J Microbiol Immunol Infect 2010;43(3):180–187

Contents lists available at ScienceDirect

Journal of Microbiology, Immunology and Infection

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

Original Article

Antimicrobial Susceptibility and Multiplex PCR Screening of *AmpC* Genes From Isolates of *Enterobacter cloacae*, *Citrobacter freundii*, and *Serratia marcescens*

Chih-Chauan Kao[a], Meei-Fang Liu[a], Chin-Fu Lin[b], Yi-Ching Huang[a], Po-Yu Liu[a], Ching-Wen Chang[b], Zhi-Yuan Shi[a,c]*

[a]Section of Infectious Diseases, Department of Internal Medicine, Taichung Veterans General Hospital, Taichung, Taiwan.
[b]Section of Clinical Microbiology Laboratory, Taichung Veterans General Hospital, Taichung, Taiwan.
[c]School of Medicine, National Yang-Ming University, Taipei, Taiwan.

BACKGROUND/PURPOSE: The emergence of multiple drug resistance in *Enterobacteriaceae* is of particular concern. The aim of this study was to evaluate the antimicrobial susceptibility and screen for the *ampC* gene in three members of the *Enterobacteriaceae* family (*Enterobacter cloacae*, *Citrobacter freundii*, and *Serratia marcescens*) found at Taichung Veterans General Hospital during the past 5 years using multiplex polymerase chain reaction (PCR).

METHODS: The susceptibility of thirty isolates from each of the three *Enterobacteriaceae* family members to five antimicrobial agents (ceftazidime, flomoxef, imipenem, moxifloxacin, and colistin) was assessed. The susceptibility was analyzed by disk diffusion, screening and confirmatory tests for extended-spectrum β-lactamases (ESBL) and minimum inhibitory concentration tests according to the recommendations of the Clinical and Laboratory Standards Institute. The detection of *ampC* genes (3 families, including DHA, EBC and CIT) was performed by multiplex PCR. To detect the coexistence of ESBL genes, PCR was performed using five primer pairs: TEM, SHV, SHV-5, CTX-M-3, and CTX-M-14.

RESULTS: Of the 90 isolates, 53 (58.9%) were positive in the screening test for ESBL. Resistance genes were detected in 12 (22.6%) of these isolates: *ampC* gene of DHA type in one *E. cloacae* isolate and EBC type in three *E. cloacae* isolates; *ampC* gene of CIT type in four *C. freundii* isolates; CTX-M-3-like in one *C. freundii* isolate and one *S. marcescens* isolate; TEM in three *E. cloacae* isolates, three *C. freundii* isolates and two *S. marcescens* isolates; SHV in one *C. freundii* isolate.

CONCLUSION: Antibiotic phenotypes cannot accurately distinguish the resistance mechanisms caused by *ampC* or ESBL, and especially in ESBL-*ampC* combinations. However, PCR is a useful technique for the identification of the different types of resistance genes.

KEYWORDS: AmpC, Citrobacter, Enterobacter, extended-spectrum β-lactamases, Serratia

*Corresponding author. Section of Infectious Diseases, Department of Internal Medicine, Taichung Veterans General Hospital, 160 Section 3, Chung-Kang Road, Taichung, 40705 Taiwan.

E-mail: cylindro@vghtc.gov.tw

Article History:
Received: Apr 30, 2009
Revised: May 14, 2009
Accepted: Jun 23, 2009

Copyright © 2010 Taiwan Society of Microbiology. Published by Elsevier Taiwan LLC. All rights reserved.

Introduction

Escherichia coli, Klebsiella pneumoniae, Proteus mirabilis, Enterobacter cloacae, Serratia marcescens, and *Citrobacter freundii* account for the majority of *Enterobacteriaceae* isolated from clinical specimens. The emergence of multidrug resistant *Enterobacteriaceae* is of particular concern because of the potential for widespread dissemination, and difficulties in treating infected patients.[1,2]

The majority of plasmid-mediated *ampC* genes are found in nosocomial isolates of *E. coli* and *K. pneumoniae. E. cloacae, C. freundii*, *S. marcescens*, and *Morganella morganii* are characterized by chromosomally encoded AmpC β-lactamases and possess the ability to develop resistance upon exposure to broad-spectrum cephalosporins.[3] These genes can confer broad-spectrum resistance to most β-lactams (other than cefepime and carbapenems) and hence pose a major therapeutic challenge.[4,5] According to the criteria of the Clinical and Laboratory Standards Institute (CLSI),[6] all isolates with minimum inhibitory concentrations (MICs) ≥ 2 μg/mL for ceftazidime, ceftriaxone, cefotaxime or aztreonam are potential extended spectrum β-lactamase (ESBL) producers. The CLSI recommends that a confirmatory clavulanic acid inhibition test be performed on these "screen test"-positive clinical isolates before reporting the susceptibility results for cephalosporins, and aztreonam.[6] *Enterobacteriaceae* isolates with a positive screen test of ESBL phenotype, but a negative ESBL confirmatory test, are potential candidates for production of the AmpC enzyme, either mediated by chromosomal depression or transferred by a plasmid.[7] Many clinical microbiologists appear to be unaware of the presence of plasmid-mediated AmpC β-lactamase enzymes in resistant isolates because phenotypic detection can be difficult and the strains may be misidentified as ESBL producers.[8] Many methods for the detection of ESBLs, plasmid-mediated AmpC β-lactamases, and carbapenemases have been proposed. However, some of these procedures are technically demanding and time-consuming, others are hard to interpret, and still others require specialized reagents or reagents that are difficult to obtain.[9]

The presence of multiple β-lactamases within one organism (e.g. multiple ESBLs or ESBL-AmpC combinations) can make phenotypic identification difficult.[10] Unfortunately, for these reasons, plasmid-mediated AmpC β-lactamase resistance goes undetected in most clinical laboratories.[10]

A multiplex polymerase chain reaction (PCR) method has been developed using six primer pairs to detect plasmid-mediated *ampC* genes. This technique is capable of identifying the family-specific *ampC* gene responsible for AmpC β-lactamase expression.[11]

Seventeen of 110 (15.5%) *E. cloacae* isolates from a Central Taiwan hospital were identified as ESBL-producers (predominantly SHV-12, with some isolates also producing CTX-M-3 and CTXM-9).[7] Fifteen of 123(12.2%) *S. marcescens* isolates from the same hospital were ESBL-producers, and all carried *CTX-M-3*.[12] In a study from Northern Taiwan, *SHV-12* was present in 26 isolates of *E. cloacae*, with the coexistence of *CTX-M-3* in three of the isolates.[13] However, studies of the AmpC β-lactamases present in *Enterobacteriaceae* has not been performed in Taiwan. The aim of this study was to evaluate the antimicrobial susceptibility of three members of the *Enterobacteriaceae* family (*E. cloacae*, *C. freundii*, and *S. marcescens*) found in Taichung Veterans General Hospital (TCVGH) during the past 5 years, and to screen for the related *ampC* resistant genes using multiplex PCR.

Materials and Methods

Isolates

Thirty isolates of each of the *E. cloacae*, *C. freundii*, and *S. marcescens* were collected from the Microbiology Laboratory of TCVGH between 2003 and 2007. All isolates were collected from blood specimens.

Screening test of ESBL phenotype

Screening tests were performed on all the isolates as follows: the MICs for ceftazidime were determined using the E-test (AB BIODISK, Solna, Sweden). Disk diffusion tests for ceftazidime and cefotaxime were performed according to CLSI recommendations.[6] An isolate was considered positive if the MIC for ceftazidime was ≥ 2 μg/mL, if the inhibition zone for ceftazidime was ≤ 22 mm, or the inhibition zone for cefotaxime was ≤ 27 mm.

Confirmatory test

All isolates with a positive screening test were further tested using the confirmatory test according to CLSI

recommendations.[6] An isolate was considered to have a positive confirmatory test if it showed a ≥5 mm increase in zone diameter for either ceftazidime or cefotaxime tested in combination with clavulanic acid versus its zone when tested alone.

Susceptibility test

The MICs of five antimicrobial agents; flomoxef (Shionogi, Osaka, Japan), imipenem (MSD, Rahway, NJ, USA), moxifloxacin (Bayer, Leverkusen, Germany), colistin (TTY, Hsinchu, Taiwan) and ceftazidime (GSK, UK) were assessed using the broth microdilution method according to CLSI recommendations.[6] The antibiotics were serially diluted two-fold in 50 μL of cation-adjusted Mueller-Hinton broth. The final range of antibiotic concentrations was 0.01–256 μg/mL. The bacterial suspension was prepared from actively growing bacteria in 5 mL of cation-adjusted Mueller-Hinton broth, and diluted to a bacterial cell density of 10^6 colony forming units (CFU)/mL. Five μL of bacterial suspension was then added to wells containing 100 μL of serially diluted antimicrobial agents to yield a final inoculum of approximately 5×10^4 CFU/mL. The MICs were read after overnight incubation (18–24 hours) at 35°C. All MICs were determined in duplicate. *E. coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as the quality control strains.[14]

Multiplex PCR protocol for detection of AmpC genes

Total bacterial DNA was prepared using guanidinium thiocyanate as previously described.[11,15] PCR was performed in a final volume of 50 μL. The primers used for PCR amplification are listed in Table 1.[11] Each reaction contained 45 μL of the master mix [20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 0.6 μM forward and 0.6 μM reverse primers MOXM, CITM, CITM, DHAM, and DHAM; 0.5 μM primers ACCM, ACCM, EBCM, and EBCM, 0.4 μM primers FOXM and FOXM, and 1.25 U of *Taq* DNA polymerase (QIAGEN, GmbH, Hilden, Germany)] and 5 μL of template DNA. PCR mixtures with the addition of water in place of template DNA were used as negative controls. The PCR program consisted of an initial denaturation step at 94°C for 3 minutes, followed by 25 cycles of DNA denaturation at 94°C for 30 seconds, primer annealing at 64°C for 30 seconds, and primer extension at 72°C for 1 minute. After the last cycle, a final extension step at 72°C for 7 minutes was added. PCR products (5 μL) were resolved in 2.5% agarose gel (Bio-Rad, Hercules, California, USA). Gels were stained with 10 μg/mL ethidium bromide and visualized by UV transillumination. A 100 bp DNA ladder (Gene DireX, USA) was used.[16]

Multiplex PCR for the detection of ESBL genes

To detect the coexistence of ESBL genes, three primer pairs for screening the bla_{TEM}, bla_{SHV}, and bla_{CTX-M} genes were used in the PCR reaction.[17] For further identification of the most prevalent ESBL types, the following primer pairs were used: *CTX-M-3*,[18,19] *CTX-M-14*,[19] and *SHV-5*.[20]

Results

The antibiotic susceptibility of *E. cloacae*, *C. freundii* and *S. marcescens* is summarized in Table 2. Ceftazidime had variable activity against *E. cloacae*, *C. freundii* and *S. marcescens* with susceptibility rates of 53%, 60%, and 100%, respectively. Flomoxef was less active, with susceptibility rates of 10%, 10%, and 57%, respectively. Imipenem was the most active agent, with susceptibility rates of 97%, 100%, and 87%, respectively. Moxifloxacin had moderate activity, with susceptibility rates of 53%, 87%, and 67%, respectively. Colistin was the least active, with susceptible rates of 0%, 50%, and 0%, respectively.

Table 3 shows the number of isolates with the ESBL phenotype and related resistant genes. Nineteen *E. cloacae* isolates had a positive screening test for cefotaxime by the disk diffusion. Six *E. cloacae* isolates had a positive ESBL confirmatory test, and one of these six isolates carried *ESBL* genes. Thirteen *E. cloacae* isolates had a negative ESBL confirmatory test, and two of these carried *ESBL* genes. Only three of 19 *E. cloacae* isolates were detected by ESBL confirmatory test but four isolates were noted by the multiple PCR test in the following study (Table 4). Eighteen isolates had a positive screening test for cefotaxime by disk diffusion, while 16 were positive for by ceftazidime broth microdilution. Five *C. freundii* isolates had a positive ESBL confirmatory test, and two carried an *ESBL* gene. Thirteen *C. freundii* isolates had a negative ESBL confirmatory test, and one of these 13 isolates carried *ESBL* genes. Four of 18 *C. freundii* isolates contained *ampC* genes. Among the 30 *S. marcescens* isolates, 16 had a positive screening test

Table 1. Primers used for amplification of AmpC

Gene	Accession number[a]	Primer	Sequence	Nucleotide positions	Target(s)	Product size (bp)
MOXM	D13304	forward	5′-GCT GCT CAA GGA GCA CAG GAT-3′	358–378	MOX-1, MOX-2, CMY-1,	520
		reverse	5′-CAC ATT GAC ATA GGT GTG GTG C-3′	877–856	CMY-8 to CMY-11	
CITM	X78117	forward	5′-TGG CCA GAA CTG ACA GGC AAA-3′	478–498	LAT-1 to LAT-4,	462
		reverse	5′-TTT CTC CTG AAC GTG GCT GGC-3′	939–919	CMY-2 to CMY-7, BIL-1	
DHAM	Y16410	forward	5′-AAC TTT CAC AGG TGT GCT GGG T-3′	1,244–1,265	DHA-1, DHA-2	405
		reverse	5′-CCG TAC GCA TAC TGG CTT TGC-3′	1,648–1,628		
ACCM	AJ133121	forward	5′-AAC AGC CTC AGC AGC CGG TTA-3′	861–881	ACC	346
		reverse	5′-TTC GCC GCA ATC ATC CCT AGC-3′	1,206–1,186		
EBCM	M37839	forward	5′-TCG GTA AAG CCG ATG TTG CGG-3′	1,115–1,135	MIR-1T, ACT-1	302
		reverse	5′-CTT CCA CTG CGG CTG CCA GTT-3′	1,416–1,396		
FOXM	X77455	forward	5′-AAC ATG GGG TAT CAG GGA GAT G-3′	1,475–1,496	FOX-1 to FOX-5b	190
		reverse	5′-CAA AGC GCG TAA CCG GAT TGG-3′	1,664–1,644		

[a]Sequence from Genebank and used for primer design.

for cefotaxime by disk diffusion, while one had a positive screening test for ceftazidime by broth microdilution. Four *S. marcescens* isolates had a positive ESBL confirmatory test, and two of them carried *ESBL* genes. Twelve *S. marcescens* isolates had a negative ESBL confirmatory test, and no *ESBL* genes were detected. None of these 16 *S. marcescens* isolates carried *ampC* genes.

The isolates of *E. cloacae, C. freundii, and S. marcescens* with their resistance genes and related MICs for each of the five antibiotics are listed in Table 4. The *E. cloacae* isolate, E_2, had an AmpC enzyme of the DHA type, and the *TEM* gene. The *E. cloacae* isolates, E_3 and E_{14}, had AmpC enzymes of the EBC type. The *E. cloacae* isolate, E_5, had an AmpC enzyme of the EBC type, and the *TEM* gene. The *E. cloacae* isolate, E_{19}, had only the *TEM* gene. All four of these isolates had low to high level resistance to ceftazidime with MICs ranging from 2 μg/mL to >256 μg/mL, and high level resistance to flomoxef with MICs ranging from 128 μg/mL to >256 μg/mL. The *C. freundii* isolate, C_6, had both the *TEM* and *SHV* genes. Isolates, C_{16}, C_{17}, had an AmpC enzyme of the CIT type only and isolate C_{22} had an AmpC enzyme of the CIT type, and the *TEM* and *CTX-M-3-like* genes. Isolate C_{25} had an AmpC enzyme of the CIT type and the *TEM* gene. All five isolates had low to medium level resistance to ceftazidime with MICs ranging from 2 μg/mL to >32 μg/mL, and intermediate to medium level resistance to flomoxef with MICs ranging from 32 μg/mL to 128 μg/mL. The *S. marcescens* isolate, S_5, had only the *TEM* gene. The *S. marcescens* isolate, S_7, had both the *TEM* and *CTX-M-3-like* genes. These two isolates were susceptible to ceftazidime with MICs ranging from 1 μg/mL to 2 μg/mL, and were also susceptible to flomoxef with MICs ranging from 2 μg/mL to 16 μg/mL.

Discussion

Colistin was less active against the three species of *Enterobacteriaceae* in this study, although it displayed good activity against *Acinetobacter* spp., *Klebsiella* spp., and *E. coli* in the previous study.[21] In a recent report, *Enterobacter* spp. showed low rates of susceptibility to the five fluoroquinolones: ciprofloxacin, ofloxacin, norfloxacin, levofloxacin, and gemifloxacin.[22] In contrast, moxifloxacin showed moderate activity against the three species in this study, although cross-resistance was common in the ESBL-producing *Enterobacteriaceae*.

The E_{19} isolate of *E. cloacae* carried the *TEM* gene alone, and was resistant to both ceftazidime and flomoxef

Table 2. Antibiotic susceptibility of *Enterobacter cloacae*, *Citrobacter freundii*, and *Serratia marcescens*

Antibiotics	*E. cloacae* ($n=30$)				*C. freundii* ($n=30$)				*S. marcescens* ($n=30$)			
	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	S (%)	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	S (%)	MIC range (μg/mL)	MIC_{50} (μg/mL)	MIC_{90} (μg/mL)	S (%)
Ceftazidime	0.125 to >256	2	256	53	0.25–256	2	128	60	0.06–2	0.25	0.5	100
Flomoxef	0.125 to >256	256	>256	10	0.5 to >256	64	256	10	0.5–256	16	64	57
Imipenem	0.125–8	1	2	97	0.25–2	1	2	100	1–32	4	8	87
Moxifloxacin	0.06–64	2	16	53	0.06–16	0.5	4	87	0.5–32	2	16	67
Colistin	4 to >256	8	>256	0	1–8	2	8	50	256 to >256	256	>256	0

MIC = minimum inhibitory concentration; S = susceptibility.

with MICs > 256 μg/mL. Flomoxef was less active than ceftazidime against both *E. cloacae* and *C. freundii* in this study. Flomoxef is an oxacephem antibiotic, and is supposed to be active against *Enterobacteriaceae,* producing a TEM β-lactamase. Further work is necessary to determine whether this was a new β-lactamase capable of hydrolyzing cephamycins.

Among the 19 *E. cloacae* isolates with a positive screening test for ESBL, four carried *ampC* genes of either the DHA or EBC types. Among the 18 *C. freundii* isolates with a positive screening test for ESBL, four carried an *ampC* gene of the CIT type. All these isolates showed high-level resistance to flomoxef (MIC > 128 mg/mL). Hence, flomoxef resistance in combination with a positive ESBL screening test could serve as sensitive indicator of AmpC enzyme production.

The screening and confirmatory tests have proven to be reliable for detecting the majority of conventional ESBLs, especially for variants of the TEM and SHV enzyme classes. We have tried our best to analyze the different ESBL types using six different primer pairs. The *SHV* gene was found in a *C. freundii* isolate, the *CTX-M-3-like* gene was found in a *C. freundii* isolate and in a *S. marcescens* isolate. Previous studies show that SHV-12 is the predominant *E. cloacae* ESBL type in Taiwan.[7,13,23] In contrast, the percentage of *SHV* genes found in this study was less than that found in previous studies. This discrepancy could be due to the limited number of isolates used in this study, or to the different distribution of ESBL types in different hospitals. The *TEM-1* gene was found to coexist with the *SHV-12* gene in the 13 *E. cloacae* isolates in studies by Yu et al and Ma et al.[7,23] Three *E. cloacae* isolates in this study were found to have the TEM class of β-lactamases. Identification of TEM types could be made by isoelectric focusing (IEF) and sequence analysis. However, this was beyond the scope of this study.

In a recent study conducted by the SENTRY Asia-Pacific surveillance program,[24] 52 *E. coli* isolates and 68 *K. pneumoniae* isolates with negative ESBL confirmatory tests, as well as a comparable number of isolates with confirmed ESBL-positive tests, were examined for the presence of the *TEM*, *SHV*, plasmid-borne *ampC*, and *CTX-M* genes. Interestingly, 62% of non-confirmed *E. coli* isolates and 75% of non-confirmed *K. pneumoniae* isolates harbored a plasmid-borne AmpC enzyme of the CIT or DHA type.

Table 3. Number of isolates with the extended spectrum β-lactamase phenotype and related resistant genes

	E. cloacae (*n*=30)	*C. freundii* (*n*=30)	*S. marcescens* (*n*=30)
Positive screening test			
Ceftazidime[a]	13	14	3
Cefotaxime[b]	19	18	16
Ceftazidime[c]	15	16	1
ESBL confirmatory test			
PCT (gene)	6	5	4
With ESBL genes	1	2	2
With ampC genes	0	1	0
NCT (gene)	13	13	12
With ESBL genes	2	1	0
With ampC genes	3	3	0

[a]Disk diffusion (inhibition zone ≤22 mm); [b]disk diffusion (inhibition zone≤27 mm); [c]broth dilution (MIC≥2 μg). ESBL=extended spectrum β-lactamase; PCT=positive confirmatory test; NCT=negative confirmatory test.

Table 4. The *Enterobacter cloacae*, *Citrobacter freundii*, and *Serratina marcescens* isolates with their resistance genes and related minimum inhibitory concentrations for the five antibiotics

	E. cloacae					*C. freundii*					*S. marcescens*	
	E_2	E_3	E_5	E_{14}	E_{19}	C_6	C_{16}	C_{17}	C_{22}	C_{25}	S_5	S_7
Resistance genes												
ampC	DHA	EBC	EBC	EBC	–	–	CIT	CIT	CIT	CIT		
ESBL	TEM	–	TEM	–	TEM	TEM/SHV	–	–	TEM/ CTX-M-3-like	TEM	TEM	TEM CTX-M-3-like
Confirmatory test	–	–	+	–	–	–	–	–	–	–	+	+
MICs (μg/mL)												
Ceftazidime	2	16	256	0.25	>256	2	32	2	8	8	2	1
Flomoxef	128	>256	>256	256	>256	32	128	32	128	64	16	2
Imipenem	2	2	1	2	1	1	1	2	1	1	4	2
Moxifloxacin	1	2	8	64	16	0.125	0.125	0.5	16	0.06	16	2
Colistin	8	>256	16	>256	16	8	2	2	2	4	>256	256

MIC=Minimum inhibitory concentration; ESBL=extended spectrum β-lactamase.

It is not uncommon for *Enterobacteriaceae* to carry multiple resistance genes. Among the 52 *E. coli* isolates with a non-confirmed ESBL test in the previously mentioned SENTRY Asia-Pacific surveillance program,[24] 26 isolates (50%) carried AmpC+TEM enzymes. Similarly, this study showed that one isolate of *E. cloacae* had DHA+TEM enzymes while a second had EBC+TEM enzymes; two isolates of *C. freundii* had CIT+TEM+CTX-M-3-like enzymes, and CIT+TEM enzymes respectively. For the *Enterobacteriaceae* isolates carrying both the ESBL and AmpC enzymes, the phenotype appeared to be a positive screening test and negative confirmatory test. The AmpC enzyme can hydrolyze clavulanic acid, and thus make the confirmatory test negative. We also found 12 isolates of the three species with a positive screening test in which we were unable to detect any β-lactamase genes. These

isolates may produce enzymes not covered by the selected primer pairs, or may have mutations affecting the porin channels responsible for antimicrobial uptake.

The prevalence of AmpC-mediated resistance worldwide is not well known due to the limited number of surveillance studies for AmpC-producing isolates and the difficulty in accurately detecting these resistance mechanisms.[11] The MYSTIC programs in Europe and the United States reported that the rates of *ampC* genes were much higher in *Citrobacter* spp. (9–25%) and *Enterobacter* spp. (13.8–29.2%) than in *E. coli* (0.6–2.8%) or *Klebsiella* spp. (0.5–4.5%).[25] In our study, *ampC* genes were detected in 13.3% of *E. cloacae* and *C. freundii* isolates, but not in any of the *S. marcescens* isolates.

In a previous study, the coexistence of *AmpC* (*DHA-1*, *CMY-2*, or *CMY-8*) and *ESBLs* (*CTX-M* and/or *SHV*) was detected in 35 of 99 clinical *K. pneumoniae* isolates resistant to cefoxitin and extended-spectrum cephalosporins.[26] The CMY-2-like β-lactamase was detected in 127 of 291 (43.6%) *E. coli* isolates. Among 282 *K. pneumoniae* isolates, the CMY-2-like and DHA-1-like β-lactamases were detected in 10 (3.5%) and 31 (11.0%) isolates, respectively.[27]

The CIT primer pair amplifies the family-specific genes LAT-1 to LAT-4, CMY-2 to CMY-7, and BIL-1. The DHA primer pair amplifies the family-specific genes DHA-1 and DHA-2.[11] In our study, both *CIT* and *DHA* were detected in those resistant isolates by the screening primers. However, the specific subtypes need to be sequenced further, or subjected to IEF.

In summary, *Enterobacteriaceae* carry AmpC or ESBL enzymes that can be identified by a positive screening test and elevated MIC results. Positive confirmatory tests for ESBL indicate ESBL production. However, negative confirmatory tests do not exclude ESBL, but indicate the presence of *ampC* or a combination of *ampC* and ESBL. Antibiotic phenotypes cannot accurately distinguish between the resistance mechanisms caused by *ampC* or ESBL, especially where *ampC*-ESBL combinations are concerned. However, PCR is a useful technique for the identification of the different types of resistance genes.

Acknowledgments

We thank the staff in the Microbiology Laboratory at TCVGH for the collection of bacterial isolates.

References

1. Yu WL, Chuang YC, Walther-Rasmussen J. Extended-spectrum beta-lactamases in Taiwan: epidemiology, detection, treatment and infection control. *J Microbiol Immunol Infect* 2006;39:264–77.
2. Karlowsky JA, Jones ME, Thornsberry C, Friedland IR, Sahm DF. Trends in Antimicrobial Susceptibilities among *Enterobacteriaceae* Isolated from Hospitalized Patients in the United States from 1998 to 2001. *Antimicrob Agents Chemother* 2003;47:1672–80.
3. Choi SH, Lee JE, Park SJ, Choi SH, Lee SO, Jeong JY et al. Emergence of Antibiotic Resistance during Therapy for Infections Caused by *Enterobacteriaceae* Producing AmpC β-Lactamase: Implications for Antibiotic Use. *Antimicrob Agents Chemother* 2008; 52:995–1000.
4. Odeh R, Kelkar S, Hujer AM, Bonomo RA, Schreckenberger PC, Quinn JP. Broad resistance due to plasmid-mediated AmpC beta-lactamases in clinical isolates of *Escherichia coli*. *Clin Infect Dis* 2002;35:140–5.
5. Philippon A, Arlet G, Jacoby GA. Plasmid-determined AmpC-type beta-lactamases. *Antimicrob Agents Chemother* 2002;46:1–11.
6. Clinical and Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing*. 16th Informational Supplement. M100-S16. Wayne, PA: CLSI; 2006.
7. Yu WL, Cheng KC, Chi CJ, Chen HE, Chuang YC, Wu LT. Characterization and molecular epidemiology of extended-spectrum beta-lactamase-producing *Enterobacter cloacae* isolated from a district teaching hospital in Taiwan. *Clin Microbiol Infect* 2006; 12:579–82.
8. Hanson ND. AmpC beta-lactamases: what do we need to know for the future? *J Antimicrob Chemother* 2003;52:2–4.
9. Jacoby GA, Walsh KE, Walker VJ. Identification of Extended-Spectrum, AmpC, and Carbapenem-Hydrolyzing β-Lactamases in *Escherichia coli* and *Klebsiella pneumoniae* by Disk Tests. *J Clin Microbiol* 2006;44:1971–6.
10. Thomson KS. Controversies about extended-spectrum and AmpC beta-lactamases. *Emerg Infect Dis* 2001;7:333–6.
11. Pérez-Pérez FJ, Hanson ND. Detection of Plasmid-Mediated AmpC beta-Lactamase Genes in Clinical Isolates by Using Multiplex PCR. *J Clin Microbiol* 2002;40:2153–62.
12. Yu WL, Wu LT, Pfaller MA, Winokur PL, Jones RN. Confirmation of extended-spectrum beta-lactamase-producing *Serratia marcescens*: preliminary report from Taiwan. *Diagn Microbiol Infect Dis* 2003;45:221–4.
13. Wu TL, Chia JH, Su LH, Kuo AJ, Chu C, Chiu CH. Dissemination of extended-spectrum beta-lactamase-producing Enterobacteriaceae in pediatric intensive care units. *J Clin Microbiol* 2003; 41:4836–38.
14. Hung PP, Lin YH, Lin CF, Liu MF, Shi ZY. *Chryseobacterium meningosepticum* infection: antibiotic susceptibility and risk factors for mortality. *J Microbiol Immunol Infect* 2008;41:137–44.
15. Deshpande LM, Jones RN, Fritsche TR, Sader HS. Occurrence of plasmidic AmpC type beta-lactamase-mediated resistance in

Escherichia coli: report from the SENTRY Antimicrobial Surveillance Program (North America, 2004). *Int J Antimicrob Agents* 2006;28:578–81.
16. Pitcher DG, Saunders NA, Owen RJ. Rapid extraction of bacterial genomic DNA with guanidium thiocyanate. *Lett Appl Microbiol* 1989;8:151–6.
17. Monstein HJ, Ostholm-Balkhed A, Nilsson MV, Nilsson M, Dornbusch K, Nilsson LE. Multiplex PCR amplification assay for the detection of *bla*SHV, *bla*TEM and *bla*CTX-M genes in *Enterobacteriaceae*. *APMIS* 2007;115:1400–8.
18. Cheng KC, Chuang YC, Wu LT, Huang GC, Yu WL. Clinical experiences of the infections caused by extended-spectrum beta-lactamase-producing *Serratia marcescens* at a medical center in Taiwan. *Jpn J Infect Dis* 2006;59:147–52.
19. Yu WL, Winokur PL, Von Stein DL, Pfaller MA, Wang JH, Jones RN. First description of Klebsiella pneumoniae Harboring CTX-M β-Lactamases (CTX-M-14 and CTX-M-3) in Taiwan. *Antimicrob Agents Chemother* 2002;46:1098–100.
20. Malik A, Celik EK, Bohn C, Böckelmann U, Knobel K, Grohmann E. Detection of conjugative plasmids and antibiotic resistance genes in anthropogenic soils from Germany and India. *FEMS Microbiol Lett* 2008;279:207–16.
21. Tan TY, Ng LS. Comparison of three standardized disc susceptibility testing methods for colistin. *J Antimicrob Chemother* 2006; 58:864–7.
22. Gönüllü N, Aktas Z, Salcioglu M, Bal C, Ang Ö. Comparative in vitro activities of five quinolone antibiotics, including gemifloxacin, against clinical isolates. *Clin Microbiol Infect* 2001;7: 499–503.
23. Ma L, Chang FY, Fung CP, Chen TL, Lin JC, Lu PL, et al. Variety of TEM-, SHV-, and CTX-M-type beta-lactamases present in recent clinical isolates of *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* from Taiwan. *Microb Drug Resist* 2005;11: 31–9.
24. Bell JM, Chitsaz M, Turnidge JD, Barton M, Walters LJ, Jones RN. Prevalence and Significance of a Negative Extended-Spectrum β-Lactamase (ESBL) Confirmation Test Result after a Positive ESBL Screening Test Result for Isolates of *Escherichia coli* and *Klebsiella pneumoniae*: Results from the SENTRY Asia-Pacific Surveillance Program. *J Clin Microbiol* 2007;45:1478–82.
25. Goossens H, Grabein B. Prevalence and antimicrobial susceptibility data for extended-spectrum β-lactamase- and AmpC-producing Enterobacteriaceae from the MYSTIC Program in Europe and United States (1997–2004). *Diagn Microbiol Infect Dis* 2005;53:257–64.
26. Yan JJ, Ko WC, Wu HM, Tsai SH, Shuang CL, Wu JJ. Complexity of *Klebsiella pneumoniae* isolates resistant to both cephamycins and extended-spectrum cephalosporins at a teaching hospital in Taiwan. *J Clin Microbiol*. 2004;42:5337–40.
27. Yan JJ, Hsueh PR, Lu JJ, Chang FY, Shvr JM, Wan JH, et al. Extended-spectrum β-lactamases and plasmid-mediated AmpC enzymes among clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae* from seven medical centers in Taiwan. *Antimicrob Agents Chemother* 2006;50:1861–4.