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Molecular Characteristics and Resistant Mechanisms of Imipenem-Resistant *Acinetobacter baumannii* Isolates in Shenyang, China

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The investigation was carried out to elucidate the molecular characteristics and resistant mechanisms of imipenem-resistant *Acinetobacter baumannii*. Thirty-seven isolates were collected from January 2007 to December 2007. The homology of the isolates was analyzed by both pulsed field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). The genes of β-lactamases, *adeB*, and class 1 integron were polymerase chain reaction amplified. Genotype analysis of the 37 *A. baumannii* isolates by PFGE revealed the circulation of four PFGE types (A-D) ; the A- and B-type accounted for 48.6% and 40.5%, respectively. MLST showed the existence of three allelic profiles. The agar dilution method was carried out to determine the MIC of imipenem, in the absence or presence of carbonyl cyanide m-chlorophenylhydrazone (CCCP, 10 μg/ml). The MICs of the strains to imipenem were between 16 μg/ml and 128 μg/ml. When CCCP was added, a MIC decrease of at least four-fold was observed in 20 isolates, which belonged to the A- or C-type. *AdeB* and bla_{PER-1} genes were each detected in 35 isolates, bla_{OXA-23} gene in 34 isolates and bla_{OXA-58}-like gene in 24 isolates. All isolates harbored bla_{OXA-51}-like genes. No isolates carried the bla_{IMP-1} gene. Integron was detected in 25 isolates, which mediated the resistance to aminoglycosides and rifampin. The epidemiologic data suggested that the increasing infection of *A. baumannii* in our hospital was mainly caused by the inter-hospital spread of two epidemic clones. The AdeABC efflux system may be the important factor that leads to the high level of imipenem-resistance in PFGE A-type.

***Keywords*:** *A. baumannii*, PFGE, MLST, β-lactamase, efflux system, integron

Acinetobacter baumannii is an opportunistic pathogen that is responsible for a large percentage of nosocomial infections including pneumonia, bacteremia, skin infections, wound infections, and urinary tract infections. The bacterium is characterized by its ability to evolve resistance to all clinically relevant drugs (Van *et al*., 2004). In China, a national surveillance program involving 10 geographically disparate hospitals revealed an increased *A. baumannii* resistance to carbapenems from 4.5% in 2003 to 18.2% in 2004 (Wang *et al*., 2005). Not unexpectedly, clinical outbreaks attributable to imipenem-resistance *A. baumannii* strains have been increasing in frequency (Héritier *et al*., 2005; Jeon *et al*., 2005; Vahaboglu *et al*., 2006). Acquired carbapenem resistance in *A. baumannii* is often associated with acquired carbapenemase production, including the production of OXA-23-, OXA-24-, OXA-51-, and OXA-58-type class D carbapenemases or the IMP-, VIM-, and SIM-type metallo-lactamases (Brown and Amyes, 2006; Poirel and Nordmann, 2006). Recently, a novel resistance mechanism, the AdeABC efflux system, was identified in a multidrug-resistant (MDR) *A. baumannii* strain (Magnet *et al*., 2001). This system was proven to be associated with the activity of some agents, including β-lactamases, fluoroquinolones, tetracycline and, most recently, tigecycline. Carbonyl cyanide m-chlorophenylhydrazone (CCCP) is an effective pump inhibitor. The other factor widely influencing the acquisition of resistance is the acquisition of genetic elements. Among these elements, plasmids and integrons have been reported to be mostly associated with the resistance of imipenem. The objectives of the present study were to investigate the molecular epidemiology of imipenem-resistant *A. baumannii* in our hospital in China and to characterize the imipenem-resistant mechanisms of the major epidemic clones.

Materials and Methods

Bacterial identification and susceptibility testing

Thirty-seven clinical isolates of *A. baumannii* were collected from January 2007 to December 2007 from clinical samples at the First Hospital of China Medical University. These included sputum specimens (27 isolates), secretion specimens (5 isolates), drainage fluid specimens (2 isolates), urine specimens (2 isolates), and a blood specimen (1 isolate). All 37 isolates could be defined as pathogenic bacteria that were directly responsible for the particular infection. The strains were propagated at 37°C in Luria-Bertani broth. Isolates were identified and initial antimicrobial susceptibilities were determined by the Vitek system (bioMérieux, France). *In vitro* susceptibilities (minimal inhibitory concentration; MIC) were determined by a standard agar dilution method. Susceptibilities were interpreted according to Clinical and Laboratory Standards Institute guidelines (CLSI, 2006). For imipenem, the resistance breakpoint was defined as a MIC 16 μg/ml. Imipenem was purchased from Merck (Whitehouse Station, USA). *Escherichia coli* ATCC 25922 and

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Pseudomonas aeruginosa ATCC 27853 were used as control strains.

Pulsed field gel electrophoresis (PFGE)

The 37 *A. baumannii* clinical isolates were also characterized by PFGE as described previously (Poirel and Nordmann, 2006). For this analysis, the restriction enzyme *Apa*I was used according to the manufacturer's specification (Bio-Rad, USA). DNA restriction fragments were separated in a CHEF-MAPPER system (Bio-Rad) for 20 h at 14°C and 6 V/cm, with pulse times ranging from 5-8 sec. Strain relatedness was assigned as previously described (Tenover *et al*., 1995).

Multilocus Sequence Typing (MLST)

The housekeeping genes for the MLST scheme included citrate synthase (*gltA*), DNA gyrase subunit B (*gyrB*), glucose dehydrogenase B (*gdhB*), homologous recombination factor (*recA*), 60-kDa chaperonin (*cpn60*), glucose-6-phosphate isomerase (*gpi*), and RNA polymerase 70 factor (*rpoD*) (Table 1). Amplifications of these genes were carried out as described previously (Sergio *et al*., 2005). The amplified products were submitted to a MLST Web site (http://www.mlst.net) to compare them with international strain data. For each locus, distinct allele sequences were assigned an arbitrary allele number for identification, and these were in-frame internal fragments of the gene that contained an exact number of codons. Each isolate was characterized by a pattern of numbers defining its allelic profile or sequence type.

Polymerase chain reaction (PCR) amplification

DNA templates were the genomic DNA, and were prepared using the Biospin Bacteria Genomic DNA Extraction kit (Bioer, China) as recommended by the manufacturer. All genes were amplified by PCR, with previously described primers, conditions (Lévesque *et al*., 1995; Poirel *et al*., 1999; Riccio *et al*., 2000; Afzal-Shah *et al*., 2001; Vahaboglu *et al*., 2006), and reaction system (Tian *et al*., 2008), and were directly purified from the reaction mixture with the PCR Purification kit (Sangon, China) according to the manufacturer's recommendation. The reaction products were run in an automated DNA sequencing ABI PRISM 3730 sequencer (Applied Biosystems, USA). All PCR products were sequenced in both directions.

Efflux mechanism

To determine the presence of an efflux mechanism involved in the resistance isolates, an MIC assay was performed. MICs were determined out using an established agar dilution method. MIC changes were observed in either the absence or the presence of CCCP (10 μg/ml, Sigma-Aldrich, USA) according to the guideline established by CLSI. An inoculum of 0.5 Mcfarland Standard of each isolate was inoculated onto MH medium containing serial dilutions of imipenem. Four-fold or more reduction of imipenem MICs in the absence or the presence of CCCP was defined as a positive phenotype for efflux (Shi *et al*., 2005). *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as the positive controls.

Results

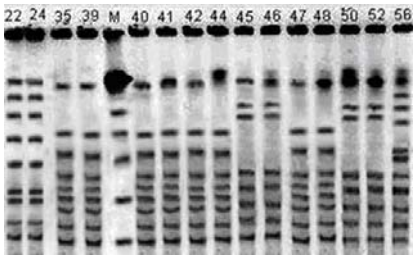
Molecular epidemiological typing by PFGE and MLST

All 37 imipenem-resistant isolates were genotyped by *Apa*I digestion-PFGE analysis and categorized into four types (A-D). The A-type and B-type accounted for 48.6% (n=18) and 40.5% (n=15), respectively, with two or more subtypes evident for some (Table 2). PFGE patterns of the representative *A. baumannii* isolates are shown in Fig. 1. Of all the isolates, most were collected from the intensive care unit.

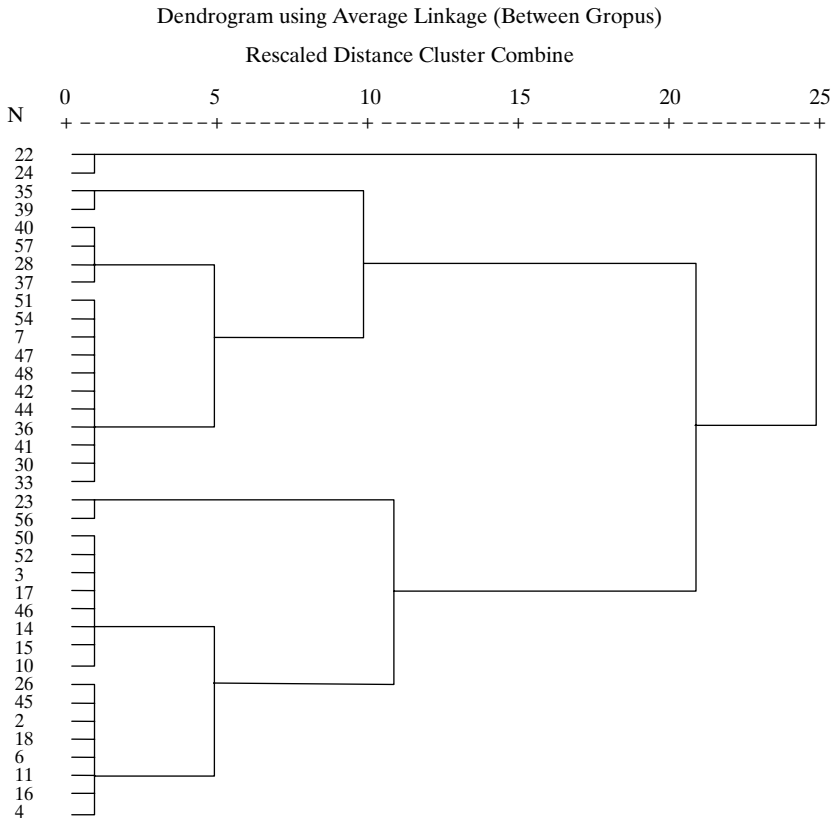
Overall, the MLST results showed the existence of three allelic profiles: 1-3-3-2-2-7-3 (A-type), 1-3-3-2-2-11-3 (B- and D-type), and 1-3-3-2-2-14-3 (C-type). The PFGE B-type and D-type had the same allelic profile. The MLST data for all isolates were in high concordance with the epidemiological typing results generated by PFGE. No isolates with identical

Table 1. Details of loci and oligonucleotide primers used in the present study

Locus	Primer	Sequences	Use
gltA	CitratoF1	AATTTACAGTGGCACATTAGGTCCC	Amplification/sequencing
	CitratoR12	GCAGAGATACCAGCAGAGATACACG	Amplification/sequencing
gyrB	APRU F	TGTAAAACGACGGCCAGTGCNGGRTCYTTYTCYTGRCA	Amplification
	M13[-21]	TGTAAAACGACGGCCAGT	Sequencing
	UP1E R	CAGGAAACAGCTATGACCAYGSNGGNGGNAARTTYRA	Amplification
	M13 F	CAGGAAACAGCTATGACC	Sequencing
gdhB	GDHB 1F	GCTACTTTTATGCAACAGAGCC	Amplification
	GDH SECF	ACCACATGCTTTGTTATG	Sequencing
	GDHB 775R	GTTGAGTTGGCGTATGTTGTGC	Amplification
	GDH SEC R	GTTGGCGTATGTTGTGC	Sequencing
recA	RA1	CCTGAATCTTCYGGTAAAAC	Amplification/sequencing
	RA2	GTTTCTGGGCTGCCAAACATTAC	Amplification/sequencing
cpn60	CPN 3F2	ACTGTACTTGCTCAAGC	Amplification/sequencing
	CPN R2	TTCAGCGATGATAAGAAGTGG	Amplification/sequencing
Gpi	GPI F1	AATACCGTGGTGCTACGGG	Amplification/sequencing
	GPI R1	AACTTGATTTTCAGGAGC	Amplification/sequencing
rpoD	70F RPOD	ACGACTGACCCGGTACGCATGTAYATGMGNGARATGGGNACNGT	Amplification
	70FS	ACGACTGACCCGGTACGCATGTA	Sequencing
	70R RPOD	ATAGAAATAACCAGACGAAGTTNGCYTCNACCATYTCYTTYTT	Amplification
	70RS	ATAGAAATAACCAGACGTAAGTT	Sequencing



Note: M, low-range PFGE DNA marker; the numbers above represent the number of the strains.



Note: N represents the number of the strains.

Fig. 1. PFGE patterns obtained with *Apa*I

PFGE patterns had different sequence types. A novel sequence type for the C-type was detected for *A. baumannii* (undesignated ST).

Efflux mechanism

Antibiotic susceptibility testing revealed that all the strains were resistant to imipenem. As shown in Table 2, the MICs of all the isolates to imipenem were between 16 μg/ml and 128 μg/ml, and were mostly 32 or 64 μg/ml. When CCCP (10 μg/ml) was present, at least a four-fold decrease of MICs was observed in 20 isolates (54.1%), consistent with a CCCP-mediated inhibition of the efflux system of *A. baumannii*. Eighteen of these 20 isolates belonged to PFGE A-type, with the remaining two being PFGE C-type. All strains grew in the presence of only 10 μg/ml CCCP.

Detection of β-lactamase and *adeB* genes

PCRs for the detection of different β-lactamase genes were performed with all 37 isolates (Table 2). The entire $bla_{\text{OXA-51}}$-like gene sequence was detected in all isolates, confirming the presence of the $bla_{\text{OXA-66}}$ gene. Among the 37 isolates, 24 carried the $bla_{\text{OXA-58}}$ gene, and 34 carried the $bla_{\text{OXA-23}}$ gene. The *adeB* and $bla_{\text{PER-1}}$ genes were the most prevalence, both being detected in 35 isolates, whereas the $bla_{\text{IPM-1}}$ gene was not detected.

Integron detection

There were 25 isolates (67.6%) positive for the class 1 integron (Table 2). Sequencing analysis showed that all the integrons (about 1,350 bp) carried *arr-3* and *aacA4*, which mediated the resistance to aminoglycosides and rifampin, respectively. Clone A (except for one strain) of *A. baumannii*

harbored the same class 1 integron, while most of the clone B isolates did not carry the integron (Table 2).

Discussion

To better understand the epidemiology and, in particular, the mode of spread of *A. baumannii*, a number of molecular typing systems have been developed (Gerner-Smidt, 1992; Dijkshoorn *et al*., 1996; Grundmann *et al*., 1997; Yoo *et al*., 1999; Koeleman *et al*., 2001). PFGE restriction analysis of chromosomal bacterial DNA has been used with excellent results in epidemiologic studies of numerous *A. baumannii* outbreaks and is currently regarded as the gold standard for epidemiologic typing (Gouby *et al*., 1992). Presently, genotype analysis of the 37 *A. baumannii* strains by PFGE revealed the circulation of four different PFGE types (A-D), with the A- and B-type clones being most prevalent. The results of this study support the contention that clonal spread was the main

Table 2. Results of PFGE and detection of all genes

Number of strains	MIC (μg/ml)		Detection of genes						PFGE	MLST
	IMP	IMP+CCCP	*adeB*	OXA-23	OXA-51	OXA-58	PER-1	Integron		
7	32	0.015	+	+	+	-	+	+	A1	1
30	64	0.015	+	+	+	+	+	+	A1	1
33	64	16	+	+	+	+	+	+	A1	1
36	64	0.015	+	+	+	-	+	+	A1	1
41	32	0.015	+	+	+	+	+	+	A1	1
42	32	8	-	+	+	+	+	+	A1	1
44	64	8	+	+	+	-	+	+	A1	1
47	64	8	+	+	+	+	+	+	A1	1
48	64	0.015	+	+	+	+	+	+	A1	1
51	64	0.015	+	+	+	+	+	+	A1	1
54	64	8	+	+	+	-	+	+	A1	1
28	128	0.03	+	-	+	-	-	+	A2	1
37	64	8	+	+	+	+	+	+	A2	1
40	32	1	+	+	+	-	+	+	A2	1
57	32	0.015	+	+	+	-	+	-	A2	1
35	64	16	+	+	+	+	+	+	A3	1
39	64	16	+	+	+	+	+	+	A3	1
25	16	4	+	-	+	+	-	+	A4	1
2	64	32	+	+	+	-	+	-	B1	2
4	64	32	+	+	+	+	+	-	B1	2
6	32	32	+	+	+	+	+	-	B1	2
11	32	32	+	+	+	+	+	+	B1	2
16	32	32	+	+	+	+	+	+	B1	2
18	32	16	+	+	+	-	+	-	B1	2
26	32	32	+	+	+	+	+	+	B1	2
45	64	32	+	+	+	+	+	+	B1	2
3	64	32	+	+	+	+	+	-	B2	2
10	64	32	+	+	+	+	+	-	B2	2
14	32	32	+	+	+	-	+	+	B2	2
15	32	16	+	+	+	+	+	-	B2	2
17	64	32	+	+	+	+	+	-	B2	2
46	64	32	+	+	+	+	+	-	B2	2
52	64	32	+	+	+	+	+	-	B2	2
23	16	0.03	+	+	+	-	+	-	C	3
56	64	16	+	+	+	+	+	+	C	3
22	32	32	+	-	+	-	+	+	D	2
24	32	16	-	+	+	-	+	+	D	2

Note: In the MSLT results, 1 represents the allelic profile of 1-3-3-2-2-7-3, 2 represents the allelic profile of 1-3-3-2-2-11-3, and 3 represents the allelic profile of 1-3-3-2-2-14-3.

reason for the increasing trend of imipenem resistance in our hospital. Patient transfer and hospital staff contact may have enhanced the spread of imipenem-resistant *A. baumannii* among different wards. Early recognition of the presence of imipenem-resistant *A. baumannii* clones is useful for preventing their spread within the hospital environment (Cetin *et al*., 2009).

For a global epidemiologic analysis, however, comparison of the results obtained at different laboratories would be required, but poor interlaboratory reproducibility remains a critical and unresolved issue. MLST offers the possibility to transfer typing data from laboratory to laboratory or compare results via the internet (http://www.mlst.net), thus providing a powerful tool for global epidemiologic studies, as well as for the population biology of bacterial species (Maiden *et al*., 1998; Grundmann *et al*., 2002; Homan *et al*., 2002). Overall, the present MLST results showed the existence of three allelic profiles. The MLST data for all isolates were in high concordance with the epidemiological typing results generated by PFGE. Isolates with differently related PFGE patterns (e.g., B-type and D-type) had the same allelic profile. No isolates with identical PFGE pattern had a different sequence type. A novel sequence type was detected for *A. baumannii* (single locus variant, undesignated sequence type). This indicates that some stimulus may lead to the emergence of new characteristics of regional strains. So, increased awareness of such strains is prudent.

Different mechanisms are involved in the resistance to imipenem in *A. baumannii*. β-Lactamase is one important factor. Here, five different β-lactamases were detected in all the strains, including OXA-23, OXA-51, and OXA-58 enzymes, PER-1, and IMP-1. The OXA-23 enzyme was first described in an *A. baumannii* strain in 1985, and subsequently disseminated globally (Dalla-Costa *et al*., 2003; Jeon *et al*., 2005). OXA-23 was the most prevalent carbapenemase among imipenem-resistant isolates in China. The OXA-51-type subgroup shares less than 63% amino acid identity with other class D enzymes, which occur naturally in that species and which are usually weakly expressed. PER-1 is an extended-spectrum-lactamase that is active against penicillins, cefotaxime, ceftazidime, and aztreonam, but with no significant activity against carbapenems. It has been widely detected in *A. baumannii* in Turkey and Korea. The three genes were all prevalent in the presently-studied region, similar to an earlier study (Wang *et al*., 2007). OXA-58 was first found in 2003 in France. It was subsequently reported from different parts of the world (Bertini *et al*., 2006; Coelho *et al*., 2006; Pournaras *et al*., 2006). Plasmid-borne $bla_{\text{OXA-58}}$ genes contribute significantly to carbapenem resistance in *A. baumannii* (Bertini *et al*., 2006). Other unpublished data have not detected the plasmid; however, due to this plasmid-borne location, the distribution of this gene should be monitored.

Unlike OXA-type β-lactamases, the IMP-type β-lactamases have much stronger carbapenem-hydrolysing activity. But, IMP-type β-lactamases were rarely detected presently, so only IMP-1 was chosen for this research. No IMP-1 gene was found.

Because of the weak hydrolyzing activity of β-lactamase, it can only lead to low-level clinically relevant imipenem-resistance. High-level resistance demonstrated the existence of other resistant mechanisms. The AdeABC efflux pump, which belongs to the resistance-nodulation-division (RND) family, has been found only in *A. baumannii*, and the *adeB* gene is essential for the efflux function (Marchand *et al*., 2004). This efflux pump, which can be coupled with β-lactamases, can lead to high-level imipenem-resistance. CCCP was proven to be an effective inhibitor. Presently, the *adeB* gene was detected in the vast majority of the *A. baumannii* population. When CCCP was added, the expression of the efflux pump between the four clones was obviously different. Of all the 20 strains positive for the efflux pump, 18 strains belonged to PFGE A-type, and the other two belonged to the C-type. The other main clones, B-type, were all negative for the efflux pump. The MIC of the A-type (MIC_{90}, 64 μg/ml) was higher than that of the B-type (MIC_{90}, 32 μg/ml), providing evidence that the AdeABC efflux pump may participate in imipenem-resistance together with β-lactamases in the A- and C-type clones.

Integron typing is a valuable tool for molecular epidemiology. In this study, the integron-borne gene cassettes were found mainly in the A-type, but no β-lactamases were detected. This indicates that resistant genes may be transferred at the clone level.

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