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Original Article

Characterization and distribution of drug resistance associated *β-lactamase, membrane porin* and *efflux pump* genes in MDR *A. baumannii* isolated from Zhenjiang, China

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Abstract: Background: *Acinetobacter baumannii* (*A. baumannii*), especially the multidrug resistant *A. baumannii* (MDR-AB) is becoming a common opportunistic pathogen in hospital, and constitutes significant public health threats. This study aimed at investigating the relationship between drug resistance with expression of class A-D β-lactamase genes, mutation in membrane porin and over-expression of efflux pump genes among *A. baumannii* isolated from Zhengjiang, China. Methods: Antibiotic susceptibility assays were performed using Kirby-Bauer disc diffusion method. PCR was used to detect *β-lactamase* genes and *carO, oprD, adeR, ade*S. Real-time PCR was used to assess the mRNA expression level of efflux pump gene *adeB*. The software of DNAMAN was applied to assemble *oprD* and *carO* sequences, and the sequences were compared with those retrieved from GenBank (http://www.ncbi.nlm.nih.gov/). Results: 27 isolates (61.4%) in this study were MDR-AB, in which five β-lactamases including *TEM, CTX-M-2, ADC, OXA-23* and *OXA-51* were found, and the positive rate was 96.3% (26), 14.8% (4), 92.6% (25), 88.9% (24) and 92.6% (25), respectively. In addition, the expression level of *adeB* mRNA was significantly increased in MDR-AB, it might due to *adeR* mutation. Some mutations were also found in *carO* and *oprD*. Conclusion: MDR-AB showed high relationship with β-lactamase, mutation in membrane porin and overexpression of *adeB*, which may directly relates to the mutation in regulating gene *adeR*.

Keywords: *A. baumannii*, multidrug resistant, β-lactamase, membrane porin, efflux pump

Introduction

A. baumannii is a kind of nosocomial opportunistic pathogen, which can cause series of diseases, including pneumonia, skin and soft tissue infections, urinary tract infections and bacteraemia in immunocompromised patients [1], especially those in the intensive care units. Moreover, MDR-AB have emerged swiftly all over the world [2-5], bringing a knotty problem which is becoming much more difficult to cure in patients who have been infected. Thus, this pathogen possesses huge potential threat to human health, and its resistant mechanisms urgently need to be understood.

Although the mechanism of resistance in *A. baumannii* has largely been reported in recent years, which are associated with β-lactam antibiotics resistance includes producing varies enzymes, mutation in membrane porin, changing in conformation of PBP and over-expression of efflux pumps. In this study, we aim to characterize the frequency of class A~D β-lactamases, mutation in the membrane porin encoding genes and efflux pump relative gene among *A. baumannii* which were isolated in Zhenjiang, China.

Material and methods

Bacterial strains

44 strains of *A. baumannii* used in this study were isolated from inpatients with infectious diseases in the Affiliated Hospital of Jiangsu

Table 1. Primers used in PCR assay

Target gene		Primer sequence	Size (bp)
Membraneporin	*carO*	P1: ATGAAAGTATTACGTGTTTTAGTGACAAC;	729
		P2: TTACCAGTAGAATTCNACACCAACT	
	cprD	P1: ATGCTAAAAGCACAAAAACTTACATTAGCA;	1320
		P2: TTAGAATAATTTCACAGGAATATCTAAGAA	
Class A β-lactamases	*TEM*	P1: AGGAAGAGTATGATTCAACA;	535
		P2: CTCGTCGTTTGGTATGGC	
	SHV	P1: TGCGCAAGCTGCTGACCAGC;	305
		P2: TTAGCGYTGCCAGTGCTCGA	
	CTX-M-1 group	P1: ATGGTTAAAAAATCACTGCGYCAGTTC;	876
		P2: TCACAAACCGTYGGTGACGATTTTAGCCGC	
	CTX-M-2 group	P1: ATGATGACGCAGAGCATTCGCCGCTCA;	876
		P2: TCAGAAACCGTGGGTTACGATTTTCGC	
	CTX-M-9 group	P1: ATGGTGACAAAGAGAGTGCAACGG;	876
		P2: TTACAGCCCTTCGGCGATGATTCTCGC	
	PER	P1: AGTCAGCGGCTTAGATA;	978
		P2: CGTATGAAAAGGACAATC	
	GES	P1: ATGCGCTTCATTCACGCAC;	846
		P2: CTATTTGTCCGTGCTCAGG	
	VEB	P1: GCGGTAATTTAACCAGA;	961
		P2: GCCTATGAGCCAGTGTT	
	CARB	P1: AAAGCAGATCTTGTGACCTATTC;	588
		P2: TCAGCGCGACTGTGATGTATAAAC	
Class B β-lactamases	*IMP*	P1: CGGCCKCAGGAGMGKCTTT;	587
		P2: AACCAGTTTTGCYTTACYAT	
	VIM	P1: ATTCCGGTCGGMGAGGTCCG;	633
		P2: GAGCAAGTCTAGACCGCCCG	
	SIM	P1: ACAAGGGATTCGGCATCGTT;	355
		P2: TTATCTTGAGTGTGTCCTGG	
Class C β-lactamases	*DHA* group	P1: AACTTTCACAGGTGTGCTGGGT;	405
		P2: CCGTACGCATACTGGCTTTGC	
	ADC Group	P1: GGTATGGCYGTGGGBGTYATTC;	739
		P2: CTAAGASTTGGTCRAARGGT	
Class D β-lactamases	*OXA-1* group	P1: CTGTTGTTTGGGTTTCGCAAG;	440
		P2: CTTGGCTTTTATGCTTGATG	
	OXA-2 group	P1: CAGGCGCYGTTCGYGATGAGTT;	233
		P2: GCCYTCTATCCAGTAATCGCC	
	OXA-10 group	P1: GTCTTTCRAGTACGGCATTA;	822
		P2: GATTTTCTTAGCGGCAACTTA	
	OXA-20 group	P1: TTGATAATCCGATTTCTAGCAC;	801
		P2: CTAGTTGGGTGGCAAAGCAT	
	OXA-23 group	P1: ATGAATAAATATTTTACTTGCTATGTG;	822
		P2: TTAAATAATATTCAGCTGTTTTAATGA	
	OXA-24 group	P1: CAAGAGCTTGCAAGACGGACT;	420
		P2: TCCAAGATTTTCTAGCRACTTATA	
	OXA-51 group	P1: ATGAACATTAAAGCACTCTTACTT;	825
		P2: CTATAAAATACCTAATTGTTCTAA	
	OXA-58 group	P1: TCGATCAGAATGTTCAAGCGC;	530
		P2: ACGATTCTCCCCTCTGCGC	

Efflux pump regulatory gene	*adeR*	P1: GCTTGAGCGACTTCTTTTGAAT; P2: CTAATCCAGCCTTTTTCAATCG	1134
	*ade*S	P1: CCCCTAGCTGTAAAAGATGACG; P2: ACCAATGGGGTCAAATACACA	1354

Table 2. Primer sequence of efflux pump genes and 16SrRNA

Target gene	Primer sequence	Size (bp)
adeB	P1: GGATTATGGCGACAGAAGGA	104 bp
	P2: AATACTGCCGCCAATACCAG	
16SrRNA	P1: GTAGCGGTGAAATGCGTAGA	85 bp
	P2: CTTTCGTACCTCAGCGTCAG	

University and the First People's Hospital of Zhengjiang during November 2012 to May 2013. Sputum constituted the most specimens in this study, which was about 90%. Isolates were identified by VITEK 2 compact automatic bacteria identification system (French). The bacteria were incubated on blood culture media and preserved at -80°C until they were used.

Antibiotic susceptibility testing

Antibiotic susceptibility assays were performed using Kirby-Bauer disc diffusion method. In brief, over night bacterial cultures were diluted to 0.5 with normal saline in McFar land, and then sub-cultured in 5 mL Mueller-Hinton broth (Bosai Biotechnology Co. Ltd). The measured inhibition zones were judged according to the CLSL 2014 standard. *E. coli* (ATCC25922) and *P. aeruginosa* (ATCC27853) were used as control strains. The following antibiotics were tested: Imipenem, Gentamicin, Amikacin, Ceftazidime, Ceftriaxone, Cefepime, Ciprofloxacin, Ampicillin/sulbactam, Sulfamethoxazole, Cefoperazone/sulbact, Piperacillin/tazobactam, Minocycline (Oxoid company, England).

PCR analysis

PCR was used to detect the β-lactamase genes *carO, oprD, adeR* and *ade*S. Primers for *carO, oprD, adeR* and *ade*S were synthesized by Wuxi Clone Gen-Tech Institute in China, others were purchased from Shanghai Hanyubiotech Co., Ltd. Among these, the primers for *adeR* and *ade*S were used to amplify the full-length fragment of efflux pump regulatory genes *adeR* and *ade*S. The PCR kits were provided by Takara Company. All of the reagents used in this experiment were used following the manufacturer's instructions. Primers used are listed in **Table 1**.

Sequencing and analyzing for carO and oprD

(www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.html) was used to analyze the sequences of *carO, oprD, adeR* and *ade*S. Chromas were used to read each sequence, and then BLAST online to analysis the results. Software of DNAMAN was used to assemble *oprD, adeR* and *ade*S sequences. The sequences obtained in this experiment were compared with those present in the GenBank database from strain SDF (http://blast.ncbi.nlm.nih.gov). After BLAST online, translating the sequences of *carO* and *oprD* into amino acid, and then compared with amino acid sequence of strain SDF. Sequence from strain SDF was downloaded from www.biocyc.org. The three-dimensional structure of OprD was created according to the amino acid sequence of OprD using the molecular visualization tools Swiss-Pdb Viewer 3.7.

Analysis of the mRNA level of efflux pump gene AdeB

RT-PCR was used to detect the mRNA level of efflux pump gene *adeB*. Primer of *16SrRNA* was synthesized by Shanghai Hanyubiotech Co., Ltd and Primer of *adeB* was from Hinggins et al [6]. The RT-PCR kits were provided by Takara Company. All of the reagents were used following the manufacturer's instructions. Primers used in this study were listed in **Table 2**. Relative quantification was used to analyze the data according to the formula $2^{-\Delta Ct}$ ($\Delta Ct = Ct_{unknown} - Ct_{housekeeping\ gene}$).

Statistical analysis

Data were shown as the mean ± S.E.M. The software SPSS was used to perform the statistical analysis of the data with homogeneity of variance test and non-parametric test. $P<0.05$ was considered to be statistically significant.

Results

Characterization of the isolates

MDR-AB refers to resistance to more than 3 of the 5 classes of antibiotics resistance including

Table 3. The results of antibiotic susceptibility patterns of *A. baumannii isolates*

Antibiotic	R (%)	I (%)	S (%)
Imipenem	27 (61.4)	2 (4.5)	15 (34.1)
Gentamicin	28 (63.6)	1 (2.3)	15 (34.1)
Amikacin	29 (65.9)	0 (0.0)	15 (34.1)
Ceftazidime	28 (63.6)	0 (0.0)	16 (36.4)
Ceftriaxone	31 (70.5)	1 (2.3)	12 (27.2)
Cefepime	30 (68.2)	3 (6.8)	11 (25.0)
Ciprofloxacin	28 (63.6)	0 (0.0)	16 (36.4)
Ampicillin/sulbactam	12 (27.2)	10 (22.7)	22 (50.1)
Sulfamethoxazole	29 (65.9)	2 (4.5)	13 (29.6)
Cefoperazone/sulbact	20 (45.5)	3 (6.8)	21 (47.7)
Piperacillin/tazobactam	27 (61.4)	3 (6.8)	14 (31.8)
Minocycline	18 (40.9)	6 (13.6)	20 (45.5)

The break point of Kirby-Bauer disc diffusion in *A. baumannii* to Cefoperazone/sulbact was accepted according to the standard (S: ≥21 mm; I: 16 mm-20 mm; R: ≤15 mm) [7]. S = susceptible; I = intermediate; R = resistant.

Table 4. The prevalence of β-lactamase genes in MDR-AB

β-lactamase	Positive	Negative
TEM	96.3% (26)	3.7% (1)
CTX-M-2	85.2% (23)	14.8% (4)
ADC	92.6% (25)	7.4% (2)
OXA-23	88.9% (24)	11.1% (3)
OXA-51	92.6% (25)	7.4% (2)

aminoglycoside, beta lactamase inhibitor penicillinum, cephalosporins, carbon alkene and fluoroquinolone. In this study, 27 strains of *A. baumanii* were multidrug-resistant in 44 isolates, and their antibiotic susceptibility patterns were shown in **Table 3**.

TEM, CTX-M-2, ADC, OXA-23, OXA-51 were expressed inMDR-AB

Among the β-lactamase producing MDR-AB isolates, only *TEM, CTX-M-2, ADC, OXA-23* and *OXA-51* were found in this study. It was therefore suggested that the multidrug resistance of the isolates might be associated with these β-lactamase genes (**Table 4**).

Point mutation of CarO in the isolates

Sequencing results showed that the *carO* was obtained in 25 of the 27 MDR-AB isolates, which was different from strain SDF. After translating it into amino acid sequence, there were some point mutations were found. Apart from some sense mutation, they were also deficient in 4 amino acids locus at positions 144, 145, 146 and 213 in the isolates, and the amino acid distance was showed in **Figure 1**.

Point mutation of OprD in the isolates

Like *carO* analysis, 29 of the 44 strains expressed *oprD*, and the sequences of OprD from 27 strains of MDR-AB were also showed absolutely the same in this study. Their sense mutations were T→98→A and D→278→G, and the amino acid distance was showed in **Figure 2** and the three-dimensional structure of OprD was showed in **Figure 3**.

The mutation of adeR and adeS

It was reported that *adeR* and *adeS*, as main efflux pump regulator genes, involved in the regulation of expression of AdeABC efflux pump system genes [8]. In our study, 3 strains were selected randomly from the MDR-AB strains, and the full length of their *adeR* and *adeS* genes was amplified by PCR, followed by blasting online with strain SDF genes. The result showed that there was a single mutation A→G in 2639 of *adeR*, which cause an Lys219→Glu amino acid replacement, while nonsense mutation was found in *adeS* (**Figure 4A**, **4B**).

In addition, the *adeB* gene could be detected in all MDR-AB strains. The mRNA expression level of *adeB* was also detected using qRT-PCR, and the result showed that the *adeB* mRNA in MDR-AB was significantly increased (**Figure 4C**, **4D**).

Discussion

The role of *A. baumannii* is strengthened by relatively high resistance to numerous antibiotics which is determined by both natural and acquired mechanisms. In multi-drug resistant strains of *A. baumannii*, the drugs of choice are the Carbapenems. Unfortunately, the development of resistance dose not elude even this family of antimicrobial agents, due to the production of carbapenemase or β-lactamases. In addition, resistance to a wide range of antibiotics can be caused by a single mutation in a gene. Emerging expression of β-lactamase and mutation in membrane porin undoubtedly become a significant mechanism of MDR-AB.

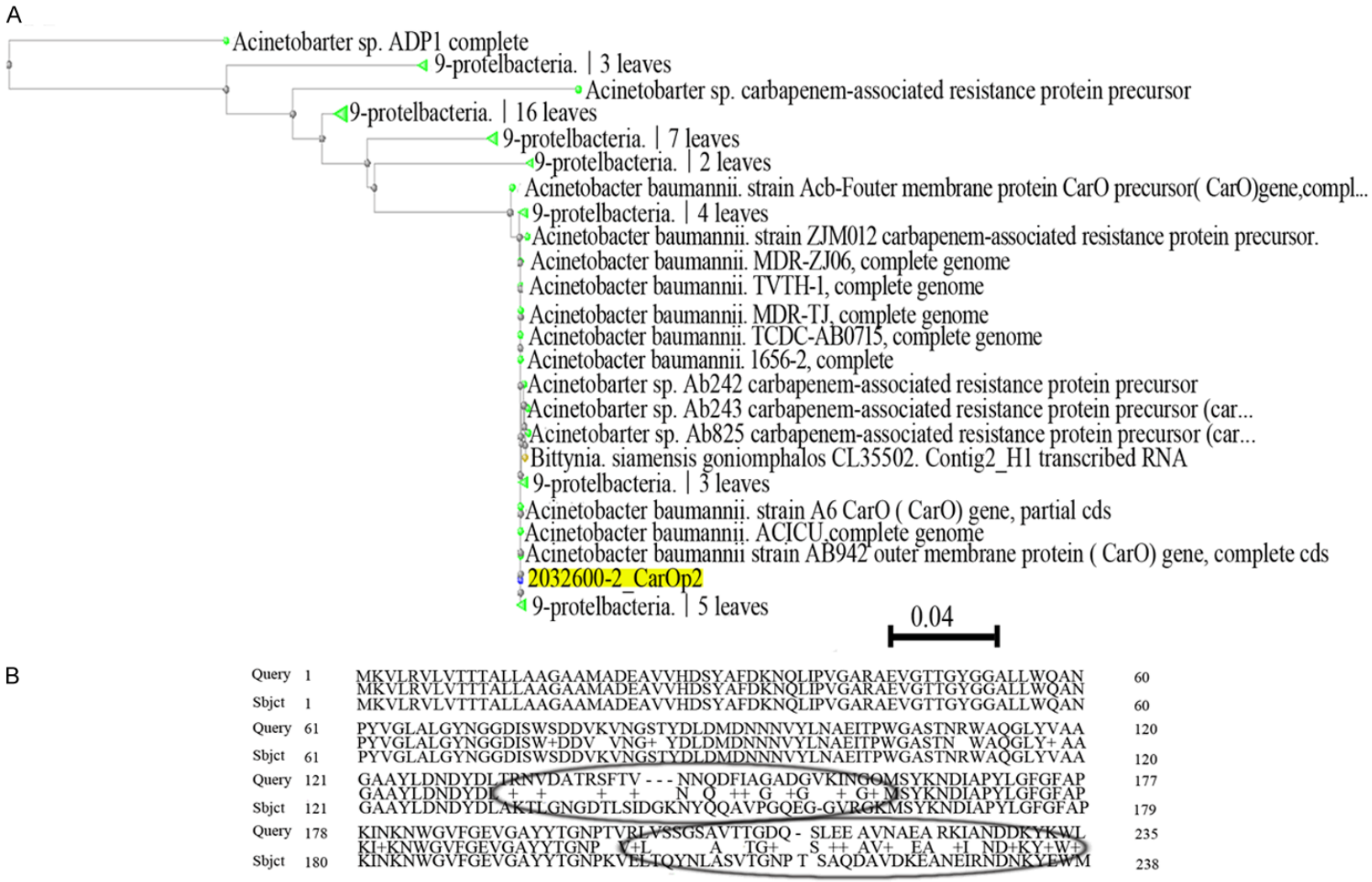

Figure 1. Homologic analysis of CarO in *A. baumannii* isolates. A. The amino acid distance based on CarO of *A. baumannii* isolates. B. The sense mutation of amino acid of CarO in MDR-AB strains compared with strain SDF.

A

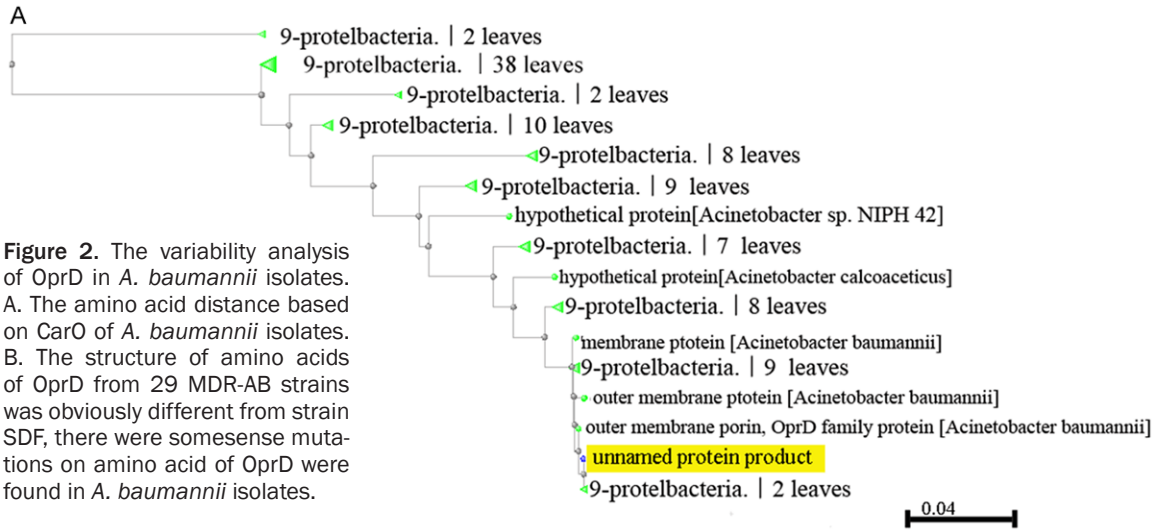

Figure 2. The variability analysis of OprD in *A. baumannii* isolates. A. The amino acid distance based on CarO of *A. baumannii* isolates. B. The structure of amino acids of OprD from 29 MDR-AB strains was obviously different from strain SDF, there were somesense mutations on amino acid of OprD were found in *A. baumannii* isolates.

B

```
Query  1    MLKAQKLTLAVLISAAIISSAQASEQSEAKGFVEDANGSILFRTGYI SRDKKQGAKDTSS  60
            MLKAQKLTLAVLISAAIISSAQASEQSEAKGFVEDANGSILFRTGY++RDKKQGAKDTSS
Sbjct  1    MLKAQKLTLAVLISAAIISSAQASEQSEAKGFVEDANGSILFRTGYLTRDKKQGAKDTSS  60

Query  61   VAQSAIVSIESGFTPGIVGFGVGVVGDGSFKIGENKNAGNQMIPKHNDGSAYDHWARGGG  120
            VAQSAIVSIESGFTPGIVGFGVGVVGDGSFKIGENKN GNQMIPKHNDGSAYDHWARGGG
Sbjct  61   VAQSAIVSIESGFTPGIVGFGVGVVGDGSFKIGENKNTGNQMIPKHNDGSAYDHWARGGG  120

Query  121  SVKARFSNTTVRYGTQVLDLPVLASNTGRMVPEYFTGTLLTSHEIKNLEVVAGKFTKDQM  180
            SVKARFSNTTVRYGTQVLDLPVLASNTGRMVPEYFTGTLLTSHEIKNLEVVAGKFTKDQM
Sbjct  121  SVKARFSNTTVRYGTQVLDLPVLASNTGRMVPEYFTGTLLTSHEIKNLEVVAGKFTKDQM  180

Query  181  SDQINTDADASGRGLDRAIVWGAKYKFNDNLNASYYGLDSKNALERHYANVNFKQPLAN  240
            SDQINTDADASGRGLDRAIVWGAKYKFNDNLNASYYGLDSKNALERHYANVNFKQPLAN
Sbjct  181  SDQINTDADASGRGLDRAIVWGAKYKFNDNLNASYYGLDSKNALERHYANVNFKQPLAN  240

Query  241  SSLTYDFSGYHTKFDANAHTYSATGTVAPNYAADGIAGEEKTNNIWAISGTYATGPHSVM  300
            SSLTYDFSGYHTKFDANAHTYSATGTVAPNYAADGIA EEKTNNIWAISGTYATGPHSVM
Sbjct  241  SSLTYDFSGYHTKFDANAHTYSATGTVAPNYAADGIADEEKTNNIWAISGTYATGPHSVM  300

Query  301  LAYQQNTGNVGYDYGQNADGFQSIYLPNSYMSDFIGNHEKSAQIQYNVDFGKLGVLPGLN  360
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Sbjct  301  LAYQQNTGNVGYDYGQNADGFQSIYLPNSYMSDFIGNHEKSAQIQYNVDFGKLGVLPGLN  360

Query  361  WTTAFVYGWDIKVRNVTDDAQEREFFNQVKYTVQSGFAKDASLRIRNSYYRASDAYQGAY  420
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Sbjct  361  WTTAFVYGWDIKVRNVTDDAQEREFFNQVKYTVQSGFAKDASLRIRNSYYRASDAYQGAY  420

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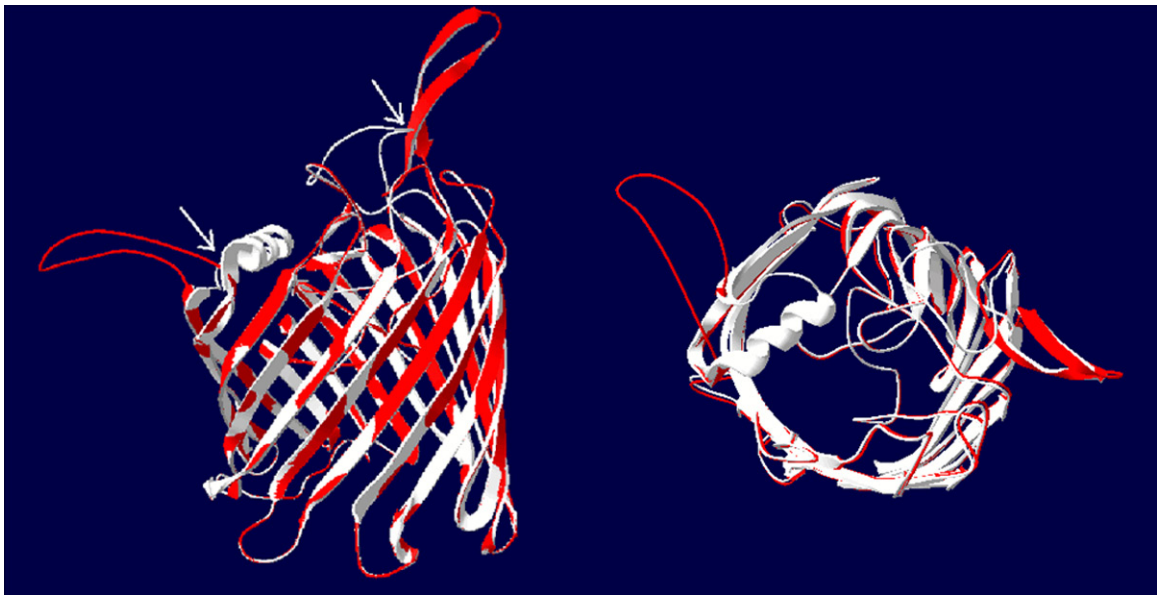

Figure 3. The three-dimensional structure of OprD. The PCR products of OprD genes from 29 MDR-AB strains were sequenced, and the homology analysis showed that there were mutations in MDR-AB strains compared with the SDF strain. It was significant difference in three dimensional structure of protein between MDR-AB and SDF strains by molecular modeling and comparison of overlapping molecular structure. The white region isthe structure of OprD and the red region is the structure of MDR-AB. Left: the frontage of β-barrel structure; Right: the side of β-barrel structure.

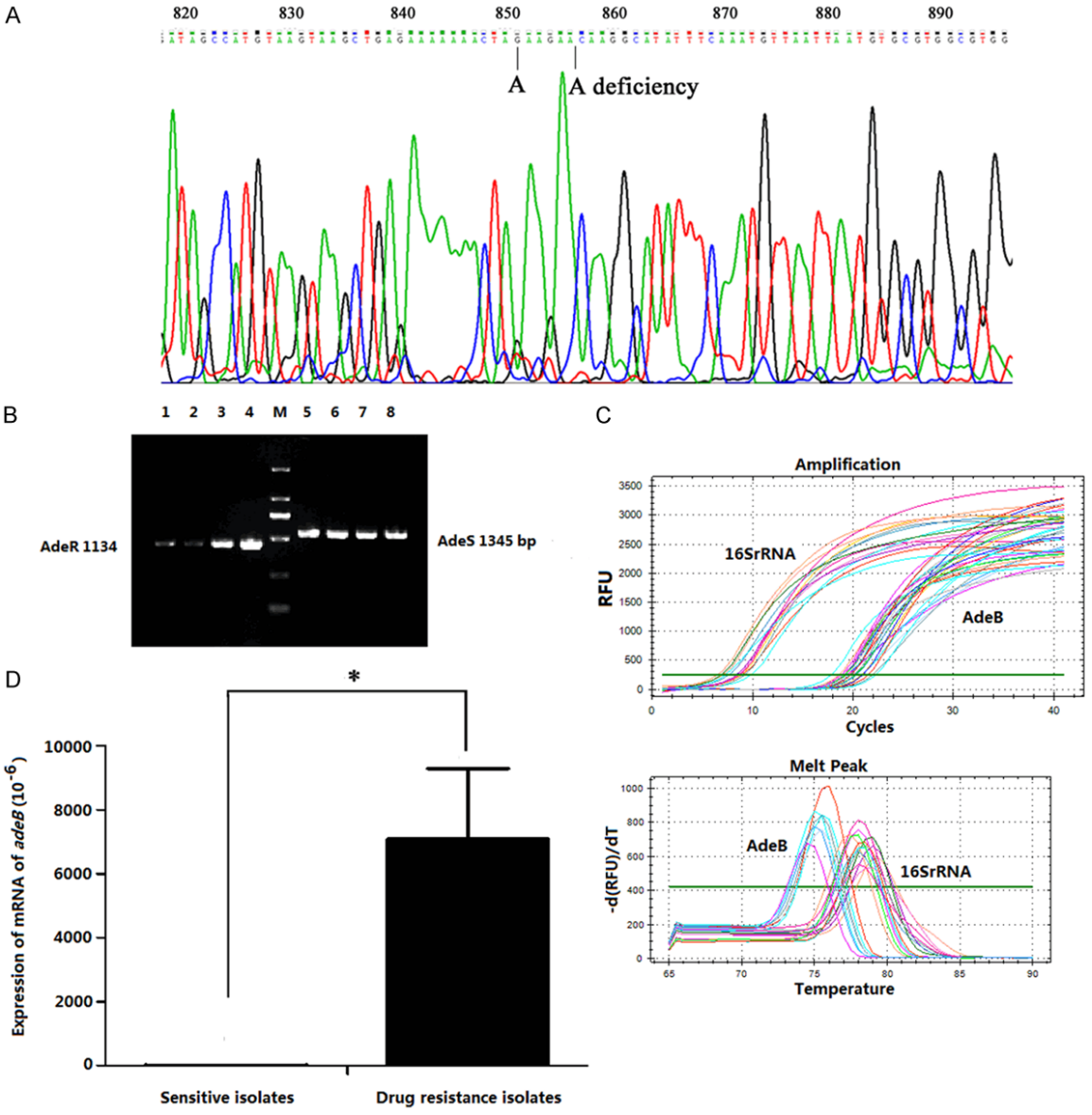

Figure 4. The expression and characteristics of efflux pump genes. PCR amplification and DNA sequence analysis were showed in (A and B), there was a single mutation A→G in 2639 of *adeR*, which causean Lys219→Glu amino acid replacement; the deficiency of base "A" in 2633 of *adeR* displayed a nonsense mutation, in addition, nonsense mutation was found in *ade*S (A and B). (C) The results of qRT-PCR for detecting mRNA levels of *16SrRNA* and *adeB*. (D) Comparison of the levels of *adeB* mRNA expression between drug sensitive isolates and MDR-AB strains. *P<0.01.

Expression of β-lactamase is often assumed to be the result of resistant to β-lactams antibiotics. According to amino acid sequence and conserved motifs, β-lactamases are defined to classes A, B, C, and D [9]. Class C β-lactams means AmpC enzymes, AmpC enzymes are cephalosporinase encoded by chromosomally in *Acinetobacter* spp and *P. aeruginosa* [10].

Chromosomal AmpC β-lactams have been identified in several works in *A. baumannii* such as Acinetobacter-derived cephalosporinase (ADC) [11, 12]. The expression of AmpC enzymes was reported to be promoted by insertion such as *IASba1* upstream the *blaampC gene* [11], and to be associated with cephalosporin resistance [13-15].

Our data has revealed that *TEM, ADC, OXA-23* and *OXA-51* expressed highly in *A. baumannii* isolates, which were more than 90%, specifically the *TEM*. Extended spectrum β-lactamases (ESBLs) identified in *Acinetobacter spp* were *TEM, SHV, PER* and *CTX* [10, 16], and the most popular was also TEM. In this study, the positive rate of *TEM* was as high as 96.3%, except one strain which did not expressed *TEM*, all the others showed obvious stripe. *TEM* could be mediated not only by chromosome, but also plasmid and *TEM-1* is reported to be associated with sulbactam resistance in *A. baumannii* [17]. CTX-M group was not found in our *A. baumannii* isolates, but it is known to be the most common type of class A β-lactamases strains in Turkey among ESBL-producing *E. coli* [18].

Carbapenem resistance in *A. baumannii* was reported to be due to the emergence and dissemination of OXA-type carbapenemase encoding genes [19], such as OXA-51-like gene [20]. Pagano et al revealed that upstream of *OXA-23*-like gene only in isolates resistant to carbapenems, whereas *ISAba1* upstream of *OXA-51*-like gene was presented in both susceptible and resistant isolates, and *ISAba1/blaOXA-51*-like gene alone could not lead to resistance to carbapenems [21]. The positive rate of *OXA-51* in this experiment was as high as 92.6% and *OXA-23* was 88.9%, and the confirming fact was that resistance rate to imipenem was up to the 96.8% among MDR-AB *strains*. Recent study showed that IMP increased carbapenemase activities in multidrug-resistant *Pseudomonas aeruginosa* [22], but no metallo-β-lactamase gene was found in those isolates.

There are limited reports on mutations in the membrane porin of *Acinetobacter baumannii*. In our experiment, the sequencing results of CarO and OprD were used to compare with strain SDF, and we found some sense mutations in most of the *A. baumannii* isolates. It can be speculated that the channel would be changed subsequently after increasing mutations in membrane porin, and could resulted in antibiotics being not able to enter or the amount entered being decreased, which will result in resistance.

Furthermore, more and more evidences have shown the relationship between multiple drug resistance of *A. baumannii* and expression of efflux system [8, 23-27]. As it is known, under antibiotic, point mutation of efflux pump regulatory gene induce the over-expression of efflux pump system, decreasing the concentration of antibiotics in *A. baumannii*. Our data showed that the expression of *adeB* had a close relationship with the mutation of *adeR*, it might expel the antibiotics from bacteria, and mediate drug resistance.

In conclusion, the mutual effect of various of drug resistance mechanisms lead to the overwhelming resistance to majority of antibiotics, and MDR-AB have populated all over the world, it not only can be spread within a country, but also disseminated from one country to another [28-30]. In this study, we found that those strains of MDR-AB have high relationship with some β-lactamase and mutations in *carO* and *oprD*, and mutation in *adeR* causing overexpression of *adeB*. Consequently, prevention the spread of β-lactamase producing isolates should be managed carefully, in order to prevent the spread of MDR-AB within hospitals.

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Disclosure of conflict of interest

None.

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