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Detection of 16S rRNA Methylase Genes in Gram-Negative Bacilli Isolated from Hospitals in Changchun, China

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ABSTRACT

Methylation of 16S rRNA is an important mechanism of aminoglycoside resistance among gram-negative pathogens. In this report, 16S rRNA methylase genes were amplified using PCR among gram-negative bacillus isolates from hospitals in the Changchun area of China and 16S rRNA methylase genotypes (*arm*A, *rmt*B, *rmt*A, *rmt*C, *rmt*D, and *npm*A) were identified by direct sequencing. Fifty of the isolates (43.1%) harbored 16S rRNA methylase genes. The common 16S rRNA methylase genes were *arm*A and *rmt*B (12.1% and 31.0%, respectively), whereas the *rmt*A, *rmt*C, *rmt*D, and *npm*A genes were absent from the sample. It suggests that the predominant 16S rRNA methylase genes among gram-negative bacilli in the Changchun area are *arm*A and *rmt*B.

Keywords: 16S rRNA; Methylases; Gram-Negative Bacilli

1. Introduction

Aminoglycosides have strong antibacterial activity against gram-negative bacilli and gram-positive bacilli. Aminoglycosides are well received by clinicians because of their broad antimicrobial spectrum and efficacy. The irrational use of antibiotics is causing increasingly acute problems associated with antimicrobial resistance, however. The methylation of 16S rRNAs in gram-negative bacilli is one of the mechanisms underlying strong resistance to aminoglycosides. Recent studies [1-2] showed that 16S rRNA methylase could methylate the 30S ribosomal subunit in gram-negative bacilli. 16S rRNA methylase can protect the target sites of the 30S ribosomal subunit, preventing the aminoglycosides from combining with the 30S ribosomal subunit.

Following the first discovery of 16S rRNA methylase gene *arm*A in France [3], other methylases such as *rmt*B, *rmt*A, and *rmt*C were found among gram-negative pathogens [4,5]. The predominant methylase genes in southern China are *arm*A and *arm*B [6]; however, the prevalence of 16S rRNA methylases among clinical isolates of gram-negative bacilli in Changchun, Northeast of China, has not been previously assessed. 16S rRNA methylase mediates high-level resistance to aminoglycosides in gram-negative isolates, and some spices in gram-negative bacilli are major causes of nosocomial infections [7]. The aim of this study was to investigate the prevalence of 16S rRNA methylases in aminoglycoside-resistant isolating from three hospitals in Changchun and to characterize the host bacteria.

2. Materials and Methods

2.1. Isolates and Drugs

One hundred and sixteen strains of gram-negative bacilli were isolated from China-Japan Union Hospital of Jilin University (Changchun, China), the Affiliated Hospital to Changchun University of Chinese Medicine (Changchun, China), and the Clinical Laboratory of Jilin Province People's Hospital (Changchun, China). The clinical isolates consisted of 33 *Escherichia coli* strains, 25 *Klebsiella pneumoniae* strains, 14 *Enterobacter cloacae* strains, 15 *Acinetobacter baumannii* strains, 19 *Pseudomonas aeruginosa* strains, and 10 *Serratia marcescens* strains. Identification of these isolates was done using the VITEK-32 system (Mérieux, France). Amikacin was provided by the National Institute for the Control of Pharmaceutical and Biological Products (batch number 130335-200204) (Bei-

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jing, China). Gentamicin was supplied by Beijing Dingguo Changsheng Biotech Co., Ltd. (batch number 1B310330) (Beijing, China).

2.2. Testing for Antibiotic Susceptibility by Agar Dilution

The susceptibilities of the 116 strains to amikacin and gentamicin were determined by agar dilution. Isolates that were able to grow at antibiotic concentrations above 16 mg/L were regarded as being resistant to the antibiotics; and those that were not able to grow at concentrations above 2 mg/L were regarded as sensitive.

2.3. Extraction of Bacteria DNA

One milliliter of bacterial culture was placed in a 1.5 mL centrifuge tube and centrifuged at 10,000 rpm for 1 min. The supernatant was removed, and 100 μL TE Buffer (1 mol/L Tris-HCl, pH 8.0; 500 mmol/L EDTA, pH8.0) was added. Then, an equal volume of mixed phenol, chloroform, and isoamyl alcohol (25:24:1) was added. Vortex oscillation was then performed for 30 s, and the mixture was subsequently centrifuged at 10,000 rpm for 5 min. The resulting supernatant was then stored at −20˚C until later use as the template for genetic testing.

2.4. Primer Design and Detection of 16S rRNA Methylase Genes

Primers were self-designed for six different 16S rRNA methylase gene sequences available from GenBank. The primer sequences, target genes, and primer lengths are shown in **Table 1**. The conditions for producing PCR products with lengths greater than 500 bp were: 2 min at 93˚C; 35 cycles of 1 min at 93˚C, 1 min at 55˚C, and 1 min at 72˚C; followed by 5 min at 72˚C. The conditions for producing PCR products with lengths less than 500 bp were: 5 min at 93˚C; 35 cycles of 30 s at 93˚C, 30 s at 55˚C, and 1 min at 72˚C; followed by 5 min at 72˚C. After 2% agarose gel electrophoresis, the PCR products were observed under a gel imager and photos were taken.

2.5. PCR Product Sequencing

The PCR products were sent to Beijing Genomics Institute, (Beijing, China) for sequencing. The sequences were detected using the Chromas software and compared with those released by GenBank.

3. Results

3.1. Results of Antibiotic Susceptibility Testing

Among the 116 gram-negative bacilli, 16 *E. coli* strains, 1 *K. pneumoniae* strain, 3 *E. cloacae* strains, 11 *A. baumannii* strains, 10 *P. aeruginosa* strains, and 9 *S. marcescens* strains showed resistance to amikacin concentrations exceeding 16 mg/L (**Table 2**). Likewise, 26 *E. coli* strains, 11 *K. pneumoniae* strains, 8 *E. cloacae* strains, 15 *A. baumannii* strains, 17 *P. aeruginosa* strains, and 10 *S. marcescens* strains exhibited resistance to gentamycin concentrations exceeding 16 mg /L (**Table 3**).

Table 1. Primer sequences for the six known 16S rRNA methylase genotypes.

Target gene	Primer sequences (5' → 3')	Product length (bp)
*arm*A	P1: ATGGAT AAGAATGATGTTGTTAAG P2: TTAT T TCTGAAATCCACTAGT AATTA	774
*rmt*A	P1: ACTGTGATGGGATACGCGTC P2:AGCGATATCCAACACACGATGG	315
*rmt*B	P1: ATGAACATCAACGATGCCCTC P2:TTATCCATTCTTTTTTATCAAGTATAT	756
*rmt*C	P1: ATGAAAACCAACGATAATTATC P2:TTACAATCTCGATACGATAAAATAC	846
*rmt*D	P1:ATGAGCGAACTGAAGGAAAAACTGCT P2:TCATTTTCGTTTCAGCACGTAAAACAG	744
*npm*A	P1:TTGGGTACTGGAGACGGTAG P2: CAGCT TTGTATTGT TCGCTC	421

3.2. Results of 16S rRNA Methylase Genetic Testing

Fifty (43.1%) of the 116 isolates harbored 16S rRNA methylase genes. The common 16S rRNA methylase genes were *arm*A (12.1%, 14/116) and *rmt*B (31.0%, 36/ 116). All of the isolates were negative for the *rmt*A, *rmt*C, *rmt*D, and *npm*A genotypes (**Table 4**). Additionally, three of the *S. marcescens* strains harbored both *arm*A and *rmt*B and were highly resistant to both amikacin and gentamicin. An electrophoregram of the products of PCR amplification of *arm*A and *rmt*B is shown in **Figures 1** and **2**.

3.3. Sequencing of 16S rRNA Methylase

The results of the direct sequencing of the *arm*A and *rmt*B genes isolated from our sample were compared with the corresponding sequences in the GenBank database, and the sequences in our sample were found to be identical to those in the database (*arm* A:HQ204573.1; *rmt* B:FJ539137.1).

4. Discussion

Aminoglycosides exert their antibacterial action by binding to the highly conserved A site of the 16S rRNA of the bacterial 30S ribosomal subunits, interfering with protein synthesis with subsequent bacterial death. Furthermore, aminoglycosides have a broad antimicrobial

Table 2. Susceptibilities of gram-negative bacilli to amikacin.

Drug-resistant isolate	S strains (%)	R strains (%)
Escherichia coli	17 (51.5)	16 (48.5)
Klebsiella pneumoniae	24 (96.0)	1 (4.0)
Enterobacter cloacae	11(78.6)	3 (21.4)
Acinetobacter baumannii	4 (26.7)	11 (73.3)
Pseudomonas aeruginosa	9 (47.4)	10 (52.6)
Serratia marcescens	1 (10.0)	9 (90.0)
Total	66 (56.9)	50 (43.1)

S: Sensitive; R: Resistant.

Table 3. Susceptibilities of gram-negative bacilli to gentamicin.

Drug-resistant isolate	S strains (%)	R strains (%)
Escherichia coli	7 (21.2)	26 (78.8)
Klebsiella pneumoniae	14 (56.0)	11 (44.0)
Enterobacter cloacae	6 (42.9)	8 (57.1)
Acinetobacter baumannii	0 (0.0)	15 (100)
Pseudomonas aeruginosa	2 (10.5)	17(89.5)
Serratia marcescens	0 (0.0)	10 (100)
Total	29 (25.0)	87 (75.0)

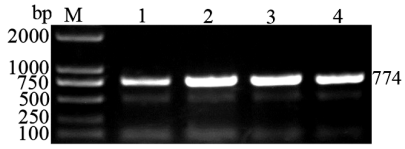
S: Sensitive; R: Resistant.

Table 4. Detection results of 16S rRNA methylase genes.

Drug-resistant isolates	*arm*A (%)	*rmt*B (%)
Escherichia coli	1 (3.0)	18 (54.5)
Klebsiella pneumoniae	0 (0.0)	0 (0.0)
Enterobacter cloacae	1 (7.1)	0 (0.0)
Acinetobacter baumannii	5 (33.3)	0 (0.0)
Pseudomonas aeruginosa	0 (0.0)	14 (73.7)
Serratia marcescens	7 (70.0)	4 (40.0)
Total	14 (12.1)	36 (31.0)

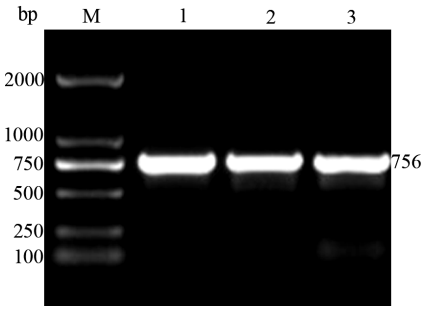
Note: *rmt*A, *rmt*C, *rmt*D, and *npm*A are negative.

spectrum and produce synergistic effects with other kinds of antibiotics. Because of massive use of antibiotics including aminoglycosides, problems related to bacterial resistance to aminoglycosides are becoming very serious. Such resistance is achieved by enzymatic modification, changes in cellular membrane permeability, efflux pump activity, the phoP-phoQ system, or 16S rRNA methylase activity [10-12]. 16S rRNA methylase is usually encoded



M: DNA Marker; Lanes 1-4: *arm*A.

Figure 1. Electrophoregram of PCR amplified products of 16S rRNA methylase gene *arm*A.



M: DNA Marker; Lanes 1-3: *rmt*B.

Figure 2. Electrophoregram of PCR amplified products of 16S rRNA methylase gene *rmt*B.

by plasmids, and its transfer via plasmids has led to the rapid spread of 16S rRNA methylase genes among bacilli [13,15], causing great difficulties for clinical treatment.

Our study showed that 43.1% and 75% of a sample of 116 gram-negative bacillus isolates were resistant to amikacin and gentamicin, respectively. Except for the low resistance rates among the *K. pneumoniae* and *E. cloacae* strains, the resistance rates among the other strains of gram-negative bacilli were all greater than 45%, indicating high rates of aminoglycoside resistance among gram-negative bacilli in the Changchun area. In terms of the 16S rRNA methylase genetic testing, the frequency of *rmt*B (31.0%) in our sample was higher than that of *arm*A (12.1%). Furthermore, *rmt*B is mainly distributed among *E. coli* (54.5%) and *P. aeruginosa* (73.7%) strains; whereas *arm*A is mainly distributed among *A. baumannii* (33.3%) and *S. marcescens* (70%) strains. We did not detect any 16S rRNA methylase genes among 25 *K. pneumoniae* strains; and we only found 1 *rmt*A gene among 14 strains of *E. cloacae* (7.1%). The three *S. marcescens* strains that carried both *arm*A and *rmt*B showed strong resistance to both amikacin and gentamicin. In our study, the frequency of aminoglycoside resistance among the 116 strains of gram-negative bacilli was higher than the frequency of 16S rRNA methylase genes, suggesting that the drug-resistant phenotypes were not totally consistent. Therefore, some of the aminoglycoside resistance in our sample was likely caused by other antibiotic resistance mechanisms such as the production of AME genes, changes in cellular membrane permeability, efflux pump activity, or the phoP-phoQ system.

In terms of the distribution of 16S rRNA methylase ge-

nes, only the *arm*A and *rmt*B genes were detected in our study, suggesting that *arm*A and *rmt*B are the two main 16S rRNA methylase genes in the Changchun area, which is consistent with relevant domestic studies [16, 17]. The *arm*A and *rmt*B genes are also the most common genotypes in other Asian countries such as South Korea [18] and Japan [19]. The most commonly detected 16S rRNA methylase genes in European countries such as Belgium [20] and Bulgaria [21] is *arm*A, whereas in Brazil it is *rmt*B [22]. According to our data, high-level aminoglycoside resistance in clinical isolates conferred by 16S rRNA methylase is of great concern in Changchun area. Hence, clinicians must pay more attention to the rational use of such drugs to reduce the frequencies of drug-resistant bacteria under the selective pressure by antibiotics.

5. Conclusion

In conclusion, our experimental results suggest that the predominant 16S rRNA methylase genes among gram-negative bacillus isolates from Changchun area, Northeast of China, are *arm*A and *rmt*B. Aminoglycoside-resistant isolates producing *arm*A or *rmt*B may become a major therapeutic threat in the future.

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