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Plasmid-Mediated 16S rRNA Methylases in Aminoglycoside-Resistant *Enterobacteriaceae* Isolates in Shanghai, China[▽]

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High-level resistance to aminoglycosides produced by 16S rRNA methylases in *Enterobacteriaceae* isolates was investigated. The prevalences of *armA* in *Escherichia coli*, *Klebsiella pneumoniae*, and *Enterobacter cloacae* were 0.6%, 3.0%, and 10%, respectively. *rmtB* was more prevalent than *armA*. Pulsed-field gel electrophoresis patterns indicated that *armA* and *rmtB* have spread horizontally and clonally.

Aminoglycosides are still widely used to treat severe bacterial infections, often together with β-lactams in synergistic combinations (14). These agents interfere with protein synthesis in bacteria by binding to the A site of the 16S rRNA of prokaryotic 30S ribosomal subunits (12). The mechanisms of resistance to aminoglycosides in pathogenic bacteria were previously believed to be restricted to the production of aminoglycoside-modifying enzymes, a decrease in intracellular antibiotic accumulation, and the substitution of ribosomal proteins or mutation of rRNA (6). Recently, a new type of mechanism, posttranscriptional methylation of 16S rRNA, has been reported (8, 10, 24), which results in high-level resistance to aminoglycoside antibiotics (1, 15). Six 16S rRNA methylase genes have been described in the world up to now: *armA* (9), *rmtA* (22), *rmtB* (21), *rmtC* (18), *rmtD* (7), and *npmA* (17).

In China, only *armA* in *Acinetobacter baumannii* from humans and *rmtB* in *Escherichia coli* and *Enterobacter cloacae* from pigs have been reported (2, 25). The aim of this study was to investigate the prevalence of the 16S rRNA methylases among clinical isolates of *Enterobacteriaceae* from Ruijin Hospital, Shanghai, People's Republic of China, and to evaluate the molecular epidemiology of these isolates.

A total of 723 nonduplicate isolates of *E. coli*, 202 isolates of *Klebsiella pneumoniae*, and 60 isolates of *E. cloacae* from patients' specimens were consecutively collected between October 2006 and May 2007 from the Department of Clinical Microbiology, Ruijin Hospital, Shanghai, People's Republic of China, and were identified with the ATB 32 GN system (bioMerieux, Marcy L'Etoile, France). *E. coli* ATCC 25922 was used as the reference strain. *E. coli* J53 resistant to sodium azide was used as the recipient in plasmid conjugation experiments.

Antibiotic susceptibilities and MICs were determined in accordance with CLSI guidelines (4).

To detect 6 16S rRNA methylase gene alleles, 142 *E. coli*, 35 *K. pneumoniae*, and 24 *E. cloacae* isolates that were resistant to gentamicin or amikacin (zone diameter of ≤12 mm for gentamicin or ≤14 mm for amikacin) were included for study. The PCR primers and method used were as previously reported (13, 17, 21, 24). Two pairs of primers were designed by GenBank numbers AB194779 and DQ914960 (Prmtc1, 5′-CGAAGAAGTAACAGCCAAAG-3′, and Prmtc2, 5′-GCTAGAGTCAAGCCAGAAAA-3′, and Prmtd1, 5′-TCATTTTCGTTTCAGCAC-3′, and Prmtd2, 5′-AAACATGAGCGAACTGAAGG-3′) to amplify the *rmtC* and *rmtD* genes, respectively. The bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, $bla_{\text{PER-1}}$, and $bla_{\text{VEB-1}}$ genes were amplified by PCR, as described previously (3, 11). A series of carbapenemase genes were amplified by PCR (20) in five isolates of imipenem-resistant *E. cloacae*. The PCR products were sequenced on both strands in an ABI Prism 377 automated sequencer (Applied Biosystems, Foster City, CA). The nucleotides were then analyzed with software available on the Internet (www.ncbi.nlm.nih.gov/).

The conjugal transfer of aminoglycoside resistance determinants was performed in broth culture with *E. coli* J53 as the recipient (5). The transconjugants were selected on agar plates containing sodium azide (100 mg/liter) supplemented with amikacin (128 mg/liter). Plasmid DNA was prepared from transconjugants using a commercial kit (QIAprep spin miniprep kit; Qiagen, Germany) and then was screened for 16S rRNA methylase and β-lactamase genes by PCR.

Pulsed-field gel electrophoresis (PFGE) was carried out with a CHEF system (CHEF MAPPER XA; Bio-Rad). The genomic DNA preparation was digested with XbaI, and PFGE was performed as described previously (16, 19). Restriction patterns were interpreted by the criteria proposed by Tenover et al. (16).

Sixteen *armA* (4 *E. coli*, 6 *K. pneumoniae*, and 6 *E. cloacae*) and 22 *rmtB* (20 *E. coli* and 2 *K. pneumoniae*) allele-positive isolates were identified by PCR and sequencing. None of the 16S rRNA methylase genes was detected in the strains susceptible to gentamicin and/or amikacin. Among the 723 *E. coli*, 202 *K. pneumoniae*, and 60 *E. cloacae* isolates, the prevalences of *armA* were 0.6%, 3.0%, and 10%, respectively, and the prevalences of *rmtB* in *E. coli* and *K. pneumoniae* were 2.8% and 1.0%, respectively. *rmtB* was found to be more prevalent than *armA* in our hospital, which is opposite to the report from Taiwan (23). In Taiwan, 94.3% of the *armA*- or *rmtB*-positive

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E. coli and *K. pneumoniae* isolates produced CTX-M β-lactamases. In our study, 81.6% (31/38), 84.2% (32/38), and 34.2% (13/38) of *armA*- or *rmtB*-positive *Enterobacteriaceae* isolates harbored $bla_{\text{CTX-M}}$, $bla_{\text{TEM-1}}$, and $bla_{\text{SHV-12}}$, respectively. Two *K. pneumoniae* isolates were detected as producing SHV-1 narrow-spectrum β-lactamases. However, in a Korean study, only one 16S rRNA methylase-positive *K. pneumoniae* isolate harbored the $bla_{\text{CTX-M}}$ allele (13).

All of the 38 isolates displayed high-level resistance to gentamicin and amikacin ($MIC_{90} > 256$ mg/liter; $MIC_{50} > 256$ mg/liter). Among them, thirty-six isolates were resistant to ciprofloxacin ($MIC_{90} = 256$ mg/liter; $MIC_{50} = 128$ mg/liter). Five isolates of *armA*-positive *E. cloacae* were resistant to imipenem (MIC > 32 mg/liter), and the carbapenemase activity of crude enzyme could be inhibited by clavulanic acid (4 μg/ml). PCR and sequencing for a series of carbapenemase genes indicated that these five isolates produced KPC-2 β-lactamase which may be responsible for the imipenem resistance.

Plasmid conjugation experiments were successful with 30 *armA*- or *rmtB*-positive isolates. The remaining eight isolates failed to transfer the *armA* or *rmtB* gene identified by PCR using their plasmids as templates, which indicated that the methylase genes are located on plasmids. PCR and sequencing showed that $bla_{\text{CTX-M-14}}$, $bla_{\text{TEM-1}}$, and $bla_{\text{SHV-12}}$ cotransferred with *armA* or *rmtB* to 11, 20, and 7 isolates, respectively, to the recipient. This demonstrated that the *armA* and *rmtB* genes can be transferred horizontally on conjugative plasmids together with β-lactamase genes. Eight isolates of the transconjugants were resistant to ciprofloxacin, which showed that fluoroquinolone resistance is also cotransferred with the *armA* or *rmtB* gene.

PFGE typing was successfully performed in these 38 isolates. *K. pneumoniae*, *E. coli*, and *E. cloacae* were divided into 4, 21, and 1 PFGE pattern, respectively, which indicated that a clonal outbreak of *armA*-positive *E. cloacae* and a horizontal spread of 16S rRNA methylases in *E. coli* and *K. pneumoniae* had likely occurred in our hospital. High-level aminoglycoside resistance in human pathogens conferred by 16S rRNA methylases is of great concern in China.

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