for clinical physicians to be able to recognize ESBL production in *E. cloacae* and *S. marcescens*. With the aid of the antibiogram-based ESBL screening method described in our study, targeting effective antimicrobials to patients harboring ESBL producers is possible. Since these specimens were isolated only from Taiwan, its applicability to other geographical populations warrants further study.

In conclusion, despite that the Clinical and Laboratory Standards Institute methods are not issued for the detection of *E. cloacae* and *S. marcescens* EBSL, we suggest a simple and reliable method for physicians to detect ESBL production among these AmpC producers. For *S. marcescens*, ESBLs commonly occurred in isolates with a type III antibiogram; in *E. cloacae*, ESBLs frequently occurred in isolates with type III or subtype IIb antibiogram. However, ESBLs also occurred in some of the subtype IIa strains. Therefore, further ESBL testing should be focused on the subtype IIa *Enterobacter* isolates. Our antibiogram-based method may simplify the screening of potential ESBL-producing populations among AmpC-producing organisms.

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