Resistant mechanisms and molecular epidemiology of imipenem-resistant *Acinetobacter baumannii*

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Abstract. The aim of the study was to investigate the resistant mechanisms and homology of imipenem-resistant *Acinetobacter baumannii* (*A. baumannii*). A total of 46 non-duplicate imipenem-resistant *A. baumannii* clinical isolates were collected from three tertiary hospitals between July, 2011 and June, 2012. The minimal inhibitory concentrations (MICs) of antimicrobial agents were determined using the agar dilution method. Phenylalanine-arginine β-naphthylamide was used to detect the presence of the efflux pump-mediated resistant mechanism. Polymerase chain reaction was employed to amplify genes associated with drug resistance, including β-lactamase genes, efflux pump genes and outer membrane protein gene *CarO*. A few amplicons were randomly selected and sequenced. Multilocus sequence analysis (MLST) was employed in typing *A. baumanni*. *A. baumannii* was resistant to imipenem, simultaneously showing resistance to several other antimicrobials. In addition, 13 *A. baumannii* were found to mediate drug resistance through operation of the efflux pump. Of the various drug resistance genes tested, $bla_{\text{OXA-51}}$ was present in 46 isolates, $bla_{\text{OXA-23}}$ gene was present in 44 isolates and bla_{NDM} gene was found in only one strain. Other drug resistant-associated genes, including bla_{KPC}, bla_{IMP}, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-58}}$, bla_{SHV}, bla_{GIM} and bla_{VIM} were not detected. Mutation of *adeS* and outer membrane protein gene *CarO* were found in a few of the imipenem-resistant isolates. The MLST analysis revealed that all 46 clinical isolates were clustered into 11 genotypes and the most frequent genotype was ST208. In conclusion, β-lactamase genes, genes involved in efflux pump and mutation of outer membrane protein encoding gene may be important in mediating imipenem resistance in *A. baumannii*. Of the 11 different genotypes, ST11 was shared by the majority of *A. baumannii*, which may be due to horizontal transfer of patients from hospitals.

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Introduction

Acinetobacter baumannii (*A. baumannii*) has emerged as a major pathogen of nosocomial infections and is associated with high rates of morbidity and mortality in recent years (1,2). A nationwide surveillance program, including hospitals from 14 geographically different regions in China revealed that the ratio of *A. baumannii* is on the increase annually (3).

Carbapenem has good antibacterial activity against *A. baumannii* and was the first choice in treatment of infection caused by *A. baumannii* in the past years (4). However, the emergence of resistance to carbapenem was reported in 1991 (5), followed by similar reports from different parts of the world (6,7). In China, 57 and 61% of *Acinetobacter spp.* (*A. baumannii* accounted for 89.6%) showed resistance to imipenem and meropenem, respectively (3). International studies in China as well as in other parts of the world focused only on evaluating the resistance of *A. baumannii* to various antimicrobials (8-10). However, to the best of our knowledge, few studies have investigated the molecular mechanism underlying drug resistance. Additionally, no data are available on the epidemiological characteristics of imipenem-resistant *A. baumannii* in Shanghai.

Thus, *A. baumannii* clinical isolates were collected from three tertiary hospitals in Shanghai and their drug resistance pattern to a spectrum of antimicrobials, molecular mechanisms (including carbapenemase, efflux pumps and membrane proteins) behind their resistance and multilocus sequence analysis (MLST) were analyzed to assess their molecular epidemiology.

Materials and methods

Bacterial strains. During the period July, 2011 to June, 2012, 46 non-duplicate imipenem-resistant *A. baumannii* strains

Table I. Gene-specific primers used in this study.

Genes	Primer sequences
recA	F: CCTGAATCTTCTGGTAAAAC
	R: GTTTCTGGGCTGCCAAACATTAC
ITS	F: CATTATCACGGTAATTAGTG
	R: AGAGCACTGTGCACTTAAG
bla_{KPC}	F: TTACTGCCCGTTGACGCCCAATCC
	R: TCGCTAAACTCGAACAGG
bla_{IMP}	F: AACCAGTTTTGCCTTACCAT
	R: CTACCGCAGCAGAGTCTTTG
bla_{NDM}	F: CCGCCCAGATCCTCAACT
	R: ATCAGGCAGCCACCAAAA
bla_{OXA-51}	F: TAATGCTTTGATCGGCCTTG
	R: TGGATTGCACTTCATCTTGG
bla_{OXA-23}	F: GATCGGATTGGAGAACCAGA
	R: ATTTCTGACCCATTTCCAT
bla_{OXA-24}	F: GGTTAGTTGGCCCCCTTAAA
	R: AGTTGAGCGAAAAGGGGATT
bla_{OXA-58}	F: AAGTATTGGGGCTTGTGCTG
	R: CCCCTCTGCGCTCTACATAC
bla_{SHV}	F: GGTTATGCGTTATATTCGCC
	R: TTAGCGTTGCCAGTGCTC
bla_{GIM}	F: AGAACCTTGACCGAACGCAG
	R: ACTCATGACTCCTCACGAGG
bla_{VIM}	F: TCCACGCACTTTCATGACGA
	R: AGACGTGCGTGACAACTCAT
adeA	F: GAAATCCGTCCGCAAGTC
	R: ACACGCACATACATACCC
adeB	F: AAAGACTTCAAAGAGCGG
	R: TCACGCATTGCTTCACCC
adeC	F: ATTTCAGGTCGTAGCATT
	R: CTTGATAAGTAGAGTAGGGATT
adeS	F: ACTGTTATCTTCTGTGGCTGTA
	R: GTGGACGTTAGGTCAAGTTCTG
adeR	F: AAACGGTTGGGAAGTATTA
	R: ATGGCTATCTACGGTTCG
CarO	F: AAGGAGAAAACGATGA
	R: TTATTACGTGGTTATGG
gltA	F: AATTTACAGTGGCACATTAGGTCCC
	R:GCAGAGATACCAGCAGAGATACACG
gyrB	F: TGAAGGCGGCTTATCTGAGT
	R: GCTGGGTCTTTTTCCTGACA
gdhB	F: ACCACATGCTTTGTTATG
	R: GTTGGCGTATGTTGTGC
recA	F: CCTGAATCTTCYGGTAAAAC
	R: GTTTCTGGGCTGCCAAACATTAC
cpn60	F: GGTGCTCAACTTGTTCGTGA
	R: CACCGAAACCAGGAGCTTTA
Gpi	F: GAAATTTCCGGAGCTCACAA
	R: TCAGGAGCAATACCCCACTC
rpoD	F: ACCCGTGAAGGTGAAATCAG
	R: TTCAGCTGGAGCTTTAGCAAT

were collected from three tertiary hospitals located in Shanghai, China.

Reconfirmation of strains. The collected strains were subjected to gram staining, biochemical tests, and *recA* gene and 16S-23S rRNA gene intergenic spacer region to reconfirm them as *A. baumannii* (7).

Antimicrobial susceptibility and efflux phenotype tests. The collected *A. baumannii* isolates were subjected to an antimicrobial susceptibility test against imipenem, meropenem, amikacin, piperacillin, ceftazidime, cefotaxime, minocycline, ciprofloxacin, ampicillin/sulbactam, sulbactam, cefoperazone/sulbactam, piperacillin/tazobactam, colistin, tigecycline and trimethoprim/sulfamethoxazole using agar dilution method. *Escherichia coli* strain ATCC25922 and *Pseudomonas aeruginosa* (*P. aeruginosa*) strain ATCC27853 were used as reference strains.

Strains in which efflux pump operation was detected by agar dilution method where imipenem- and meropenem-resistant isolates were cultured in Mueller-Hinton agar contained the efflux pump inhibitor phenylalanine-arginine β-naphthylamide (PAβN) at a final concentration of 20 mg/l (11,12). A ≥4-fold reduction of imipenem or meropenem minimal inhibitory concentrations (MICs) in the presence of PAβN possessed an operating drug efflux pump.

Analysis of genes responsible for drug resistance, drug efflux and outer membrane protein. Polymerase chain reaction (PCR) was performed for the genes, bla_{KPC}, bla_{IMP}, bla_{NDM}, bla_{OXA-51}, bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-58}, bla_{SHV}, bla_{GIM} and bla_{VIM}, *CarO*, *adeA*, *adeB*, *adeC*, *adeS* and *adeR*. Thus, obtained amplicons were subjected to sequencing analysis.

A fresh and pure bacterial colony was suspended in distilled water and boiled at 100˚C for 15 min. After centrifugation at 8,000 x g for 15 min, 1 μl of the supernatant was used for PCR analysis with the primers (Table I). PCR was performed in a total volume of 50 μl containing 0.25 μl Taq DNA polymerase (Takara Bio, Inc., Tokyo, Japan), 5 μl 10X PCR buffer (Mg^{2+} Plus), 4 μl dNTP mixture (2.5 mM each), 2.5 μl DNA template, 1 μl of each primer (20 μM), and 36.25 μl ddH_2O. The PCR thermal cycle consisted of initial denaturation at 94˚C for 5 min, followed by 30 cycles of 94˚C for 30 sec, annealing 55˚C for 1 min and 72˚C for 1 min and a final extension at 72˚C for 7 min. The PCR products were electrophoresed in 1% agarose gel and visualized under ultraviolet light, and subsequently sequenced (Sangon Biotech Co., Ltd., Shanghai, China).

MLST. Seven housekeeping genes including homologous recombination factor (*recA*), citrate synthase (*gltA*), DNA gyrase subunit (*gyrB*), glucose-6-phosphate isomerase isomerase (*gpi*), glucose dehydrogenase B (*gdhB*), 60-kDa chaperonin (*cpn60*), and RNA polymerase 70 factor (*rpoD*) were amplified in PCR using relevant primers (Table I) and appropriate thermal conditions. The amplicons were sequenced and the sequences were submitted to the MLST database (http://pubmlst.org.net) to compare them with sequences submitted from other parts of the world. Each strain was then characterized by a pattern of numbers defining its allelic profile.

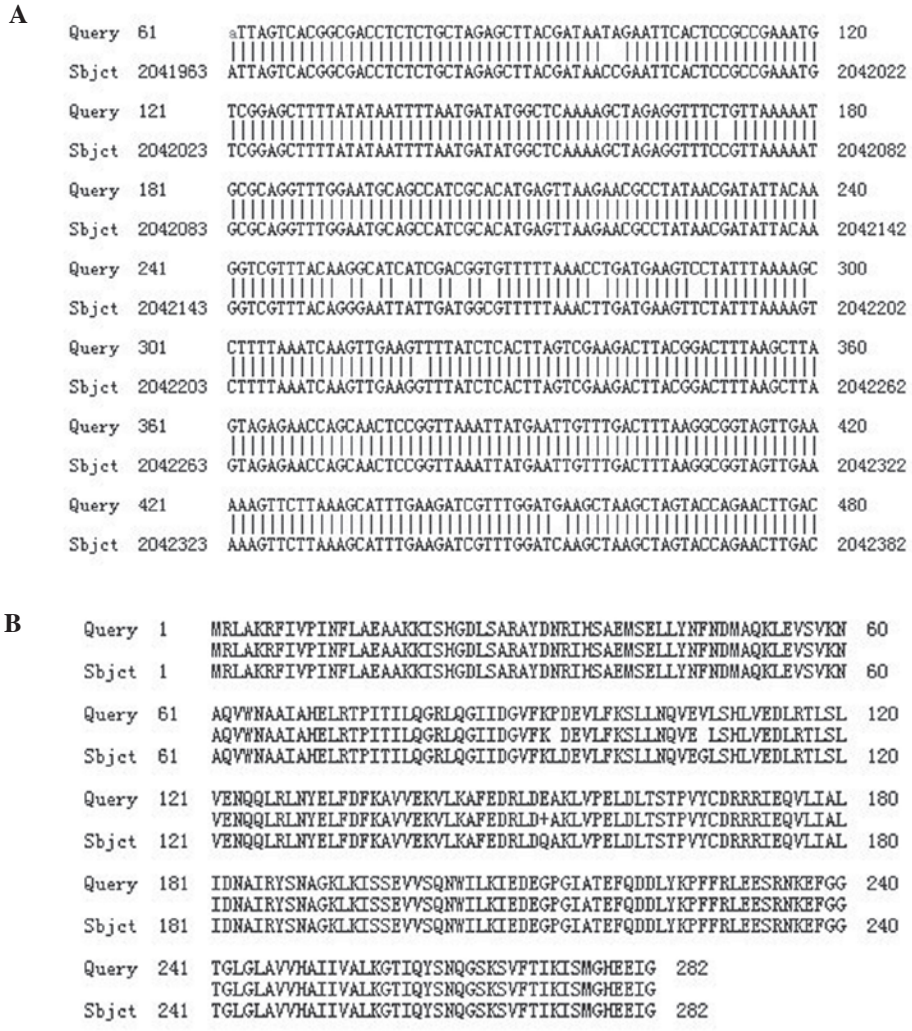

Figure 1. (A) Comparison of nucleotide sequence of *adeS* gene between resistant strain and reference strain ATCC17978. (B) Comparison of amino acid sequence of *adeS* gene between resistant strain and reference strain ATCC17978.

Results

Antimicrobial susceptibility. A. baumannii resistant to imipenem simultaneously showed resistance to several other common antimicrobials. The resistance rate was >80% for all the antimicrobials except minocycline and colistin. Antibiotic susceptibility of the 46 clinical isolates is shown in Table II. Thirteen imipenem-resistant *A. baumannii* isolates were positive for efflux pump.

Detection of genes involved in drug resistance, drug efflux and outer membrane protein. Of the various drug resistance genes tested, $bla_{\text{OXA-51}}$ was present in 46 isolates, $bla_{\text{OXA-23}}$ gene was present in 44 isolates and bla_{NDM} gene was found in only one strain. Other drug-resistant genes including bla_{KPC}, bla_{IMP}, $bla_{\text{OXA-24}}$, $bla_{\text{OXA-58}}$, bla_{SHV}, bla_{GIM} and bla_{VIM} were not detected in the isolates.

Of the five genes associated with the drug efflux pump tested, all five were found to be present in the isolates. Several mutations were found in the sequences of *adeS* gene in isolates with efflux phenotype. Differences were observed at three places when nucleotide sequences were translated into an amino acid sequence. This amino acid sequence was then compared to the amino acid sequence of the reference strain ATCC17978 (Fig. 1).

Table II. The drug-resistant rates of imipenem-resistant *Acinetobacter baumannii*.

Drug	Resistance rate No. of resistant strains (%)
Meropenem	41 (89)
Amikacin	38 (83)
Piperacillin	46 (100)
Ceftazidime	46 (100)
Minocycline	34 (74)
Ciprofloxacin	45 (98)
Ampicillin/sulbactam	43 (93)
Piperacillin/tazobactam	46 (100)
Colistin	1 (2)
Trimethoprim/sulfamethoxazole	43 (93)
Cefotaxime	45 (98)

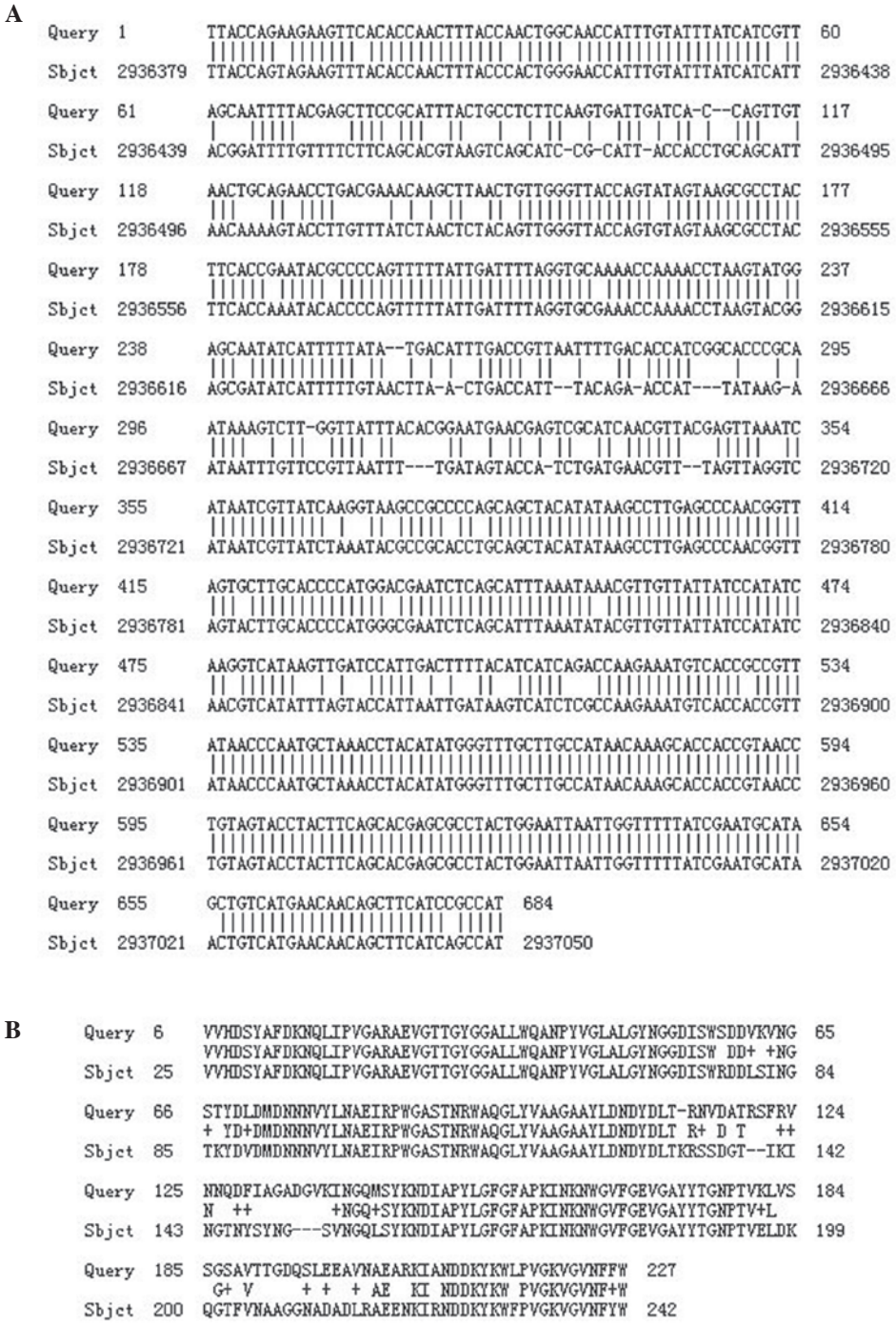

Figure 2. (A) Comparison of nucleotide sequence of *CarO* gene between resistant and reference strain ATCC17978. (B) Comparison of amino acid sequence of *CarO* gene between resistant and reference strain ATCC17978.

Similarly, the nucleotide sequence of the outer membrane protein encoding gene *CarO*, when compared with the nucleotide sequence of reference strain ATCC17978, harbored mutations that were reflected in the amino acid sequence (Fig. 2).

Genotyping of isolates by MLST. The MLST analysis revealed that the isolates were clustered in 11 different genotypes or STs. The ST208 genotype was shared by the majority of isolates (58.7%, 27/46), followed by ST191 (10.9%, 5/46) and ST451 (6.5%, 3/46). We also detected some other STs shared by certain isolates such as ST75 (2.1%, 1/46), ST90 (4.2%, 2/46), ST92 (2.1%, 1/46), ST108 (2.1%, 31/46), ST109 (2.1%, 1/46), ST172 (2.1%, 1/46), ST368 (4.2%, 2/46) and ST69 (4.2%, 2/46). These STs were grouped into the three clonal complexes, CC92, CC109 and CC28.

Discussion

A. baumannii develops resistance to imipenem through a variety of mechanisms. Carbapenemase is an important factor responsible for imipenem resistance. In the present study, common carbapenemases were detected in the

isolates, including bla_{OXA-51}, bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-58}, bla_{KPC}, bla_{IMP}, bla_{SHV}, bla_{GIM}, bla_{NDM} and bla_{VIM}. OXA-type enzymes are naturally present in *Acinetobacter spp.* and are usually expressed in small amounts (13). The expression of such genes is markedly higher under the effect of a strong promoter (insertion sequence ISAba1 is the most shared) and induce drug resistance only when combined with a reduction in outer membrane permeability and/or activation of the efflux pump (14). In the present study, bla_{OXA-51} and bla_{OXA-23} genes were prevalent among the isolates, results that are consistent with other reports (15-17). Carbapenemases that are different from OXA, such as KPC, IMP, SHV, GIM, NDM and VIM have strong carbapenem-hydrolysing activity (14). However, such types of carbapenemases were rarely detected in *A. baumannii*. The bla_{NDM} gene was identified in only one strain while the remaining resistant genes were not detected. NDM was first identified in *Escherichia coli* and *Klebsiella pneumoniae* in 2008 in India (18). This finding was followed by reports on NDM-producing *P. aeruginosa*, *Enterobacter cloacae*, *Citrobacter freundii* and *Enterococcus faecium* (19-23). In China, NDM-producing *A. baumannii* was first reported in 2011. Of the antimicrobials tested one NDM-positive isolate in the present study was identified that was multidrug-resistant, and only susceptible to amikacin, colistin and minocycline.

Drug efflux systems including AdeABC, AdeIJK, AdeDE and AdeXYZ (RND family) have been found in *A. baumannii*. Of these, the AdeABC efflux system is common in *A. baumannii* (24). This efflux pump, together with other resistant mechanisms, can lead to high-level imipenem resistance. Although mediated by the substrate, its expression may increase when a single point mutation occurs in the *adeR* or *adeS* gene (25). PAβN was proven to be an effective inhibitor of drug efflux. In the present study, *adeA*, *adeB* and *adeC* were present in all of the isolates because when PAβN was added the MICs inherent to imipenem in 13 isolates were decreased. The *adeS* gene differed from the *adeS* of standard strain and this is the possible reason for increased drug efflux associated with drug resistance.

Few studies concerning the impact of changes on membrane proteins in *A. baumannii* are available. In 2002, a laboratory in Argentina advocated for the first time that inducible resistance by imipenem can trigger loss of a 29-kDa membrane protein. In 2005, the same laboratory furthering their study, demonstrated that the outer membrane protein is encoded by the *CarO* gene and when there is an insertion mutation or any other mutation in the *CarO* gene makes it off and thus the strain become resistant to certain drugs (26). In the present study, the sequence of *CarO* gene had nucleotide insertions, deletions and point mutations in comparison with the standard strains and there were also differences in their nucleotide and amino acid sequences.

In summary, for a global epidemiologic analysis, a comparison of the results between different laboratories is required. MLST is a powerful tool used to transfer typing data and compare results via relevant databases. The MLST analysis revealed that the major epidemic clone of *A. baumannii* in Shanghai was ST208 (CC92 clone complex), which differed from the results obtained in other regions in China (27).

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