

OXA- and MBL-type Enzymes among Uncommonly Isolated *Acinetobacter* spp. in Asia-Pacific Nations: Natural Reservoir for Resistance Determinants

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ICID 2008

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AMENDED ABSTRACT

Background: *Acinetobacter* spp. other than *A. baumannii* are occasionally recovered from clinical sources, but carbapenem-hydrolyzing class D β -lactamases (CHCDB) and metallo- β -lactamase (MBL) genes have rarely been reported. The aim of this study was to identify and characterize OXA and MBL genes in *Acinetobacter* spp. Gene context of *bla*_{OXA-23}-like, namely *bla*_{OXA-133}, detected in *A. radioresistens* was also evaluated.

Methods: *Acinetobacter* spp. recovered from patients in 10 countries in the Asia-Pacific (APAC) region were tested by broth microdilution. Isolates with imipenem or meropenem MIC \geq 8 mg/L were screened for MBL- (IMP-like, VIM-like, SIM-1, GIM-1, SPM-1) and OXA- (OXA-23, -24, -58 clusters) genes. CHCDB genes and surrounding sequences were assessed by PCR using primers targeting to ISAb1, 2 or 3, or degenerate primer approach. Southern blot and hybridization of chromosomal and plasmid DNA were performed. RNA extraction followed by reverse transcriptase-PCR was performed to access expression of *bla*_{OXA-133}. Species identification was confirmed by 16S rRNA.

Results: Among 581 *Acinetobacter* spp. isolates, 26.0% carried CHCDB or MBL (0.35%) genes, from which 0.7% were found in *Acinetobacter* spp. other than *A. baumannii*. An *A. junii* with *bla*_{OXA-23} and an *A. radioresistens* with *bla*_{OXA-133} and *bla*_{OXA-58} were detected in 2 Indian centers, while an *A. johnsonii* with *bla*_{IMP-4} and *A. calcoaceticus* with *bla*_{OXA-58} were identified in the Philippines and China, respectively. ISAb1 and ISAb3 surrounded *bla*_{OXA-23} from *A. johnsonii* and *bla*_{OXA-58} from *A. radioresistens* and *A. calcoaceticus*, respectively. The *A. radioresistens* showed a putative O-sialoglycoprotein endopeptidase-encoding gene upstream of *bla*_{OXA-133}; *bla*_{OXA-133} transcriptional levels were very low; and *bla*_{OXA-133} and *bla*_{OXA-58} were located on the chromosome and plasmid, respectively. Susceptibility to polymyxin and tigecycline (\leq 2 mg/L; no breakpoint criteria) was \geq 98.9%.

Conclusion: High dissemination of CHCDB genes was detected, emphasizing their ability to spread among *Acinetobacter* spp. The data suggest *A. radioresistens* may be a natural reservoir for *bla*_{OXA-23}-like genes. *bla*_{IMP-4} has been previously detected in Hong Kong, Australia and now Philippines, highlighting spread in APAC.

INTRODUCTION

Acinetobacter spp. are opportunistic pathogens, frequently associated with patients having immuno-suppression, serious underlying diseases or invasive procedures and use of broad-spectrum antimicrobial agents. Recently, several reports have described the association of these pathogens with numerous outbreaks and a corresponding increase in resistance rates. As such, carbapenems are important therapeutic options for treating infections caused by *Acinetobacter* spp.; however, these antimicrobial agents may be hydrolyzed by Ambler class B metallo- β -lactamases (MBL) as well as carbapenem-hydrolyzing class D β -lactamases (CHCDB).

Six groups of acquired MBL-encoding genes have been identified; however, only *bla*_{IMP}- and *bla*_{VIM}-like, and more recently *bla*_{SIM-1} have been reported in *Acinetobacter* spp. Conversely, acquired CHCDB-encoding genes have been more frequently detected in *Acinetobacter* spp. and are clustered in three major subfamilies, *bla*_{OXA-23}, *bla*_{OXA-24} and *bla*_{OXA-58}-types. The objective of this surveillance study was to evaluate the occurrence of acquired CHCDB- and MBL-encoding genes among *Acinetobacter* spp. isolates recovered from medical centres in the Asia-Pacific (APAC) region. Additionally, we wished to further characterize a novel *bla*_{OXA-23}-like gene, namely *bla*_{OXA-133}, and *bla*_{OXA-58} detected concurrently in an *A. radioresistens* clinical isolate recovered from a patient in India.

MATERIALS AND METHODS

Bacterial isolates: During 2006-2007, 41 medical centers located in 10 countries in the APAC region were recruited to participate to the SENTRY Program. Species identification was performed by standard biochemical tests and Vitek System (bioMérieux; Hazelwood, Missouri, USA), when necessary. Additionally, species identification was confirmed by sequencing of the 16S ribosomal RNA gene.

Antimicrobial susceptibility testing: Isolates were tested for susceptibility by the broth microdilution procedure and interpretative criteria described by the CLSI; 2006.

Carbapenem hydrolyzing class D β -lactamase- (CHCDB) and MBL-encoding gene detection: Isolates non-susceptible to imipenem or meropenem (MIC, \geq 8 mg/L) were PCR-screened for acquired CHCDB (*bla*_{OXA-23}, *bla*_{OXA-24}, *bla*_{OXA-51} and *bla*_{OXA-58}-like) and MBL. Amplicons were sequenced on both strands.

Resistance gene location, plasmid analysis and hybridization: Total DNA from *A. radioresistens* clinical isolate (251-39C) was restriction digested with I-Ceu-I and DNA fragments and plasmids were transferred onto a nylon membrane by Southern blot. Specific labeled probes for *bla*_{OXA-133}, *bla*_{OXA-58} and 16S rRNA were used for hybridization.

Upstream DNA sequences and quantification of transcriptional levels of *bla*_{OXA-133} and *bla*_{OXA-58} in *A. radioresistens*: The upstream DNA sequences of the *bla*_{OXA-58} and *bla*_{OXA-133} in *A. radioresistens* 251-39C were characterized by PCR using primers targeting ISAb1, 2 or 3, or a degenerate primer approach. Quantification of the transcriptional levels of *bla*_{OXA-133} and *bla*_{OXA-58} were evaluated by quantitative real-time PCR (qRT-PCR).

Molecular typing: Clinical isolates were typed by PFGE.

RESULTS

- A total of 581 *Acinetobacter* spp. were recovered during 2006-2007. The vast majority were *A. baumannii* (94.3%), followed by *A. junii* (2.6%), *A. lwofii* (1.7%), *A. haemolyticus* (0.3%), *A. johnsonii* (0.3%), *A. calcoaceticus* (0.2%) and *A. radioresistens* (0.2%).
- Polymyxin-B (99.1% susceptible) and tigecycline (98.9% susceptible) were the most active agents tested (Table 1).

Table 1. Activity of antimicrobial agents tested against 581 *Acinetobacter* spp. isolates recovered from APAC region during 2006-2007^a.

Antimicrobial agent	MIC ₅₀	MIC ₉₀	Range	% susceptible/resistant ^b
Ampicillin/sulbactam	>16	>16	\leq 2->16	42.5 / 51.6
Ceftazidime	>16	>16	\leq 1->16	37.5 / 61.0
Cefepime	>16	>16	\leq 0.25->16	37.3 / 55.8
Imipenem	2	>8	\leq 0.12->8	59.6 / 37.4
Meropenem	2	>8	\leq 0.12->8	58.5 / 39.4
Levofloxacin	>4	>4	\leq 0.5->4	38.3 / 50.6
Amikacin	>32	>32	\leq 4->32	44.4 / 53.8
Gentamicin	>8	>8	\leq 2->8	36.9 / 63.9
Tigecycline	0.5	1	\leq 0.03->4	98.8 / 1.2
Polymyxin B	\leq 0.5	1	\leq 0.5->4	99.1 / 1.0

a. Includes 428 non-oxacillinase- and MBL-producing isolates, 151 oxacillinase-producing isolates and 2 MBL-producing isolates.

b. According to CLSI M100-S18, except for tigecycline, which was determined according to the Enterobacteriaceae breakpoints approved by the USA-FDA (breakpoint for susceptibility, \leq 2 mg/L; breakpoint for resistance, \geq 8 mg/L).

Table 2. Molecular epidemiology data from *bla*_{OXA}-carrying isolates at each medical centre.

Bacterial isolate	Enzyme	No. of isolates	Medical centre	Clones ^a (No. of isolates)	Country
<i>A. baumannii</i>	OXA-23	4	204	A (4)	Hong Kong
		20	217	A (16), B (2), C (2)	Singapore
		6	218	A (6)	China
		15	224	A (12), B (1), C (1), D (1)	Korea
		9	225	A (2), B (3), C (3), D (1)	Korea
		15	226	A (1), B (4), C (1), D (3), E (1), F (1), G (1), H (1), I (1), J (1)	Thailand
		8	227	A (1), B (4), C (3)	Thailand
		9	231	A (6), A1 (1), A2 (1), A3 (1)	China
		1	238	ND ^b (1)	China
		7	243	A (3), B (2), C (1), D (1)	India
		26	248	A (2), B (2), C (1), D (1), E (1), F (1), G (7), G1 (1), H (2), I (1), K (1), NT ^c (6)	India
		4	252	A (1), B (1), C (1), D (1)	India
		2	246	A (1), B (1)	India
<i>A. junii</i>	OXA-24	1	246	ND ^b (1)	India
		2	223	A (2)	Taiwan
<i>A. baumannii</i>	OXA-24	2	226	A (1), B (1)	Thailand
		1	242	ND ^b (1)	Indonesia
<i>A. baumannii</i>	OXA-58	2	232	A (2)	China
<i>A. calcoaceticus</i>	OXA-58	1	234	ND ^b (1)	China
<i>A. baumannii</i>	OXA-23 and -58	11	231	A (10), A1 (1)	China
<i>A. radioresistens</i>	OXA-133 and -58	1	251	ND ^b (1)	India
<i>A. baumannii</i>	OXA-24 and -58	1	226	ND ^b (1)	Thailand
<i>A. baumannii</i>	OXA-23, -24 and -58	3	226	A (3)	Thailand

a. Indicates the number of clones carrying oxacillinase-encoding genes within each participating medical centre.

b. ND reads "Not Determined", only one isolate.

c. Not typeable.

Table 3. *bla*_{OXA-133} and *bla*_{OXA-58} transcriptional levels analysis.

Target gene	Strain	C _T ^a	Relative expression/ target vs. reference gene ^b	Overexpression ratio ^c
<i>bla</i> _{OXA-133}	251-39C	34.74	3.97e-08	1667
<i>bla</i> _{OXA-58}	251-39C	24.00	6.62e-05	
16S rRNA	251-39C	10.91		

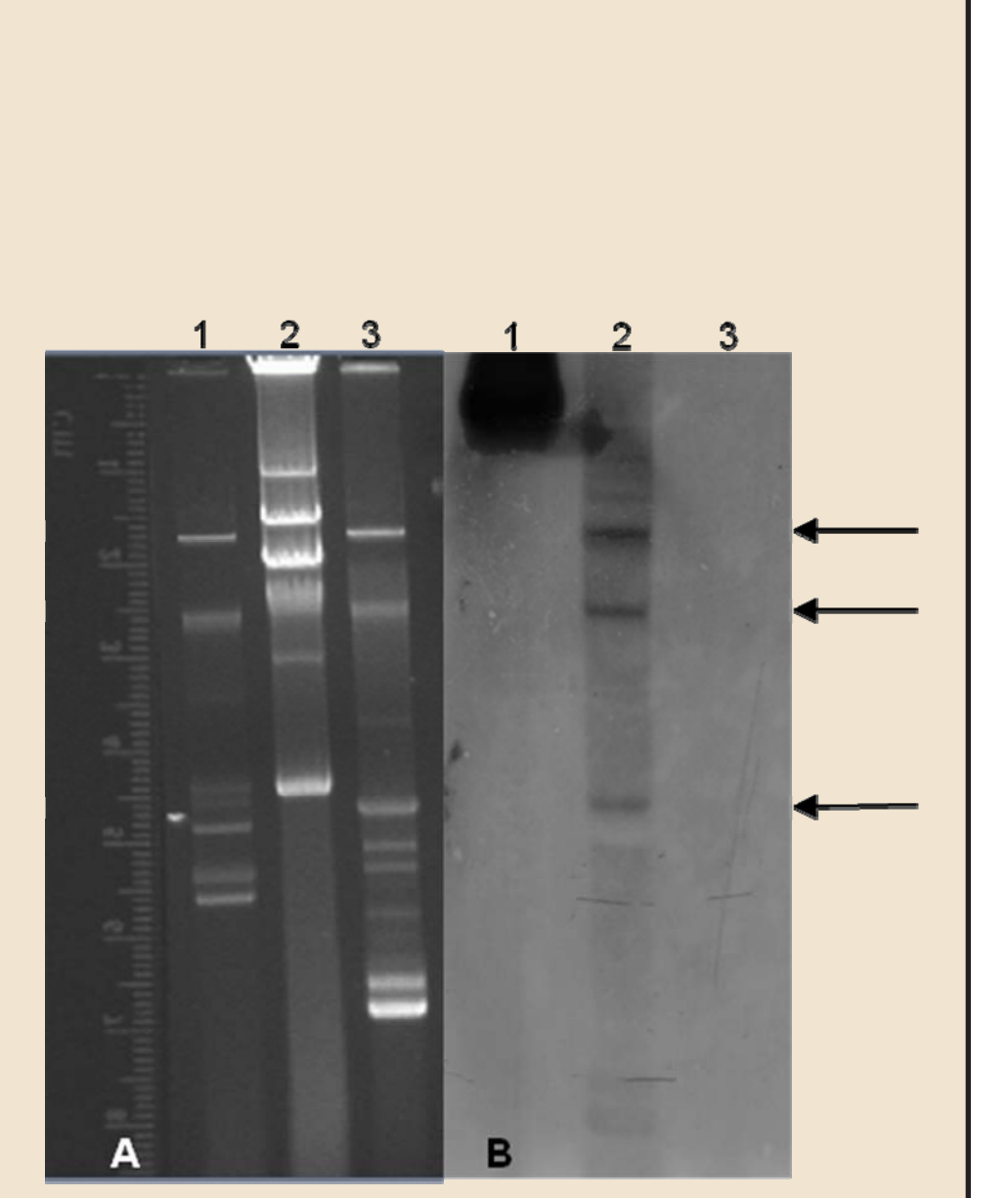
a. C_T (critical threshold cycle) numbers represent an average from triplicate tests and were determined by the detection system software.

b. Relative expression = $2^{-\Delta\Delta C_T}$, where $\Delta C_T = C_{T\text{target}} - C_{T\text{reference}}$.

c. Ratio between relative expression levels of the target genes (*bla*_{OXA-58}/*bla*_{OXA-133}).

- Among the isolates, 267 (46.0%) were non-susceptible (MIC, \geq 8 mg/L) to imipenem or meropenem.
- Among the carbapenem non-susceptible isolates, CHCDB and MBL genes were detected in 56.5% (151 isolates) and 0.8% of the isolates (two isolates), respectively. *bla*_{OXA-23} was the most common gene, which accounted for 94.0% of the CHCDB-encoding genes detected, followed by *bla*_{OXA-58} (13.2%) and *bla*_{OXA-24} (6.6%).
 - A new *bla*_{OXA-23}-like gene, namely *bla*_{OXA-133}, was detected in an *A. radioresistens* (251-39C). The putative amino acid sequence of OXA-133 showed highest identity with OXA-102 (99.6%). MBL-encoding genes were detected in one *A. johnsonii* recovered from the Philippines (*bla*_{IMP-4}) and one *A. baumannii* recovered from Korea (*bla*_{VIM-2}).
 - A. radioresistens* showed multiple plasmids. The *bla*_{OXA-58}-specific probe hybridized with 54-, 35- and 5.6-kb plasmid DNAs (Figure 1). *bla*_{OXA-133}-specific probe hybridized with the 1050-kb *A. radioresistens* total DNA fragment after I-Ceu-I restriction digestion (data not shown).
 - ISAb3 was detected upstream of *bla*_{OXA-58} gene in the *A. radioresistens* isolate. DNA sequencing of the upstream region of *bla*_{OXA-133} showed highest identity with a putative O-sialoglycoprotein endopeptidase-encoding gene. No putative promoter was located in this region and a truncated ATPase gene was located downstream.
 - Transcriptional levels of *bla*_{OXA-133} (mean C_T = 34.74) were 1667-fold lower than that of *bla*_{OXA-58} (mean C_T = 24.00; Table 3).
 - Clonal dissemination of *bla*_{OXA-23}-carrying *A. baumannii* was noted in medical centres 204, 217, 218, 224 and 231; while *bla*_{OXA-23}-carrying *A. baumannii* from the remaining centres were genetically unrelated (Table 2). Among *bla*_{OXA-24}-carrying *A. baumannii* recovered from Taiwan and Thailand, dissemination of related and unrelated clinical isolates was noted, respectively.

Figure 1. (A) Lanes 1 and 2 represent plasmid DNAs from *E. coli* strains V517 (NCTC 50193) and 39R861 (NCTC 50192) used as molecular size marker, respectively. Lane 3 represents plasmid DNAs from *A. radioresistens* clinical isolate 251-39C. (B) Southern hybridization performed with a *bla*_{OXA-58}-specific probe. Lane 1 represents the positive control. Lane 2 represents the *bla*_{OXA-58}-positive signals (horizontal arrows) obtained with 54-, 35- and 5.6-kb plasmid DNAs from *A. radioresistens* 251-39C. Lane 3 represents the negative control.



CONCLUSIONS

- This study showed low susceptibility rates among *Acinetobacter* spp. for most clinically available antimicrobial agents, except for polymyxin B and tigecycline.
- A. radioresistens* harbored a chromosomally located *bla*_{OXA-133}, suggesting this bacterial species as the source of *bla*_{OXA-23}-clusters. In addition, this strain harbored several plasmid DNAs, which included three different *bla*_{OXA-58}-carrying plasmids.
- The transcriptional levels of *bla*_{OXA-133} were much lower when compared with those of concurrently present *bla*_{OXA-58}. These results were expected (i) due to the lack of promoter upstream of *bla*_{OXA-133}, (ii) presence of multiple copies of *bla*_{OXA-58} and (iii) the presence of ISAb3 upstream of the *bla*_{OXA-58}, which provides a promoter and consequently enhances gene expression.
- The detection of *bla*_{VIM-2} and *bla*_{IMP-4} in South Korea and the Philippines, respectively, underscores their continue presence in these regions, albeit at low frequency.
- This study emphasizes (i) the natural occurrence of β -lactam resistance genes among environmental *Acinetobacter* species, (ii) the ability for exchanging foreign DNA and (iii) the diversity of resistance genes in the hospital setting.

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