Original Article
Clinical Microbiology

Ann Lab Med 2012;32:270-275
http://dx.doi.org/10.3343/alm.2012.32.4.270
ISSN 2234-3806 • eISSN 2234-3814

ANNALS OF LABORATORY MEDICINE

Characterization of Carbapenemase Genes in *Enterobacteriaceae* Species Exhibiting Decreased Susceptibility to Carbapenems in a University Hospital in Chongqing, China

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Background: Our study was to investigate the prevalence of carbapenemase genes in strains of *Enterobacteriaceae* species exhibiting decreased susceptibility to carbapenems in our hospital.

Methods: The carbapenemase producing *Enterobacteriaceae* species were confirmed by modified Hodge test (MHT) and EDTA-disc synergy test which indicating the production of class B carbapenemases. PCR and sequencing analysis were used to identify the drug-resistant genes. DNA fingerprinting based on enterobacterial repetitive intergenic consensus (ERIC)-PCR was applied to investigate the homology of *Enterobacteriaceae* species.

Results: From a collection of 1,472 *Enterobacteriaceae* species, 18 isolates with decreased susceptibility to carbapenem treatment were identified and 9 of which were positive by MHT, and 6 of which produced class B carbapenemases. PCR and sequencing analysis of the 18 isolates revealed 4 different carbapenemase genes (bla_{IMP-8}, bla_{oxa-1}, bla_{IMP-26}, and bla_{oxa-47}) in 10 isolates, with the bla_{IMP-8} and bla_{oxa-1} genes being the most common (60-70% prevalence). ERIC-PCR showed 5, 2, and 2 unique genotypes for *Enterobacter cloacae, Escherichia coli,* and *Klebsiella pneumoniae,* respectively. Three *E. coli* strains isolated from different patients from the urologic surgery department exhibited the same DNA banding pattern, suggesting a possible clonal dissemination. Majority (17/18) of the carbapenem-unsusceptible *Enterobacteriaceae* species isolates was obtained from the surgery department of our hospital.

Conclusions: The main carbapenemase genes of *Enterobacteriaceae* species in our hospital were bla_{IMP-8} and bla_{oxa-1}. Prevalence of carbapenem resistance may be existed in surgery department and infection control should be taken for preventing further dissemination of drug-resistant strains.

Key Words: *Enterobacteriaceae* species, Carbapenemases, Carbapenems

Received: January 26, 2012
Revision received: March 4, 2012
Accepted: May 25, 2012

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INTRODUCTION

Enterobacteriaceae species are among the most common nosocomial pathogens, causing serious infections in various organs and tissues. Currently, carbapenems are the most potent agents prescribed for the treatment of serious infections caused by *Enterobacteriaceae* species because of their broad spectra of antibacterial activity and their excellent stability to hydrolysis by most β-lactamases, including extended-spectrum β-lactamases (ESBLs) and AmpC cephalosporinases. However, the wide-

spread use of carbapenems has led to the emergence of carbapenem-resistant *Enterobacteriaceae* species in diverse geographic locations worldwide, and this is becoming an important therapeutic challenge in the clinic setting [1-3].

The main mechanisms of carbapenem resistance in *Enterobacteriaceae* species include the acquisition of carbapenemases and hyperproduction of AmpC cephalosporinases, in combination with porin loss [4]. Carbapenemases are members of the molecular class A, B, and D β-lactamases, which have the ability to hydrolyze penicillins, cephalosporins, monobactams, and carbapenems [4]. Class A serine carbapenemases include 3 major families of NMC/IMI, SME, and KPC enzymes and can be inhibited by clavulanic acid and tazobactam [5]. Among the class A carbapenemases, KPC-2 is the most common type reported in China [6, 7]. Class B carbapenemases, also called metallo-β-lactamases (MBLs), are resistant to the commercially available β-lactamase inhibitors, such as clavulanic acid, sulbactam, and tazobactam, but susceptible to inhibition by metal ion chelators, such as EDTA, a chelator of Zn^{2+} and other divalent cations [8]. In the past decade, a number of acquired MBLs have been identified and categorized into 2 major groups: IMP- and VIM-type enzymes. IMP-4 and IMP-8 carbapenemases have been detected in China, and these have led to a low to moderate level of carbapenem resistance in strains of *Enterobacteriaceae* species [9]. The hydrolysis of carbapenems by the class D oxacillinase family is weak and leads to reduced susceptibility to imipenem and meropenem but with the minimal inhibitory concentration (MIC) still in the susceptible range, thus potentially causing detection failures [10].

The goals of this study were to investigate the prevalence of carbapenemase genes in clinical strains of *Enterobacteriaceae* species isolated from a university hospital, and to explore the main mechanisms of decreased susceptibility to carbapenems in these clinical strains.

METHODS

1. Bacterial strains and susceptibility tests

All patient specimens utilized in this study were from The First Affiliated Hospital of Chongqing Medical University, which has 2,500 inpatient beds and is one of the largest hospitals in the southwest of China. Samples were collected from November 2009 to December 2010. The clinical isolates were identified and the susceptibility tests were performed by using the Vitek2 Compact System with GN card and ASTGN13 card (bioMérieux, Marcy l'Etoile, France). Strains of *Enterobacteriaceae* species with decreased susceptibility to carbapenems (MIC of imipenem, meropenem, or ertapenem ≥2 μg/mL) were consecutively collected and confirmed by the agar dilution method, according to the guidelines of the CLSI [11].

2. Detection of carbapenemases

Modified Hodge Tests (MHT) were carried out according to CLSI recommendations for phenotypic screening of carbapenemase producers among species of *Enterobacteriaceae* [11]. *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC BAA-1705 were used as negative and positive controls, respectively. The class A and B carbapenemases were screened by clavulanic acid-disc synergy tests and EDTA-disc synergy tests, respectively, as previously described [12, 13].

3. PCR amplification and DNA sequencing

Total DNA was extracted from all strains by 10 min boiling of bacterial culture, followed by 1 min centrifugation at 15,000 rpm. The supernatant was collected and used for PCR amplification. The main class A, class B, and class D carbapenemase genes were amplified using the primers and conditions described in the references listed in Table 1 [14-19]. In addition, 3 ESBL genes (*bla*SHV, *bla*TEM, and *bla*CTX-M) and 5 plasmid-mediated AmpC cephalosporinase genes (*bla*CYM-2, *bla*ACT-1, *bla*ACC-1, *bla*DHA-1, and *bla*FOX-1) were amplified by PCR as previously described [20, 21], and all PCR products were sequenced directly in an ABI PRISM 3730XL sequencer (Applied

Table 1. Oligonucleotides used as primers for PCR and sequencing in this study

Class	Target	Sequence (5′-3′)	Amplicon size (bp)	Reference
Class A	SME	Forward: AACGGCTTCATTTTTGTTTAG	820	14
		Reverse: GCTTCCGCAATAGTTTTATCA		
	KPC	Forward: ATGTCACTGTATCGCCGTCT	892	15
		Reverse: TTTTCAGAGCCTTACTGCCC		
Class B	IMP	Forward: CATGGTTTGGTGGTTCTTGT	488	16
		Reverse: ATAATTTGGCGGACTTTGGC		
	VIM	Forward: AGTGGTGAGTATCCGACA	280	17
		Reverse: ATGAAAGTGCGTGGAGAC		
	NDM-1	Forward1: GGCGGAATGGCTCATCACGA	287	18
		Reverse1: CGCAACACAGCCTGACTTTC		
		Forward2: CAGCACACTTCCTATCTC	293	18
		Reverse2: CCGCAACCATCCCCTCTT		
Class D	OXA	Forward: TTTTCTGTTGTTTGGGTTTT	519	19
		Reverse: TTTCTTGGCTTTTATGCTTG		

Biosystems, Foster City, CA, USA). DNA sequences were compared with known sequences in GenBank.

4. Strain genotyping

We performed genotyping on all isolates, except for *Enterobacter amnigenus*, by using enterobacterial repetitive intergenic consensus (ERIC)-PCR with primers ERIC1R (5′-ATGTAAGCTCCTGGGGATTCAC-3′) and ERIC2 (5′-AAGTAAGTGACTGGGGTGAGCG-3′) as previously described [22]. Electrophoretic banding patterns were compared by visual inspection. Isolates were considered different if their profiles differed by 2 or more bands [23].

RESULTS

1. Identification of *Enterobacteriaceae* species with decreased susceptibility to carbapenems

From a collection of 1,472 isolates of *Enterobacteriaceae* species, we identified 18 isolates with decreased susceptibility to carbapenem treatment, including 8 isolates of *Enterobacter cloacae*, 5 isolates of *E. coli*, 4 isolates of *K. pneumoniae*, and 1 isolate of *E. amnigenus* (Table 2). The rate of non-susceptibility to carbapenems was 1.22%. Fifteen of the 18 isolates (83.3%) were resistant to ertapenem (≥8 mg/L) and only 2 of the 18 isolates (11.1%) were resistant to imipenem (≥16 mg/L). All of the isolates exhibited multidrug-resistance to the antibiotics tested (data not shown).

2. Screening of carbapenemases

Of the 18 carbapenem-resistant isolates, 9 tested positive in MHT and 6 positive in the EDTA-disc synergy tests, indicating the production of class B carbapenemases in these strains (Table 2). None of them tested positive in the clavulanic acid-disc synergy tests, indicating the absence of class A carbapenemases.

3. PCR amplification and sequence analysis

Carbapenemase genes were detected in 10 of the 18 carbapenem-resistant isolates; the genes included the bla_{IMP-8} gene from 4 *E. cloacae* isolates and 2 *K. pneumoniae* isolates; the bla_{IMP-26} gene from 1 *E. cloacae* isolate; the bla_{oxa-1} gene from 5 *E. cloacae* isolates and 2 *E. coli* isolates; and the bla_{oxa-47} gene from 2 *K. pneumoniae* isolates (Table 2). We failed to detect bla_{SME}, bla_{KPC}, bla_{NDM-1}, or bla_{VIM} type carbapenemase genes in these 18 isolates. ESBL genes were found in all the isolates except for the 2 *E. cloacae* isolates that harbored the bla_{IMP-8} gene. AmpC cephalosporinase genes were detected in 7 of the 18 isolates: including the bla_{DHA-1} gene from 2 *K. pneumoniae* isolates; the bla_{ACT-1} gene from 1 *E. cloacae* isolate and 1 *E. amnigenus* isolate; and the bla_{CMY-2} gene from 3 *E. coli* isolates. All the AmpC-positive strains, except for 1 *E. cloacae* strain (No. 1), did not harbor carbapenemase genes.

4. Strain genotyping and epidemiological association

Genetic relatedness of all the isolates, except for *E. amnigenus*, was investigated by ERIC-PCR typing. Five distinct ERIC profiles were observed amongst 8 strains of *E. cloacae*, and no obvious clonal association was observed within these strains. Five strains of *E. coli* exhibited 2 distinct ERIC profiles, and the 3 strains presenting the same profile, i.e. showing clonal association, were all isolated from the urologic surgery department. Four strains of *K. pneumoniae* showed 2 ERIC profiles and an epidemiological relationship was not found (Fig. 1). However, the 2 strains with identical carbapenemase genes, ESBL and AmpC cephalosporinase, exhibited the same ERIC profile.

DISCUSSION

Since the widespread use of carbapenems in the clinic, carbapenem-resistant *Enterobacteriaceae* species have been detected increasingly worldwide, and a similar trend has been observed in China [9]. Effective and accurate screening for *Enterobacteriaceae* species with decreased susceptibility to carbapenems is important in routine clinical microbiology tests. In this study, we found that the resistance rate of ertapenem was much higher than that of imipenem (83.3% vs. 11.1%, $P<0.01$) in the *Enterobacteriaceae* species isolates studied, indicating that ertapenem was more sensitive than imipenem in the screening of carbapenem-unsusceptible *Enterobacteriaceae* species isolates [11]. Nine of 10 strains of *Enterobacteriaceae* species with carbapenemase genes tested positive in MHT, while none of the strains without any carbapenemase genes tested positive in MHT. The sensitivity and specificity of MHT were 90% and 100%, respectively. Six of 7 strains of *Enterobacteriaceae* species with class B carbapenemase tested positive in the EDTA-disc synergy tests, while none of the strains without class B carbapenemase genes tested positive in the EDTA-disc synergy tests. The sensitivity and specificity of the EDTA-disc synergy tests for MBLs detection were 85.7% and 100%, respectively.

In the 10 strains of *Enterobacteriaceae* species with carbapenemase genes, 4 strains harbored the bla_{IMP-8} and bla_{oxa-1} genes, 2 strains harbored the bla_{IMP-8} and bla_{oxa-47} genes, and 3 strains harbored the bla_{oxa-1} gene alone. These results suggest that the

http://dx.doi.org/10.3343/alm.2012.32.4.270

Table 2. Distribution and drug-resistant mechanisms of 18 strains of *Enterobacteriaceae* species

No.	Bacteria (isolation date)	Distribution	Sample	ETP (mg/L)	IMP (mg/L)	MHT	Carbapenemase genes	ESBLs genes	AmpC genes
1	*E. cloacae*	Neurosurgery	Blood	≥8	2	+	IMP-8	CTX-M14	Act-1
	(11/4/09)						OXA-1		
2	*E. amnigenus*	Center ICU	Secretion	≥8	8	-	Negative	CTX-M14	Act-1
	(12/15/09)								
3	*E. cloacae*	Hepatobiliary surgery	Blood	≥8	≤1	+	IMP-8, TEM	Negative	Negative
	(02/11/10)						OXA-1		
4	*E. cloacae*	Center ICU	Urine	4	≤1	+	IMP-8	Negative	Negative
	(04/23/10)						OXA-1		
5	*E. cloacae*	Outpatient	Secretion	≥8	8	-	Negative	CTX-M3	Negative
	(05/17/10)								
6	*E. cloacae*	Orthopaedics	Tissue	>8	≤1	+	OXA-1	Negative	Negative
	(06/09/10)								
7	*E. cloacae*	Surgery ICU	Bile	≥8	≤1	+	IMP-8	CTX-M14	Negative
	(07/22/10)						OXA-1		
8	*E. cloacae*	Hepatobiliary surgery	Secretion	≥8	2	+	IMP-26	Negative	Negative
	(10/26/10)								
9	*E. cloacae*	Center ICU	Urine	≥8	≤1	-	Negative	Negative	Negative
	(11/20/10)								
10	*E. coli*	Urologic surgery	Calculus	≥8	≤1	-	Negative	CTX-M14	CYM-2
	(04/15/10)								
11	*E. coli*	Urologic surgery	Urine	≥8	2	-	Negative	CTX-M14	CYM-2
	(04/27/10)								
12	*E. coli*	Emergency medicine	Secretion	≥8	≥16	+	OXA-1	CTX-M14	Negative
	(06/15/10)								
13	*E. coli*	Uriologic surgery	Urine	≥8	≤1	-	Negative	CTX-M14	CYM-2
	(06/19/10)								
14	*E. coli*	Orthopaedics	Secretion	4	≤1	-	OXA-1	Negative	Negative
	(03/24/10)								
15	*K. pneumoniae*	Urrologic surgery	Urine	4	2	+	IMP-8	CTX-M14,	Negative
	(12/08/09)						OXA-47	SHV11	
16	*K. pneumoniae*	Gastrointestinal surgery	Ascites	≥8	≤1	-	Negative	SHV11	DHA-1
	(03/14/10)								
17	*K. pneumoniae*	Surgery ICU	Urine	≥8	≥16	+	IMP-8	CTX-M14,	Negative
	(08/19/10)						OXA-47	SHV11	
18	*K. pneumoniae*	Ophthalmology	Secretion	≥8	≤1	-	Negative	CTX-M14,	DHA-1
	(12/06/10)							SHV11	

Abbreviations: *E. cloacae, Enterobacter cloacae; E. amnigenus, Enterobacter amnigenus; E. coli, Escherichia coli; K. pneumoniae, Klebsiella pneumoniae*; ETP, Ertapenem; IMP, Imipenem; MHT, modified Hodge test; ICU, intensive care unit.

most common carbapenemase-type genes in our hospital were bla_{IMP-8} (6/9) and bla_{oxa-1} (7/9). Class A carbapenemase genes and VIM and NDM-1 β-lactamase genes were not detected in our hospital.

The bla_{IMP-8} gene, first found in *K. pneumoniae* in 2001, is very closely related to bla_{IMP-2} in DNA sequence, with only 4 nucleotide

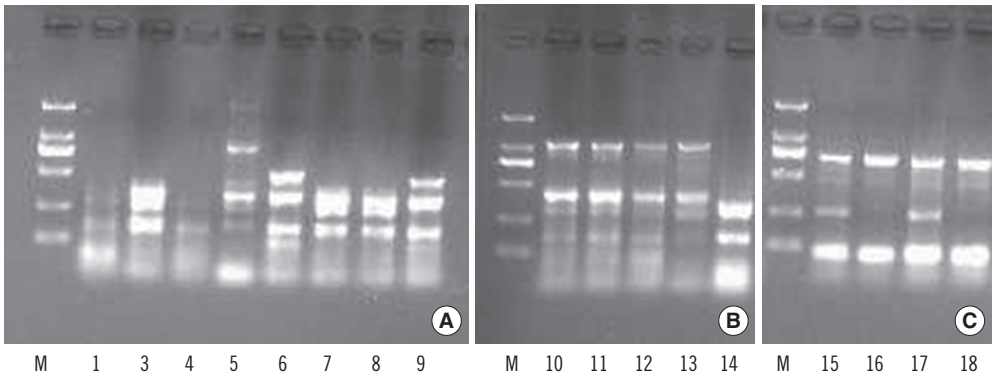

Fig. 1. Representative gel showing banding profiles by ERIC-PCR. (A) *Enterobacter cloacae*; (B) *Escherichia coli*; (C) *Klebsiella pneumoniae*. The number below each lane corresponds to the strain number in Table 2. M: DNA molecular weight marker.

differences between them. This results in 2 amino acid changes. In contrast to the bla_{IMP-2} gene, which is located on the chromosome, the bla_{IMP-8} cassette is located on the plasmid, which would facilitate the spread of the resistance gene [24] and infection outbreak [25, 26]. Like bla_{IMP-8}, class D carbapenemase can only weakly hydrolyze imipenem. All strains with bla_{OXA} carbapenemase genes in our study were susceptible to imipenem except for one *E. coli* isolate (No.12), suggesting that this strain might have other drug-resistant mechanisms, such as loss or lower expression of major porins. In this study, we simultaneously detected bla_{IMP-8} and bla_{OXA-1} in 4 strains of *E. cloacae*. This observation has not been previously reported in China.

Biochemical analysis showed that CMY-2 and ACT-1 β-lactamase had a high catalytic efficiency towards imipenem, with low observed *Km* values. CMY-2, ACT-1, and DHA-1 β-lactamases conferred a high level of resistance to ceftazidime and cefotaxime and significantly reduced the susceptibility to imipenem once expressed in *E. coli* HB4, whilst FOX-1 and ACC-1 enzymes did not confer resistance to imipenem [21]. In our study, 6 of the 9 MHT-negative strains expressed AmpC cephalosporinase genes, including bla_{ACT-1} in 1 *E. cloacae* strain, bla_{CMY-2} in 3 *E. coli* strains, and bla_{DHA-1} in 2 *K. pneumoniae* strains. The bla_{FOX-1} and bla_{ACC-1} genes were not found. This observation suggests that AmpC cephalosporinase hyperproduction may contribute to the resistance observed in these isolates [4]. Five strains were not found to contain any carbapenemase or AmpC cephalosporinase genes, implying that resistance in these strains may involve other mechanisms not investigated in this study.

ERIC-PCR studies demonstrated that the infections caused by 8 strains of *E. cloacae* were spontaneous, because of the absence of genetic relatedness between these strains. Three of 5 *E. coli* strains isolated from the urologic surgery ward showed the same genotype, suggesting a possible clonal dissemination of 1 strain. Although we did not find an obvious clonal association among 4 strains of *K. pneumoniae*, the finding of 2 strains with completely identical ERIC profile and drug-resistant gene profile suggests there is a possibility for these to become a clonal prevalence.

Interestingly, strains expressing the bla_{IMP} carbapenemase genes were not isolated from patients hospitalized in any department in the hospital except the department of surgery, and all the patients with positive of bla_{IMP} carbapenemase genes had received surgery. Surgery has been reported to be an important risk factor for the acquisition of MBL producers [25]. The finding of carbapenem-resistant isolates only in the department of surgery in our hospital may have important implications for the prevention and dissemination control of these drug-resistant pathogens. Because it is likely that the strains of *Enterobacteriaceae* species with decreased susceptibility to carbapenems might be prevalent in the department of surgery in our hospital, strict infection control measures should be implemented in order to prevent infection dissemination.

Authors' Disclosures of Potential Conflicts of Interest

No potential conflicts of interest relevant to this article were reported.

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