Distribution of carbapenemases and efflux pump in carbopenems-resistance Acinetobacter baumannii

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Acinetobacter baumannii has emerged as an important pathogen related to serious infections and nosocomial outbreaks around the world. The aim of this study was to detect the distribution of carbapenemases and efflux pump in carbopenems-resistance *Acinetobacter baumannii*(CRAB). In this study, 100 isolates of CRAB were collected from clinical specimens. Agar dilution was conducted to determine the minimum inhibitory concentrations (MICs) to 15 kinds of antibiotic. Genes of carbapenemases and efflux pumps were amplified by PCR. The expression difference of pump genes was also analyzed by real-time PCR between CRAB and carbopenems- sensitive *Acinetobacter baumannii* (CSAB). We found that most antibiotics, including aminoglycosides, fluoroquinolones and cephalosporins showed high MIC values in CRAB. While, all isolates were sensitive to polymyxin B. Among CRAB, 54, 32 and 16 isolates were positive for SHV-12 ¸ PER-1 and TEM-1, respectively. 86 isolates were positive for OXA-23. 55, 33 and 5 isolates carried adeB, adeJ and adeE genes. The expression level of adeB in CRAB was ten times higher than that in CSAB. Moreover, isolates with single adeE gene were detected for the first time in *Acinetobacter baumannii*.

Title Page

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Abstract

Acinetobacter baumannii has emerged as an important pathogen related to serious infections and nosocomial outbreaks around the world. The aim of this study was to detect the distribution of carbapenemases and efflux pump in carbopenems-resistance *Acinetobacter baumannii*(CRAB). In this study, 100 isolates of CRAB were collected from clinical specimens. Agar dilution was conducted to determine the minimum inhibitory concentrations (MICs) to 15 kinds of antibiotic. Genes of carbapenemases and efflux pumps were amplified by PCR. The expression difference of pump genes was also analyzed by real-time PCR between CRAB and carbopenems- sensitive *Acinetobacter baumannii* (CSAB). We found that most antibiotics, including aminoglycosides, fluoroquinolones and cephalosporins showed high MIC values in CRAB. While, all isolates were sensitive to polymyxin B. Among CRAB, 54, 32 and 16 isolates were positive for SHV-12，PER-1 and TEM-1, respectively. 86 isolates were positive for OXA-23. 55, 33 and 5 isolates carried adeB, adeJ and adeE genes. The expression level of adeB in CRAB was ten times higher than that in CSAB. Moreover, isolates with single adeE gene were detected for the first time in *Acinetobacter baumannii*.

Key words

carbopenems-resistance *Acinetobacter baumannii*; carbapenemases; efflux pump; resistant mechanism

Introduction

Acinetobacter baumannii is a gram-negative, nonfermentative bacillus that is widely distributed in the hospital environment and can cause a series of nosocomial infections[1], such as bacteremia, urinary tract infections, secondary meningitis, surgical site infections and ventilator-associated pneumonia. Treatment failure and death caused by *Acinetobacter baumannii* infections or diseases are common. One of the main reasons is that clinical isolates are frequently resistant to many commonly used antimbiotics. In particular, the appearance of carbopenems-resistancein strains poses great challenge to clinical treatment. The resistance mechanisms involve production of carbapenemases, decreased outer membrane permeability and overexpression of active efflux pump.

The carbapenemases reported in *Acinetobacter baumannii* include: (a) extended spectrum-β-lactamases （ESBL）, such as PER , SHV, TEM,CTX-M; (b) metallo-β-lactamases, such as IMP and VIM; (c)OXA-type enzymes, such as oxa-23 and oxa-24.

Active efflux pump, as an important resistance mechanism, attracts more and more attentions in recent years. At present, the efflux pump systems found in Acinetobacter spp include AdeABC, AdeIJK, AdeDE and AbeM. The first three belong to resistance nodulation division(RND) family. [2-5] Currently, The AdeABC was only detected in *Acinetobacter Baumannii*, while, AdeDE was mainly found in Acinetobacter genomic DNA group 3 (GDG3)[6].

The typical structure of RND family comprises the following[7]: a transporter protein, which is located in the inner membrane; a membrane fusion protein(MFP); and an outer membrane protein channel (OMP), which is located in the outer membranes. In AdeABC, AdeB is the transporter protein, AdeA is the MFP, and AdeC is the OMP. The expression of adeABC is regulated by adeR and adeS. However, the OMP of AdeDE hasn't been detected.

In this study, we assessed the distribution of the above-mentioned carbapenemases and efflux pumps in CRAB .

MATERIALS AND METHODS

Bacterial strains and growth conditions. During the period January to December in 2014, A total of 100 non-duplicate isolates of CRAB(resistant to imipenem and meropenem simultaneously) were collected from clinical specimens in a teaching Hospital with >1000 beds. All isolates were identified with the VITEK 32 system(bioMerieux Vitek Systems Inc, France), Pseudomonas aeruginosa ATCC27853 (Clinical Laboratory Center, Shandong) as control. These isolates were routinely grown at 37°C in blood agar. This research was approved by Ethical Committee of Rushan People's Hospital, Binzhou Medical University.

Antimicrobial susceptibility test. Antibiotic susceptibility test was determined by disk diffusion on Mueller-Hinton agar (Oxoid Ltd, England) according to the Clinical and Laboratory Standards Institute (CLSI, 2014) guidelines. Meropenem, amikacin, ceftazidime, cefoxitin, cefoperazone, cefoperazone, sulbactam, piperacillin, tazobactam, ciprofloxacin, levofloxacin, sulfamethoxazole, polymyxin B powder were purchased from The National Institute For Food and Drug Control. Imipenem and cefepime were presented by Sino-American

Shanghai Squibb Pharmaceuticals Ltd. and MSD Pharmaceutical Co. Ltd. , respectively.

Polymerase chain reaction (PCR) and nucleotide sequencing. DNA template was extracted as described previously[8]. PCR was done with a 50 µl reaction mixture containing 5×buffer 10µl, 2mmol/L $MgCl_2$ 4µl, 2.5mmol/L dNTPs misture 1µl, 10µmol/L each primer (Table 1) 0.5µl, DNA template 2.5µl, Taq enzyme 0.5µl, dH_2O 31µl. The PCR protocol was as follows: An initial denaturation step at 94°C for 5 min, followed by 30 cycles of 1 min at 94°C, 45 sec at 55°C, and 1 min at 72°C. A final extension step of 5 min at 72°C was performed. The presence and sizes of amplicons were assessed by electrophoresis in 1.5% agarose gels stained with ethidium bromide. The PCR products were purified and sequenced by ShineGene Bio-Technologies Inc., Shanghai, China.

Real-time PCR. 10 isolates of CRAB and 1 CSAB，which was also sensitive to most aminoglycosides, fluoroquinolones and cephalosporins, were selected to assess differences in adeB, adeJ genes expression, respectively. DNA-free RNA templates were prepared using RNA isolation kit (ShineGene Bio-Technologies Inc., Shanghai, China). RNA concentration and quality were assessed with a spectrophotometer at wavelengths of 260 and 280 nm. RNA was reverse transcribed by reverse transcription kit (Takara Biotechnology Co., LTD, China) according to the manufacturer's instructions. Real-time PCR assays were carried out with SYBR Premix Ex TaqTM kit (Takara Biotechnology Co., Ltd.) in a total of 20 µl reaction system, contained SYBR Premix Ex Taq 10 µl, ROX Reference Dye II 0.4µl, each primer (Table 1) 0.4 µl, cDNA 2µl and dH_2O 6.8µl. The PCR was performed on ABI 7500 Real-time PCR System. The condition was 95°C for 30s, followed by 40 cycles at 95°C for 5s and 60°C for 34s. 16sRNA was used as a housekeeping gene to normalize levels of each gene transcripts.

Results

Antimicrobial susceptibility pattern. The susceptibility test results of the 100 isolates were shown in Table 2. 55%, 76%, 81% and 82% isolates were resistant to cefoperazone-sulbactam, amikacin, cefoperazone, and levofloxacin, respectively. The antibiotics of MIC50>128 µg/ml included imipenem, ciprofloxacin, piperacillin, piperacillin/tazobactam, cefotaxime, cefoxitin, cefepime and sulfamethoxazole. Polymyxin was 100% susceptible in all isolates.

Detection of genes amplication. The positive rates of PER, SHV, TEM were 54%, 32% and 16%, respectively. 86% were positive to OXA-23. 55% and 33% isolates carried adeB and adeJ. 2 isolates carried 6 kinds of genes above. 5 isolates carried PER,SHV,OXA-23,adeB and adeJ at the same time. Five solates with single adeE gene were detected for the first time in *Acinetobacter baumannii*(Figure 1). OXA-24, IMP, VIM, CTX-M and abeM were not detected.

Sequencing analysis. PCR amplified products were purified and sequenced. Sequences were compared with genebank by blast. The identity of PER、SHV、TEM、OXA-23、adeB、adeJ、adeE were 99%、100%、99%、100%、100%、98% and 95% with corresponding genes in genebank.

Real-time RT-PCR. The relative expression of adeB in CRAB was 10.4 to 62.3 times higher than that in CSAB. The differences in expression levels were statistically significant ($P<0.05$). While, no significant difference was detected in adeJ(Shown in Table 3).

Discussion

Carbopenems is considered one of the most effective antibiotics to control the clinical *Acinetobacter baumannii* infection. CRAB has been spreaded all over the world since the first reported in 1994 in New York city. *Acinetobacter baumannii*, Once resistant to carbapenem, may often be resistant to other antibiotics and pose great challenge to clinical treatment. Our study has confirmed that the CRAB was also highly resistant to aminoglycosides, quinolones, cephalosporins and sulfa. However, all isolates detected were sensitive to polymyxin B. The combination of cefoperazone and sulbactam showed higher activity than cefoperazone alone (Table 2), which is basically consistent with the previous report[9]. Additionally, the resistance rate (56%) to amikacin is low relatively because of the less use of aminoglycoside in our hospital.

Resistance mechanism of *Acinetobacter baumannii* is complex. The mechanisms of generally fall into 3 categories: (1) antimicrobial-inactivating enzymes (2) reduced permeability of the outer membrane (3)overexpression of efflux pumps. For the first category, *Acinetobacter Baumannii* possess a wide array of carbapenemases that hydrolyze and confer resistance to penicillins, cephalosporins, and carbapenems, such as some ESBLs, MBLs and class D enzymes. In this article, a series of enzymes including PER, SHV, TEM and oxa-23 were detected. MBLs, the most significant threats, were not found. As reported by Bou G[10], the hydrolytic enzymes exhibit only low catalytic efficiency for the carbapenems. The enzymes could not explain the high resistance pattern. We speculated that other mechanism may participate in the carbapenems resistance together.

Active efflux system is another important resistance mechanism in *Acinetobacter baumannii*, which attracts more and more concern in recent years. It was reported that AdeABC system could significantly contribute for resistance meropenem[11]. In this research, we found that 55% and 33% isolates of CRAB carried adeB and adeJ, respectively. The realtime PCR confirmed expression level of adeB in CRAB was ten times higher than that in CSAB. No difference was detected in adeJ. It suggests that overexpression of AdeABC pumps can also result in resistance to imipenem in *Acinetobacter baumannii* isolates. 2 isolates carried 6 kinds of genes. 5% isolates carried PER,SHV,OXA-23,adeB and adeJ at the same time. The MIC values of these isolates were higher than those with one gene alone. It was consistent with previous report[12]. We speculate synergy among these mechanisms may result in high-level resistance to carbopenems.

AdeDE was first detected from Acinetobacter GDG3 in Hong Kong by Chau et al[4], and later, found in GDG 13TU and 17[6]. Lin et al[13] also reported that AdeDE was mainly in Acinetobacter GDG 3 and did not exist with AdeABC efflux pump, which was only found in *Acinetobacter baumannii* previously, and then speculated AdeABC and AdeDE efflux pumps may be likely species-specific. In this study, we found that 5 isolates were positive for adeE for the first time and negative for other genes by PCR, which demonstrated that adeE could also exist in *Acinetobacter baumannii*.

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Conflict of Interest Statement

We have no financial or commercial conflicts of interest to declare.

References

[1] Dijkshoorn L, Nemec A, Seifert H. An increasing threat in hospitals: multidrug-resistant *Acinetobacter baumannii*. Nat Rev Microbiol 2015;5:939-951.

[2] Magnet S, Courvalin P, Lamhert T. Resistance- nodulation-cell division-type efflux pump involved in aminoglycoside resistance in *A.baumannii* strain BM4454. Antimicrob Agents Chemother 2001;45:3375-3380.

[3] Damier-Piolle L, Magnet S, Bremont S, Lambert T, Courvalin P. AdeIJK,a resistance-nodulation-cell division pump effluxing multiple antibiotics in *Acinetobacter baumannii*. Antimicrob Agents Chemother 2008;52(2):557-62.

[4] Chau, S. L., Chu, Y. W., Houang, E. T. S.. Novel Resistance-Nodulation-Cell Division Efflux System AdeDE in Acinetobacter Genomic DNA Group 3. Antimicrob Agents Chemother 2004;48(10): 4054–4055.

[5] Su X Z, Chen J, Mizushima T, Kuroda T, Tsuchiya T. AbeM, an H+-coupled *A.baumannii* multidrug efflux pump belonging to the MATE family of transporters. Antimicrob Agents Chemother 2005;49: 4362–4364.

[6] Chu YW, Chau SL, Houang ET. Presence of active efflux systems AdeABC,AdeDE in different Acinetobacter spps. J Med Microbiol 2006;55: 477-478.

[7] Freire MP, Pierrotti LC, Oshiro IC, Bonazzi PR, de Oliveira LM, Machado AS et al. Carbapenem Resistant *A.baumannii* Acquired before Liver Transplantation: Impact on recipient outcomes. Liver Transpl 2015;19:22-24.

[8] Li JC, Rayner R, Nation RL, Owen RJ, Owen RJ, Spelman D, Tan KE et al. Heteroresistance to colistin in multidrug-resistant *A. baumannii*. Antimicrob Agents Chemother 2015;50:2946-2950.

[9] Zhou H, Yang Q, Yu YS, Wang H, Yang Q, Chen M et al. Clonal spread of carbopenems-resistancein *A.baumannii* among different cities of China. J Clin Microbiol 2007;45(12):4054-4057.

[10] Bou G, Oliver A, Martinez-Beltran J: OXA-24, a novel class D β-lactamase with carbapenemase activity in an *Acinetobacter baumannii* clinical strain. Antimicrob Agents Chemother 2000;44:1556-1561.

[11] Rumbo C, Gato E, Lopez M, Ruiz de Alegria C, Fernandez-Cuenca F, Martinez-Martinez L et al. Contribution of efflux pumps, porins, and β-lactamases to multidrug resistance in clinical isolates of *Acinetobacter baumannii*.Antimicrob Agents Chemother 2013;57(11):5247-5257.

[12] Little ML, Qin X, Zerr DM, Weissman SJ. Molecular diversity in mechanisms of carbapenem resistance in paediatric *Enterobacteriaceae.* Int J Antimicrob Agents 2012;39(1):52-57.

[13] Lin L, Ling BD, Li XZ. Distribution of the multidrug efflux pump genes, adeABC, adeDE and adeIJK, and class 1 integron genes in multiple-antimicrobial -resistant clinical isolates of *Acinetobacter baumannii- Acinetobacter calcoaceticus* complex. Int J Antimicrob Agents 2009;33(1):27-32.

198

199 Table 1 The sequences of Oligonucleotide primers

Primers（5’-3’）	Primer sequences	Expected fragment(bp)
PER P1	ATGAATGTCATTATAAAAGC	925
PER P2	AATTTGGGCTTAGGGCAGAA	
TEM P1	TAGGCTGCACGAGTGGGTTA	560
TEM P2	TACTTGATGCCGGGAAGCTA	
CTX-M P1	ACGCTACCCCTGCTATTT	780
CTX-M P2	GCTTTCCGCCTTCTGCTC	
SHV P1	ATTTGTCGCTTCTTTACTCGC	425
SHV P2	CCCGCAGATAAATCACCACAAT	
IMP P1	ATCCAAGCAGCAAGCGCGTTA	879
IMP P2	AGGCGTGCTGCTGCAACGACTTGT	
VIM P1	AGTGGTGAGTATCCGACAG	261
VIM P2	ATGAAAGTGCGTGGAGAC	
OXA-23 P1	GATGTGTCATAGTATTCGTCG	1069
OXA-23 P2	TCACAACAACTAAAAGCACTG	
OXA-24 P1	GTACTAATCAAAGTTGTGAA	800
OXA-24 P2	TTCCCCTAACATGAATTTGT	
adeB P1	TTAACGATAGCGTTGTAACC	391
adeB P2	TGAGCAGACAATGGAATAGT	
adeJ P1	ATTGCACCACCAACCGTAAC	305
adeJ P2	TAGCTGGATCAAGCCAGATA	
abeM P1	GTAGGTGTAGGCTTATGGA	703
abeM P2	GTACCGAAGTGACTGAAAT	
adeE P1	GAGCTGAGGATTCTCTATGT	504
adeE P2	AGTGTGCTCACCATATAGTC	
16S rRNA P1	GGAGGAAGGTGGGGATGACG	241
16S rRNA P2	ATGGTGTGACGGGCGGTGTG	

200

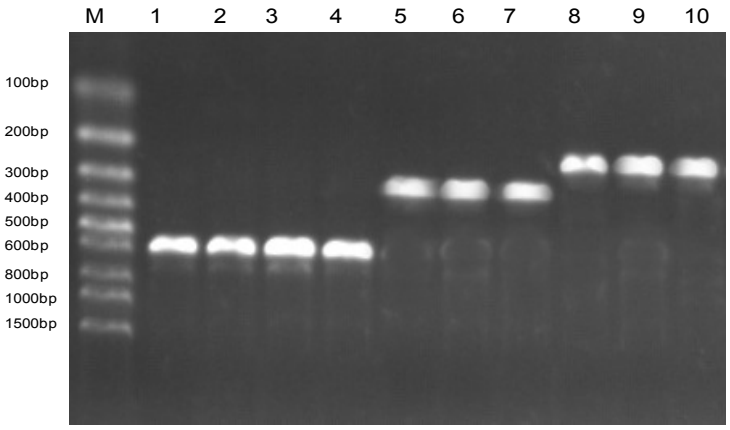
201

Table2 Resistance rate of 100 isolates of CRAB

antibiotics	MIC range	MIC_{50}	MIC_{90}	R%
Imipenem	32-512	256	>256	100
Meropenem	8-256	64	128	100
Amikacin	2-512	64	>256	76
Ciprofloxacin	256-512	256	>256	100
Levofloxacin	0.5-128	8	32	82
Piperacillin	256-512	>256	>256	100
Piperacillin-Tazobactam	2-512	128	256	88
Cefoxitin	64-512	>256	>256	100
Cefotaxime	256-512	>256	>256	100
Cefoperazone	0.5-256	64	256	81
Cefoperazone-Sulbactam	1-512	16	128	55
Ceftazidime	1-256	32	256	92
Cefepime	256-512	>256	>256	100
Sulfamethoxazole	64-512	>256	>256	100
Polymyxin B	0.0625-2	0.25	1	0

224 Table3 The relative expression of adeB and adeJ in CRAB compared to CSAB

NO.	adeB	adeJ
1	25.8	3.1
2	36.9	2.2
3	10.4	0.9
4	56.2	0.7
5	29.1	1.1
6	33.7	0.3
7	62.3	1.5
8	31.2	1.8
9	28.3	0.2
10	56.5	1.1
$\bar{x}$	37.04	1.29



M: marker; 1-4: adeE; 5-7: adeB; 8-10: abeJ;

Figure 1 Electrophoresis of adeE，adeB and abeJ genes