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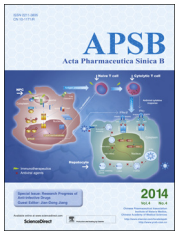

ORIGINAL ARTICLE

Genetic basis of high level aminoglycoside resistance in *Acinetobacter baumannii* from Beijing, China

Lu Nie[a,b,†], Yuemeng Lv[a,†], Min Yuan[a,c], Xinxin Hu[a], Tongying Nie[a], Xinyi Yang[a], Guoqing Li[a], Jing Pang[a], Jingpu Zhang[a], Congran Li[a], Xiukun Wang[a,*], Xuefu You[a,*]

[a]*Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100050, China*
[b]*The First People's Hospital of Foshan, Foshan 528000, China*
[c]*State Key Laboratory for Infectious Diseases Prevention and Control, National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention, Beijing 102206, China*

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KEY WORDS

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HLAR;
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Abstract The objective of this study was to investigate the genetic basis of high level aminoglycoside resistance in *Acinetobacter baumannii* clinical isolates from Beijing, China. 173 *A. baumannii* clinical isolates from hospitals in Beijing from 2006 to 2009 were first subjected to high level aminoglycoside resistance (HLAR, MIC to gentamicin and amikacin > 512 μg/mL) phenotype selection by broth microdilution method. The strains were then subjected to genetic basis analysis by PCR detection of the aminoglycoside modifying enzyme genes (*aac(3)-I*, *aac(3)-IIc*, *aac(6′)-Ib*, *aac(6′)-II*, *aph(4)-Ia*, *aph(3′)-I*, *aph(3′)-IIb*, *aph(3′)-IIIa*, *aph(3′)-VIa*, *aph(2″)-Ib*, *aph(2″)-Ic*, *aph(2″)-Id*, *ant(2″)-Ia*, *ant(3″)-I* and *ant(4′)-Ia*) and the 16S rRNA methylase genes (*armA*, *rmtB* and *rmtC*). Correlation analysis between the presence of aminoglycoside resistance gene and HLAR phenotype were performed by SPSS. Totally 102 (58.96%) HLAR isolates were selected. The HLAR rates for year 2006, 2007, 2008 and 2009 were 52.63%, 65.22%, 51.11% and 70.83%, respectively. Five modifying enzyme genes (*aac(3)-I*, detection rate of 65.69%; *aac(6′)-Ib*, detection rate of 45.10%; *aph(3′)-I*, detection rate of 47.06%; *aph(3′)-IIb*,

*Corresponding authors. Tel.: +86 10 67061033; fax: +86 10 63017302.
E-mail addresses: xiukunwang@139.com (Xiukun Wang), xuefuyou@hotmail.com (Xuefu You).
†These authors made equal contributions to this work.

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detection rate of 0.98%; *ant(3″)-I*, detection rate of 95.10%) and one methylase gene (*armA*, detection rate of 98.04%) were detected in the 102 *A. baumannii* with *aac(3)-I*+*aac(6′)-Ib*+*ant(3″)-I*+*armA* (detection rate of 25.49%), *aac(3)-I*+*aph(3′)-I*+*ant(3″)-I*+*armA* (detection rate of 21.57%) and *ant(3″)-I*+*armA* (detection rate of 12.75%) being the most prevalent gene profiles. The values of chi-square tests showed correlation of *armA*, *ant(3″)-I*, *aac(3)-I*, *aph(3′)-I* and *aac(6′)-Ib* with HLAR. *armA* had significant correlation (contingency coefficient 0.685) and good contingency with HLAR (kappa 0.940). The high rates of HLAR may cause a serious problem for combination therapy of aminoglycoside with β-lactams against *A. baumannii* infections. As *armA* was reported to be able to cause high level aminoglycoside resistance to most of the clinical important aminoglycosides (gentamicin, amikacin, tobramycin, etc), the function of aminoglycoside modifying enzyme gene(s) in *A. baumannii* carrying *armA* deserves further investigation.

1. Introduction

Acinetobacter baumannii is a notorious Gram-negative pathogen found in clinical settings due to its epidemic tendency and multidrug resistance (MDR)[1,2]. It can cause serious infections like ventilator associated pneumonia (VAP), skin and soft tissue infection, wound infection, secondary meningitis, blood infection, etc[2,3]. Since *A. baumannii* is commonly resistant to clinically available antimicrobial agents, including β-lactams, aminoglycosides and fluoroquinolones, the selection of appropriate antibiotics is increasingly limited[3–5].

Aminoglycosides, which bind specifically to 16S rRNA in the 30S ribosomal subunits to inhibit protein synthesis, are often used in combination with broad spectrum β-lactams to treat Gram-negative bacterial infections[6–8]. Resistance to aminoglycosides is most commonly caused by aminoglycoside modifying enzymes, including acetyltransferases, phosphotransferases and nucleotidyltransferases[9,10]. More recently, 16S rRNA methylases, ArmA, RmtA, RmtB, RmtC, RmtD, RmtE and NpmA have been reported among Enterobacteriaceae, *Pseudomonas* spp. and *Acinetobacter* spp.[11,12]. Aminoglycoside modifying enzymes differ in aminoglycosides that they may modified, whereas 16S rRNA methylases confer high-level resistance to almost all aminoglycosides except streptomycin[11,13,14].

The purpose of our study was to investigate the genetic basis of high level aminoglycoside resistance in *A. baumannii* clinical isolates from hospitals in Beijing, China.

2. Materials and methods

2.1. Bacterial Strains

173 *A. baumannii* clinical isolates collected from hospitals in Beijing, China between 2006 and 2009 were included in the current study, including 57 isolates in 2006, 23 isolates in 2007, 45 isolates in 2008 and 48 isolates in 2009. The strains were identified further in our laboratory by VITEK 2-compact bacteria identification system (Bio-Merieux Company) and by sequence analysis of the conserved region of 16S rRNA gene. *Escherichia coli* ATCC 25,922 and *A. Baumannii* ATCC 19606 were standard strains from American Type Culture Collection (ATCC).

2.2. Antimicrobial susceptibility to gentamicin and amikacin

The antimicrobial susceptibility of the isolates to gentamicin and amikacin were determined by microdilution method in CAMH broth (cation-adjusted Mueller–Hinton broth) according to CLSI recommendation. Three concentrations (1024, 512 and 256 μg/mL) were included in the experiment. The strains were recognized as high level aminoglycoside resistant (HLAR) if the MICs against gentamicin and amikacin were both higher than 512 μg/mL. *E. coli* ATCC 25922 and *A. Baumannii* ATCC 19606 were used as controls.

2.3. Polymerase chain reaction amplification of the aminoglycoside resistance genes

Polymerase chain reaction (PCR) was performed in a total volume of 25 μL containing one single colony, 0.6 μmol/L of each primer and 12.5 μL of 2 × Go Taq Green Master Mix (Promega). The genes encoding the following aminoglycoside modifying enzymes were investigated: acetyltransferases AAC(3)-I, AAC(3)-IIc, AAC(6′)-Ib and AAC(6′)-II; phosphotransferases APH(4)-Ia, APH(3′)-I, APH (3′)-IIb, APH(3′)-IIIa, APH(3′)-VIa, APH(2″)-Ib, APH(2″)-Ic and APH(2″)-Id; nucleotidyltransferases ANT(2″)-Ia, ANT(3″)-I, ANT (4′)-Ia. The 16S rRNA methylase genes investigated included *armA*, *rmtB* and *rmtC*. The primer sequences, expected amplicon sizes and the annealing temperatures for PCR are shown in Table 1. The amplification reaction with a DNA thermal cycler (Perkin-Elmer Cetus, Foster City, CA) consisted of a predenaturation at 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, 55 or 58 °C for 30 s, extension at 72 °C for 1 min, and a final elongation at 72 °C for 5 min.

2.4. Correlation analysis between aminoglycoside resistance gene and HLAR phenotype

The correlations of aminoglycoside resistance gene with HLAR phenotype were statistically analyzed by chi-square test using SPSS 13.0. Based on the nature of the data, Pearson's chi-square test was used in correlation analysis of *aac(3)-I*, *aac(6′)-Ib*, *aph (3′)-I* or *ant(3″)-I* with HLAR phenotype, and Fisher's exact test was used in correlation analysis of *aac(3)-IIc*, *aac(6′)-II*, *aph(3′)-IIb* or *armA* with HLAR phenotype, respectively. Correlations were evaluated by *P* values. Contingency coefficient and kappa values obtained for each gene. The gene was considered correlated with HLAR if *P* value <0.05, and no correlation if *P* value ≥ 0.05. Contingency coefficient was used to measure the extent of the correlation, and higher value suggested stronger correlation. Kappa value was the scale of the correlation agreement (≥ 0.75, good agreement; $0.75 >$ kappa value ≥ 0.4, general agreement; <0.4, poor agreement).

Table 1 The primer sequences, amplicon sizes and annealing temperatures for PCR.

Gene	Primer sequence	Amplicon size (bp)	Annealing temperature (°C)	Ref.
Aminoglycoside modifying enzyme gene				
ant(2″)-Ia	F: 5′-GCTCACGCAACTGGTCCA GA-3′ R: 5′-GGCACGCAAGACCTCAACCT-3′	719	58	15
ant(3″)-I	F: 5′-TGATTTGCTGGTTACGGTGAC-3′ R: 5′-CGCTATGTTCTCTTGCTTTTG-3′	284	55	16
ant(4′)-Ia	F: 5′-CTGCTAAATCGGTAGAAGC-3′ R: 5′-CAGACCAATCAACATGGCACC-3′	172	55	17
aac(3)-I	F: 5′-TTACGCAGCAGCAACGATGT-3′ R: 5′-GTTGGCCTCATGCTTGAGGA-3′	402	58	15
aac(3)-IIc	F: 5′-ACGCGGAAGGCAATAACGGA-3′ R: 5′-TAACCTGAAGGCTCGCAAGA-3′	854	55	15
aac(6′)-Ib	F: 5′-CATGACCTTGCGATGCTCTA-3′ R: 5′-GCTCGAATGCCTGGCGTCTT-3′	490	58	15
aac(6′)-II	F: 5′-TTCATGTCCGCGAGCACCCC-3′ R: 5′-GACTCTTCCGCCATCGCTCT-3′	178	55	18
aph(2″)-Ib	F:5′-CTTGGACGCTGAGATATATGAGCAC-3′ R:5′-GTTTGTAGCAATTCAGAAACACCCTT-3′	867	55	19
aph(2″)-Ic	F: 5′-CCACAATGATAATGACTCAGTTCCC-3′ R: 5′-CCACAGCTTCCGATAGCAAGAG-3′	444	55	19
aph(2″)-Id	F: 5′-GTGGTTTTTACAGGAATGCCATC-3′ R:5′-CCCTCTTCATACCAATCCATATAACC-3′	641	55	16
aph(3′)-I	F: 5′-ATGTGCCATATTCAACGGGAAACG-3′ R:5′-TCAGAAAAACTCATCGAGCATCAA-3′	816	55	16
aph(3′)-IIb	F: 5′-ATGCATGATGCAGCCACCTCC-3′ R: 5′-CTAGAAGAACTCGTCCAATAGCCT-3′	804	55	17
aph(3′)-IIIa	F: 5′-GGCTAAAATGAGAATATCACCGG-3′ R: 5′-CTTTAAAAAATCATACAGCTCGCG-3′	278	55	17
aph(3′)-VIa	F: 5′-ATACAGAGACCACCATACAGT-3′ R: 5′-GGACAATCAATAATAGCAAT-3′	234	55	16
aph(4)-Ia	F: 5′-CTGAACTCACCGCGACGTCT-3′ R: 5′-TCCACTATCGGCGAGTACTT-3′	977	58	15
16S rRNA methylase gene				
rmtB	F: 5′-GCTTTCTGCGGGCGATGTAA-3′ R: 5′-ATGCAATGCCGCGCTCGTAT-3′	173	55	20
rmtC	F: 5′-CGAAGAAGTAACAGCCAAAG-3′ R: 5′-ATCCCAACATCTCTCCCACT-3′	711	55	20
armA	F: 5′-ATTCTGCCTATCCTAATTGG-3′ R: 5′-ACCTATACTTTATCGTCGTC-3′	315	55	20

3. Results and discussion

3.1. Antimicrobial susceptibility to gentamicin and amikacin

Antimicrobial susceptibility of *A. baumannii* to gentamicin and amikacin was determined by broth microdilution method, and the results are summarized in Table 2. Totally 102 isolates showed high level aminoglycoside resistance (HLAR) with a HLAR rate of 58.96%. The HLAR rates for year 2006, 2007, 2008 and 2009 were 52.63%, 65.22%, 51.11% and 70.83%, respectively. The high rates of HLAR might cause a serious problem for combination therapy of aminoglycoside with β-lactams against *A. baumannii* infections.

3.2. Polymerase chain reaction amplification of the aminoglycoside resistance genes

Totally 15 aminoglycoside modifying enzyme genes and three 16S rRNA methylase genes were investigated in all of the isolates. The results are shown in Table 3. Of the 15 aminoglycoside modifying enzyme genes investigated, seven were detected in the current *A. baumannii* isolates, with positive rates of 66.47%, 45.09%, 34.10%, 32.37%, 0.58%, 0.58% and 0.58% for *ant(3″)-I*, *aac(3)-I*, *aph(3′)-I*, *aac(6′)-Ib*, *aac(3)-IIc*, *aac(6′)-II* and *aph(3′)-IIb*, respectively. Among the positive aminoglycoside modifying enzyme genes, five were detected in the 102 HLAR isolates, with positive rates of 95.10%, 65.69%, 47.06%, 45.10% and 0.98% for *ant(3″)-I*, *aac(3)-I*, *aph(3′)-I*, *aac(6′)-Ib* and *aph(3′)-IIb*, respectively. The high detection rates of *ant(3″)-I*, *aac(3)-I*, *aph(3′)-I* and *aac(6′)-Ib* genes in the HLAR strains of our study were consistent with those reported by Cho et al.[21]. Among the three methylase genes, only *armA* was detected with a positive rate of 59.54% for all the strains and 98.04% in the 102 HLAR isolates (100 out of 102 strains showed positive results). *rmtB* or *rmtC* genes were not able to be detected in the current isolate group. These results were also in accordance with other reports which found *armA* to be the only 16S rRNA methylase gene detected in high level aminoglycoside resistant *A. baumannii* [13,14,21,22].

Aminoglycoside resistance genes were also detectable in non-HLAR strains, though at relatively low rates. The presence of the

Table 2 High level aminoglycoside resistance among 173 *Acinetobacter baumannii* clinical isolates from Beijing, China.

Year	Total strains	Strains of S[a]		Strains of I[b]		Strains of R[c]		Strains of non-HLAR[d]	Strains of HLAR	HLAR rate (%)
		Amk[e]	Gm[f]	Amk	Gm	Amk	Gm			
2006	57	27	22	0	0	30	35	27	30	52.63
2007	23	8	7	0	1	15	15	8	15	65.22
2008	45	22	14	0	0	23	31	22	23	51.11
2009	48	14	10	0	0	34	38	14	34	70.83
Total	173	71	53	0	1	102	119	71	102	58.96

[a]S: susceptible
[b]I: intermediate
[c]R: resistance
[d]HLAR: high level aminoglycoside resistance as demonstrated by MICs to amikacin and gentamicin higher than 512 μg/mL, non-HLAR: non-high level aminoglycoside resistance
[e]Amk: Amikacin
[f]Gm: Gentamicin

aminoglycoside resistance gene in non-HLAR strains suggested that complicated regulation mechanisms were involved in the onset of the HLAR phenotype.

3.3. *Aminoglycoside resistance gene profile*

The aminoglycoside resistance gene profiles of the 102 HLAR *A. baumannii* are shown in Table 4. As demonstrated, *aac(3)-I+aac(6′)-Ib+ant(3″)-I+armA*, *aac(3)-I+aph(3′)-I+ant(3″)-I+armA* and *ant(3″)-I+armA* were the most prevalent resistance gene profiles, with positive rates of 25.49%, 21.57% and 12.75%, respectively. Resistance gene profiles of secondary high detection rates were *aac(3)-I+aac(6′)-Ib+aph(3′)-I+ant(3″)-I+armA*, *aac(3)-I+ant(3″)-I+armA* and *aph(3′)-I+ant(3″)-I+armA*, and the corresponding positive rates were 8.82%, 7.84% and 7.84%. Other resistance gene profiles included *aac(6′)-Ib+aph(3′)-I+ant(3″)-I+armA* (detection rate of 4.90%), *aph(3′)-I+armA* (detection rate of 3.92%), *aac(6′)-Ib+ant(3″)-I+armA* (detection rate of 3.92%), *aac(3)-I+aac(6′)-Ib+ant(3″)-I* (detection rate of 0.98%) and *aac(3)-I+aph(3′)-IIb+aac(6′)-Ib+ant(3″)-I+armA* (detection rate of 0.98%).

As shown in Table 4, the methylase gene *armA* was detected along with aminoglycoside modifying enzyme genes for most of the isolates investigated except 2 (one showed positive result for *aac(3)-I+aac(6′)-Ib+ant(3″)-I* gene, and the other showed no positive result for all the 18 aminoglycoside resistance genes). As *armA* was reported to be able to cause high level aminoglycoside resistance to most of the clinical important aminoglycosides (gentamicin, amikacin, tobramycin, etc.)[1], the function of aminoglycoside modifying enzyme gene(s) in *A. baumannii* carrying *armA* deserves further investigation. The HLAR isolate with negative results for all of the 18 aminoglycoside resistance genes also needs our further study.

3.4. *Correlation analysis between aminoglycoside resistance gene and HLAR phenotype*

Data were statistically analyzed by chi-square test using SPSS 13.0, and the results are summarized in Table 5. The values of chi-square test showed *armA*, *ant(3″)-I*, *aac(3)-I*, *aph(3′)-I* and *aac(6′)-Ib* associated with HLAR. A contingency coefficient of 0.685 showed that *armA* was significantly correlated with HLAR. The contingency coefficients for *ant(3″)-I*, *aac(3)-I*, *aph(3′)-I* and *aac(6′)-Ib* were 0.588, 0.444, 0.311 and 0.310, respectively. Kappa values were further used to scale the correlation agreement. Among the 5 correlative genes, *armA* had good contingency (kappa value of 0.940), *ant(3″)-I* and *aac(3)-I* had general contingency (kappa values of 0.717 and 0.477), whereas *aph(3′)-I* and *aac(6′)-Ib* had poor consistency (kappa values of 0.289 and 0.282).

4. Conclusions

A. baumannii clinical isolates collected between 2006 and 2009 from the hospitals in Beijing, China showed high levels of aminoglycoside resistance. Several resistance genes were detected in *A. baumannii* clinical isolates, and coexistence of resistance genes was found in most strains. Correlation analysis demonstrated that *armA* gene was closely related to HLAR. The high rates of HLAR in these clinical isolates may cause a serious problem for combination therapy of aminoglycoside with *β*-lactams against *A. baumannii* infections.

Table 3 Distribution of aminoglycoside resistance genes in 173 *Acinetobacter baumannii* isolates.

Result	Aminoglycoside resistant genes							
	armA	*aac(3)-I*	*aac(3)-IIc*	*aac(6′)-Ib*	*aac(6′)-II*	*aph(3′)-I*	*aph(3′)-IIb*	*ant(3″)-I*
Positive isolates from HLAR	100	67	0	46	0	48	1	97
Positive rate from HLAR (%)	98.04	65.69	0	45.10	0	47.06	0.98	95.10
Positive isolates from non-HLAR	3	11	1	10	1	11	0	18
Positive rate from non-HLAR (%)	4.23	15.49	1.41	14.08	1.41	15.49	0	25.35
Total positive isolates	103	78	1	56	1	59	1	115
Positive rate (%)	59.54	45.09	0.58	32.37	0.58	34.10	0.58	66.47

Table 4 Aminoglycoside resistance gene profiles of the 102 HLAR *Acinetobacter baumannii.*

Aminoglycoside resistance gene profile	No. of isolate	Positive rate (%)
aac(3)-I+ aac(6′)-Ib+ant(3″)-I+armA	26	25.49
aac(3)-I+ aph(3′)-I+ant(3″)-I+armA	22	21.57
ant(3″)-I+armA	13	12.75
aac(3)-I+aac(6′)-Ib+aph(3′)-I+ant(3″)-I+armA	9	8.82
aac(3)-I+ant(3″)-I+armA	8	7.84
aph(3′)-I+ant(3″)-I+armA	8	7.84
aac(6′)-Ib+aph(3′)-I+ant(3″)-I+armA	5	4.90
aph(3′)-I+armA	4	3.92
aac(6′)-Ib+ant(3″)-I+armA	4	3.92
aac(3)-I+aac(6′)-Ib+ant(3″)-I	1	0.98
aac(3)-I+ aph(3′)-IIb+aac(6′)-Ib+ant(3″)-I+armA	1	0.98
None of 18 aminoglycoside resistance genes	1	0.98

Table 5 Correlation analysis between aminoglycoside resistance gene and HLAR (chi-square test).

Aminoglycoside resistant genes	*P* value	Contingency coefficient	Kappa value
armA	0.000	0.685	0.940
aac(3)-I	0.000	0.444	0.477
aac(3)-IIc	0.410	0.091	−0.012
aac(6′)-Ib	0.000	0.310	0.282
aac(6′)-II	0.410	0.091	−0.012
aph(3′)-I	0.000	0.311	0.289
aph(3′)-IIb	1.000	0.063	0.008
ant(3″)-I	0.000	0.588	0.717

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