Original Article

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

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KEY WORDS

Acinetobacter baumannii; HLAR; Aminoglycoside modifying enzyme; 16S rRNA methylase; Correlation analysis

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′)-IIb, aph(4)-Ia, aph (3′)-I, aph(3′)-IIb, aph(3′)-IIIa, aph(3′)-Vla, 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(3)-IIC, detection rate of 45.10%; aph(3′)-I, detection rate of 47.06%; aph(3′)-IIb, detection rate of 45.10%; aph(3′)-IIIa, detection rate of 38.62%; ant(2″)-Ia, detection rate of 59.92%; ant(3′)-I and ant(4″)-Ia, detection rate of 47.06%; av(3′)-Vla, detection rate of 38.62%; armA, detection rate of 51.11%) and the 16S rRNA methylase genes (rmtB and rmtC) were significantly associated with HLAR phenotype.
1. Introduction

Acinetobacter baumannii is a notorious Gram-negative pathogen found in clinical settings due to its epidemic tendency and multidrug resistance (MDR)\(^2\). It can cause serious infections like ventilator associated pneumonia (VAP), skin and soft tissue infection, wound infection, secondary meningitis, blood infection, etc.\(^3\,^4\). 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\(^3\,^4\). The antimicrobial susceptibility of the isolates to gentamicin and amikacin were determined by microdilution method in CAMH broth with MICs against gentamicin and amikacin both higher than 512 m\(\mu\)g/mL. E. coli ATCC 25922 and A. Baumannii ATCC 19606 were used as controls.

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 m\(\mu\)g/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 m\(\mu\)g/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 \(\mu\)L containing one single colony, 0.6 \(\mu\)mol/L of each primer and 12.5 \(\mu\)L of 2 \(\times\) 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’)-Ib, APH(3’)-IIa, APH(3’)-IIb, APH(3’)-Ic and APH(3’)-IId; 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 arm(a)-I with HLAR phenotype, and Fisher's exact test was used in correlation analysis of aac(3’)-Ic, aac(6’)-II, aph(3’)-Ib 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 \(\geq 0.05\). 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 (\(\geq 0.75\), good agreement; \(0.75 > \text{kappa value} \geq 0.4\), general agreement; \(< 0.4\), poor agreement).

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results are shown in Table 3. Of the 15 aminoglycoside modifying rRNA methylase genes were investigated in all of the isolates. The Totally 15 aminoglycoside modifying enzyme genes and three 16S aminoglycoside resistance genes 58.96%. The HLAR rates for year 2006, 2007, 2008 and 2009 were level aminoglycoside resistance (HLAR) with a HLAR rate of 3.2. Polymerase chain reaction amplification results are summarized in Table 2. Totally 102 isolates showed high amikacin was determined by broth microdilution method, and the A. baumannii Antimicrobial susceptibility of 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(6')-Ic, 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, aac(6')-Ic, aac(6')-II 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.1. 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 3,14,21,22.

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

| Gene                      | Primer sequence                        | Amplicon size (bp) | Annealing temperature (°C) | Ref. |
|---------------------------|----------------------------------------|--------------------|----------------------------|------|
| Aminoglycoside modifying enzyme gene | | | | |
| ant(2")-Ia                | F: 5'-GCTCACGCAACTGTGATCCAA GA-3'   | 719                | 58                         | 15   |
|                           | R: 5'-GACCCAGAGACCTCAACCT-3'         |                    |                            |      |
| ant(3")-I                 | F: 5'-TGATTGCTGTTACGTTGC-3'          | 284                | 55                         | 16   |
|                           | R: 5'-CGCTATGTCCTCTGTTGCTT-3'         |                    |                            |      |
| ant(4")-Ia                | F: 5'-CTGCTAAATCGTGAGAACG-3'         | 172                | 55                         | 17   |
|                           | R: 5'-CAGACCAAACCACCTGACACC-3'       |                    |                            |      |
| aac(3)-I                  | F: 5'-TTTACAGCAGACGACAGGT-3'         | 402                | 58                         | 15   |
|                           | R: 5'-GTGGCTCATGGCTTGAAGGA-3'        |                    |                            |      |
| aac(6')-Ib                | F: 5'-ACGCGGAAGGCAAATAACGG-3'        | 854                | 55                         | 15   |
|                           | R: 5'-TAACCTGAAGGCTCGAAGA-3'         |                    |                            |      |
| aac(6')-II                | F: 5'-TTGATGCTGGAGACACC-3'           | 55                 | 18                         |      |
|                           | R: 5'-GACCTCTGGGCGATCT-3'            |                    |                            |      |
| aph(2")-Ib                | F: 5'-CTTGGAGCGTGTATATGAGCG-3'       | 867                | 55                         | 19   |
|                           | R: 5'-GTTTGAGCAGAATTCGAAACCCCT-3'    |                    |                            |      |
| aph(2")-Ic                | F: 5'-CCACAAATGATATTGACTGAT-3'       | 55                 | 19                         |      |
|                           | R: 5'-CCAGACCTGGCATAGACAGA-3'        |                    |                            |      |
| aph(2")-Id                | F: 5'-GTGTTTTTTCAGGAAAGGATCC-3'      | 641                | 55                         | 16   |
|                           | R: 5'-CCCTCTTATACCAAGCTATATAAC-3'    |                    |                            |      |
| aph(3")-I                 | F: 5'-ATGGGCTATTTCAACAGGGAAAAG-3'    | 816                | 55                         | 16   |
|                           | R: 5'-TCAGAAATCCTATGCCATGCACCA-3'    |                    |                            |      |
| aph(3")-IIb               | F: 5'-ATGCAATGCTGACGCCCT-3'          | 804                | 55                         | 17   |
|                           | R: 5'-CTAGAAGAATCTCGTCAAAGCCT-3'     |                    |                            |      |
| aph(3")-IIIa              | F: 5'-GGCTAAAATGAGGATAGTCGCG-3'      | 278                | 55                         | 17   |
|                           | R: 5'-CTTTAAAAATCATAGACGTCGCCG-3'    |                    |                            |      |
| aph(3")-Vla               | F: 5'-ATACAGGACCCACCTACGAT-3'        | 234                | 55                         | 16   |
|                           | R: 5'-GGCAATCAATAATGCAAT-3'          |                    |                            |      |
| aph(4")-Ia                | F: 5'-CTGAACTATCCGGCAGCTCT-3'        | 977                | 58                         | 15   |
|                           | R: 5'-TCGACATGCTGCGCGAGTACTT-3'      |                    |                            |      |
| 16S rRNA methylase gene   | rmtB                                   | 173                | 55                         | 20   |
|                           | F: 5'-GCTTCTCCCGGGCGGTAA-3'          |                    |                            |      |
|                           | R: 5'-ATCGAATGCGGCGTCGTAT-3'         |                    |                            |      |
|                           | rmtC                                   | 711                | 55                         | 20   |
|                           | F: 5'-CGAAGAATGAACTACGCGAAG-3'       |                    |                            |      |
|                           | R: 5'-ATGCAAGAATCTATCCCTCGACT-3'     |                    |                            |      |
|                           | armA                                   | 315                | 55                         | 20   |
|                           | F: 5'-ATTCTGCTATATAATGG-3'           |                    |                            |      |
|                           | R: 5'-ACCTATAATATATATCGTGC-3'        |                    |                            |      |

Table 1 The primer sequences, amplicon sizes and annealing temperatures for PCR.
aminoglycoside resistance gene in non-HLAR strains suggested that complicated regulation mechanisms were involved in the onset of the HLAR phenotype.

3.3. Aminoglycosides 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-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)-Ib-aph(3′)-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 armA, 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.), 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 aminoglycosides 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, aac(6′)-Ib and aph(3′)-I 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 aminoglycosides 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 Acinetobacter baumannii.

| Aminoglycoside resistance gene profile | No. of isolate | Positive rate (%) |
|---------------------------------------|----------------|-------------------|
| aac(3)-I+ aac(6′)-II+ant(3″)-I+armA   | 26             | 25.49             |
| aac(3)-I+ aac(6′)-II+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              |
| aac(6′)-Ib+aph(3′)-I+ant(3″)-I+armA   | 5              | 4.90              |
| aac(6′)-Ib+ant(3″)-I+armA            | 4              | 3.92              |
| aac(3)-I+aac(6′)-Ib+ant(3″)-I        | 4              | 3.92              |
| aac(3)-I+aac(6′)-Ib+ant(3″)-I        | 1              | 0.98              |
| aac(3)-I+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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