16S Ribosomal RNA Methylation: Emerging Resistance Mechanism against Aminoglycosides

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Methylation of 16S ribosomal RNA (rRNA) has recently emerged as a new mechanism of resistance against aminoglycosides among gram-negative pathogens belonging to the family Enterobacteriaceae and glucose-nonfermentative microbes, including Pseudomonas aeruginosa and Acinetobacter species. This event is mediated by a newly recognized group of 16S rRNA methylases, which share modest similarity to those produced by aminoglycoside-producing actinomycetes. Their presence confers a high level of resistance to all parenterally administered aminoglycosides that are currently in clinical use. The responsible genes are mostly located on transposons within transferable plasmids, which provides them with the potential to spread horizontally and may in part explain the already worldwide distribution of this novel resistance mechanism. Some of these organisms have been found to coproduce extended-spectrum $\beta$-lactamases or metallo-$\beta$-lactamases, contributing to their multidrug-resistant phenotypes. A 2-tiered approach, consisting of disk diffusion tests followed by confirmation with polymerase chain reaction, is recommended for detection of 16S rRNA methylase–mediated resistance.

Aminoglycosides continue to play an important role in the management of serious infections caused by gram-negative pathogens, often in combination with broad-spectrum $\beta$-lactams. They bind specifically to the aminoacyl site (A-site) of 16S rRNA within the prokaryotic 30S ribosomal subunits and interfere with protein synthesis [1]. The most commonly encountered mechanism of resistance to aminoglycosides is enzymatic inactivation, which is mediated by 3 classes of enzymes: acetyltransferases, nucleotidyltransferases, and phosphotransferases [2]. They are further divided into subclasses that are based on the site of modification and the spectrum of resistance within the class of antimicrobials. Other known mechanisms of aminoglycoside resistance include defect of cellular permeability, active efflux, and, rarely, nucleotide substitution of the target molecule [1].

Aminoglycosides are produced by species of actinomycetes, such as Streptomyces species and Micromonospora species. These actinomycetes are intrinsically resistant to the aminoglycosides that they produce [3]. In many cases, this resistance is caused by ribosomal protection through methylation of specific nucleotides within the A-site of 16S rRNA, which hampers binding of aminoglycosides to the 30S ribosomal subunits and serves as a means of self-protection. Until recently, this resistance mechanism was believed to be absent in clinically relevant species.

However, clinical strains of Pseudomonas aeruginosa and Klebsiella pneumoniae that produced 16S rRNA methylases were reported in 2003 [4, 5]. These enzymes were found to confer extraordinarily high levels of resistance to clinically useful aminoglycosides, such as amikacin, tobramycin, and gentamicin. Since 2003, the literature on this newly recognized resistance mechanism has grown rapidly, documenting identification of new enzymes and their spread to different species in various parts of the world. In the present article, we will first review aminoglycoside resistance caused by 16S rRNA methylation in aminoglycoside-producing actinomycetes. We will then discuss the current knowledge of this emerging, plasmid-mediated resistance mechanism that is found among gram-negative path-
Figure 1. Dendrogram of 16S rRNA methylases of gram-negative bacteria and of representative actinomycetes. KsgA is a nonresistance 16S rRNA methylase intrinsic in *Escherichia coli*. The protein sequences were obtained from the databases of GenBank, European Molecular Biology Laboratory, and DNA Data Bank of Japan and were aligned using ClustalW [11].

MODIFICATION OF RNA IN BACTERIA

The ribosome is a large enzyme consisting of multiple proteins and RNA components [6]. In bacteria, it comprises 30S and 50S subunits, the former containing 16S rRNA and the latter containing 23S and 5S rRNAs. Posttranscriptional modification events of RNAs, such as methylation of nucleosides, take place following the generation of initial RNA transcripts. They are predominantly reported in tRNA, but they are also reported in rRNA. *Escherichia coli*, for example, is known to contain 10 methylated nucleosides in 16S rRNA and 14 methylated nucleotides in 23S rRNA [7]. The primary roles of rRNA methylation likely include modulation of rRNA maturation, stabilization of rRNA structures, and alteration of translation rates. For instance, mutants of *E. coli* that were deficient in the production of KsgA (RsmA) showed an increased leakiness of nonsense and frameshift mutants and alteration in decoding fidelity at both the A-site and peptidyl-tRNA site of 16S rRNA [8, 9]. In addition, some of the posttranscriptional methylation events are known to confer resistance to antimicrobials that target rRNA.

RESISTANCE IN ACTINOMYCETES MEDIATED BY 16S RNA METHYLATION

A number of actinomycetes are known to be intrinsically resistant to aminoglycosides that they produce themselves. The mechanisms of resistance include inactivation of aminoglycosides by production of aminoglycoside-modifying enzymes and protection of 16S rRNA within the 30S ribosome subunit by production of 16S rRNA methylase. The latter mechanism results in high-level resistance to multiple aminoglycosides. It represents an efficient means to avoid inhibition of their own protein synthesis and is prevalent among aminoglycoside-producing actinomycetes (figure 1) [3].

Two sites of methylation within 16S rRNA that lead to different aminoglycoside-resistance phenotypes have been identified [10]. One group of 16S rRNA methylases, such as that produced by the istamycin producer *Streptomyces tenjimariensis*, methylates residue A1408 (figure 2). Another group of 16S rRNA methylases, exemplified by those produced by gentamicin-producer *Micromonospora purpurea*, methylates residue G1405. The former confers resistance to kanamycin and apramycin but not gentamicin, whereas the latter confers resistance to kanamycin and gentamicin but not apramycin. Both of these residues are located within the A-site–decoding region of 16S rRNA.
gentamicin; Kan, kanamycin.

A1408 have only been reported in actinomycetes. Apr, apramycin; Gen, gentamicin; Kan, kanamycin.

MEDIATED BY 16S RRNA METHYLATION RESISTANCE IN GRAM-NEGATIVE PATHOGENS

Figure 2. The positions of modifications in the aminoacyl site (A-site) decoding region in 16S rRNA (modified from Beaucleur et al. [10] with permission). ArmA is known to methylate G1405 [19]. Based on the common Kan$^R$-Gen$^R$ phenotype, the other methylases in gram-negative organisms likely methylate the same residue. Methylocases that modify A1408 have only been reported in actinomycetes. Apr, apramycin; Gen, gentamicin; Kan, kanamycin.

rRNA, where aminoglycosides are known to bind and interfere with accurate translation through blocking translocation of peptidyl-tRNA from the A-site to the peptidyl-tRNA site [12].

RESISTANCE IN GRAM-NEGATIVE PATHOGENS MEDIATED BY 16S rRNA METHYLATION

Because it became evident that many aminoglycoside-producing actinomycetes used ribosomal resistance afforded by methylation of 16S rRNA, the question was raised as to why the same resistance mechanism was not identified in clinically relevant species. It was speculated that such resistance mechanisms could exist but were possibly missed because of limited screening methods, because the resistance pattern could mimic that of organisms producing multiple aminoglycoside modifying enzymes. In 2002, a gene encoding 16S rRNA methylase, later designated ArmA, was deposited to the European Molecular Biology Laboratory and GenBank as part of a plasmid sequence from *Citrobacter freundii* in Poland (accession number AF550415). No additional findings have been published to date.

Then, in 2003, an aminoglycoside-resistant *P. aeruginosa* clinical isolate from Japan was reported to produce 16S rRNA methylase [5]. The deduced amino acid sequence of this new enzyme, designated RmtA, shared modest (up to 35%) identity with 16S rRNA methylases of various actinomycetes. RmtA displayed methylation activity against 16S rRNA of 30S ribosomal subunits derived from a susceptible strain of *P. aeruginosa*. When rmtA was cloned and expressed in *E. coli* and *P. aeruginosa*, it was found to confer a high degree of resistance to all 4,6-disubstituted deoxystreptamines, which include gentamicin, tobramycin, and amikacin. As described above, a putative 16S rRNA methylase gene was initially found in a *C. freundii* clinical isolate from Poland. This gene was also identified in *K. pneumoniae* from France [4]. The gene product ArmA was also shown to confer high-level resistance to 4,6-disubstituted deoxystreptamines. The identity of the amino acid sequence of ArmA with those of RmtA and other 16S rRNA methylases from actinomycetes was only modest, ranging between 30% and 35%. The structural gene of RmtA was associated with a genetic element that resembled a mercury-resistance transposon Tn5041 on a transferable plasmid [13]. The guanine cytosine content of rmtA was 55%, suggesting its origin from some guanine cytosine–rich microbe, including actinomycetes. The structural gene for ArmA was reported to be located on functional composite transposon Tn1548 [14]. The guanine cytosine content of armA was 30%, suggesting that it was derived from some microbe with lower guanine cytosine content. These findings point to the possibility that these genes were acquired horizontally from diverse nonpathogenic environmental microbes, but their exact origins remain unknown.

Several other 16S rRNA methylases were subsequently discovered among gram-negative bacteria, and a total of 5 are known to date (figure 1). RmtB was identified in *Serratia marcescens* from Japan [15]. RmtB is closest to RmtA, sharing 82% identity at the amino acid level. The structural gene for RmtB was located adjacent to a Tn3–like transposon on a large transferable plasmid. RmtC was found in a *Proteus mirabilis* clinical strain from Japan that was rather distant in phylogeny from the 3 enzymes already reported [16]. The structural gene for RmtC is also located next to a transposon-mediated recombination system termed ISm1, and the methylase gene was shown to be mobilizable from plasmid to plasmid [17]. The most recently identified 16S rRNA methylase is RmtD, which shares moderate identity (40%–42%) with RmtA and RmtB [18]. RmtD was found to be produced by a *P. aeruginosa* clinical strain from Brazil, which also produced metallo-β-lactamase SPM-1. This particular strain was, therefore, highly resistant to carbapenems as well as to aminoglycosides.

These newly identified 16S rRNA methylases in gram-negative bacilli all confer resistance to 4,6-disubstituted deoxystreptamines, including gentamicin, tobramycin, and amikacin, but not including 4,5-disubstituted deoxystreptamines, such as neomycin, 4-monosubstituted deoxystreptamines, such as paromomycin, or streptomycin, which lacks a deoxystreptamine ring. The level of resistance to tobramycin appears to be slightly lower than the level of resistance to other 4,6-disubstituted deoxystreptamines when the responsible genes are cloned and expressed in experimental strains of *E. coli*, such as XL1-Blue, DH5α, or INVαF (MIC, 64–256 μg/mL) (table 1). However,
Data on the prevalence of aminoglycoside resistance mediated by 16S rRNA methylation among gram-negative bacilli is still scarce. The prevalence of RmtA among clinical isolates of *P. aeruginosa* in Japan was estimated to be at least 0.4% during a national surveillance when screened by high-level multiple-aminoglycoside resistance followed by PCR confirmation [5]. Subsequently, RmtB has been detected in various species belonging to the family *Enterobacteriaceae*, including *K. pneumoniae*, *Klebsiella oxytoca*, *E. coli*, and *C. freundii* in Japan, Taiwan, South Korea, China, and Belgium [20—25]. ArmA, which was initially found in *C. freundii* and later characterized in *K. pneumoniae*, has also been identified in clinical isolates of *E. coli*, *S. marcescens*, *Enterobacter cloacae*, *Salmonella enterica*, *Shigella flexneri*, and *Acinetobacter* species from various countries in East Asia and Eastern and Western Europe (table 2) [14, 20–23]. A recent report from a university hospital in Taiwan estimated the prevalence of ArmA and RmtB to be 0.9% and 0.3%, respectively, among *K. pneumoniae* and *E. coli* when screened by resistance to amikacin and confirmed by PCR [22]. RmtA and RmtD have only been reported from *P. aeruginosa* in Japan and Brazil, respectively [5, 18, 21]. These findings indicate that 16S rRNA methylase genes are already disseminated globally among pathogenic gram-negative bacilli, although the overall prevalence appears to remain low.

Strains producing 16S rRNA methylase have been reported from livestock, as well. Plasmid-mediated armA and rmtB genes have been identified from *E. coli* in swine from Spain and China, respectively [26, 25]. A large amount of aminoglycosides, including kanamycin, gentamicin, apramycin, and streptomycin, has been consumed in veterinary medicine. This may have served as a selective pressure for enteric gram-negative organisms to acquire 16S rRNA methylase genes, possibly from non-pathogenic environmental actinomycetes that intrinsically produced aminoglycosides or similar 16S rRNA inhibitors, and then maintain and spread them to humans through the food supply chains. Monitoring for high-level aminoglycoside resistance among gram-negative pathogens mediated by this new resistance mechanism would, therefore, be important in livestock breeding environments, as well.

### Table 1. Aminoglycoside resistance pattern conferred by G1405 16S rRNA methylase.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Susceptibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>4,6-disubstituted DOS</td>
<td>Highly resistant</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Highly resistant</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>Resistant, highly resistant</td>
</tr>
<tr>
<td>Amikacin</td>
<td>Highly resistant</td>
</tr>
<tr>
<td>4,5-disubstituted DOS; neomycin</td>
<td>Susceptible</td>
</tr>
<tr>
<td>Monosubstituted DOS; apramycin</td>
<td>Susceptible</td>
</tr>
<tr>
<td>No DOS; streptomycin</td>
<td>Susceptible</td>
</tr>
</tbody>
</table>

**NOTE.** DOS, deoxystreptamine.

### Table 2. Genetic association and geographic distribution of 16S rRNA methylase genes.

<table>
<thead>
<tr>
<th>16S rRNA methylase gene</th>
<th>Guanine content, %</th>
<th>Molecular weight of product, kDa</th>
<th>IS or transposon</th>
<th>Associated β-lactamase genes</th>
<th>Bacterial species (country or countries)</th>
<th>Country, reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rmtA</td>
<td>55.4</td>
<td>27.4</td>
<td>IS6100, αγ element, Tn4051</td>
<td>Pseudomonas aeruginosa (J)</td>
<td>J [5, 21]</td>
<td></td>
</tr>
<tr>
<td>rmtB</td>
<td>55.6</td>
<td>27.4</td>
<td>Tn3</td>
<td>bla&lt;sub&gt;TEM-1&lt;/sub&gt;, bla&lt;sub&gt;CTX-M-14&lt;/sub&gt;</td>
<td>Serratia marcescens (J), <em>Escherichia coli</em> (J, T, C, Be), Klebsiella pneumoniae (J, T, K), Klebsiella oxytoca (J), Citrobacter freundii (K)</td>
<td>J [15, T [22], K [20, 23], C (DG345788), Be [24]</td>
</tr>
<tr>
<td>rmtC</td>
<td>41.1</td>
<td>32.1</td>
<td>ISEc1</td>
<td>Proteus mirabilis (J)</td>
<td>J [16]</td>
<td></td>
</tr>
<tr>
<td>rmtD</td>
<td>59.3</td>
<td>27.7</td>
<td>ISCR</td>
<td>bla&lt;sub&gt;TEM-1&lt;/sub&gt;</td>
<td>P. aeruginosa (Br)</td>
<td>Br [18]</td>
</tr>
<tr>
<td>armA</td>
<td>30.4</td>
<td>30.2</td>
<td>IS26, Tn1548</td>
<td>S. marcescens (J, F, K), C. freundii (P, F, K, Bu), Citrobacter amalonaticus (Be), K. pneumoniae (F, S, T, K, Bu, Be), K. oxytoca (Bu), E. coli (J, T, S, F, Bu, Be), <em>Enterobacter cloacae</em> (F, K, Be), <em>Enterobacter aerogenes</em> (Bu, Be), P. mirabilis (F), <em>Salmonella enterica Enteritidis</em> (Bu), S. enterica Oranienburg (P), Shigella flexneri (Bu), Acinetobacter species (J, K)</td>
<td>P (AY522431) [14], Bu [14], F [14], S [26], Be [24], J [21], T [22], K [20, 23]</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** DQ345788 and AY522431 are European Molecular Biology Laboratory and GenBank accession numbers. Be, Belgium; Br, Brazil; Bu, Bulgaria; C, China; F, France; I, India; IS, insertion sequence; J, Japan; K, South Korea; P, Poland; S, Spain; T, Taiwan.
CLINICAL IMPLICATION OF AMINOGLYCOSIDE RESISTANCE DUE TO 16S rRNA METHYLATION

Despite its currently low prevalence, the global spread of gram-negative bacilli producing 16S rRNA methylase is concerning for several reasons. First, these gram-negative bacilli show a very high level of resistance to most clinically useful aminoglycosides, including gentamicin, tobramycin, and amikacin, which cannot be overcome by dose adjustments. Second, all of the structural genes of known 16S rRNA methylases are associated with mobile genetic elements, such as transposon, some of which have been proven functional, providing them with the means to spread horizontally to other strains and species. Third, these organisms appear to possess a high potential for developing multidrug resistance, especially via acquisition of various β-lactamase genes. Of 35 ArmA- and RmtB-positive clinical isolates, 33 produced CTX-M- or SHV-type extended-spectrum β-lactamases (ESBLs) in a Taiwanese university hospital [22]. Similar observations have also been made in South Korea [20]. It has been reported that the structural gene for ArmA, the most prevalent methylase thus far, is located on a composite transposon Tn1546 on a transferable plasmid and is frequently associated with CTX-M-3–type ESBL genes [14]. Production of CTX-M-9–type ESBLs is seen in many strains with RmtB [22] (K. Yamane, unpublished data). RmtD was initially reported in a P. aeruginosa strain that co-produced SPM-1 metallo-β-lactamase, which is endemic in Brazil. The latter combination would render ineffective a potent double-coverage regimen of carbapenem plus aminoglycoside. Currently, there are no data regarding clinical outcome in patients infected with these organisms. Nonetheless, it would be prudent to pay careful attention to the antibiogram and to maintain a low threshold to screen for ESBL production when these 16S rRNA methylase–producing gram-negative bacteria are encountered in clinical situations. Contact precautions should be used for patients when coproduction of 16S rRNA methylase and ESBL or metallo-β-lactamase is highly suspected or confirmed.

DETECTION OF AMINOGLYCOSIDE RESISTANCE DUE TO 16S rRNA METHYLATION

Screening for 16S rRNA methylase–producing organisms may be considered for epidemiologic purposes when nosocomial or foodborne spread of such bacteria is suspected. Detection of this resistance mechanism may pose a challenge in clinical laboratories. Gram-negative bacilli commonly produce aminoglycoside-modifying enzymes, such as acetyltransferases, nucleotidytranferases, and phosphotransferases. When >1 of these enzymes are produced in single organisms, they could readily become resistant to multiple aminoglycosides. The hallmark of resistance mediated by 16S rRNA methylase that methylates residue G1405 is the very high level of resistance to all parenterally formulated aminoglycosides (MIC is typically ≥256 μg/mL), except streptomycin. This, however, may not be discernible in routine susceptibility testing conducted in the clinical laboratory, especially when automated susceptibility testing systems are used that only measure MICs close to the breakpoints of each aminoglycoside. One unique characteristic of these methylases is the high-level resistance to arbekacin that they confer. Arbekacin is a semisynthetic aminoglycoside derived from dibekacin [27]. It has activity against staphylococci,
as well as against gram-negative bacteria, and it is currently approved only for treatment of multidrug-resistant *Staphylococcus aureus* infections in Japan. It is generally stable against the actions of aminoglycoside-modifying enzymes, with the exception of the bifunctional enzyme AAC(6′)/APH(2′′), which is known to be produced by some multidrug-resistant *S. aureus* and enterococcal strains, and it may result in low-level arbekacin resistance. However, arbekacin is not readily available in many instances. Therefore, we propose the following approach in screening for 16S rRNA methylase production (figure 3). When a strain belonging to the family *Enterobacteriaceae* or glucose nonfermentative species, such as *P. aeruginosa* or *Acinetobacter* species, meets the criteria set forth by the Clinical and Laboratory Standards Institute for resistance to multiple aminoglycosides, disk diffusion test using gentamicin, amikacin, and arbekacin (if available) may be performed. Production of 16S rRNA methylase is suspected when no or little inhibitory zone is observed with any of the aminoglycoside disks. The addition of an arbekacin disk is desirable, because it raises the positive predictive value of this method to ≥90%, compared with ~60% when performed only with amikacin [20]. Alternatively, if the MICs of these aminoglycosides are to be used, a cutoff value of 256 μg/mL appears to provide excellent positive predictive value. All of the RmtA-, RmtB-, RmtC-, RmtD- and ArmA-producing isolates that we have tested to date have had MICs of these aminoglycosides ≥256 μg/mL—an observation confirmed elsewhere [20]. Currently, PCR is the only confirmatory method available. Multiplex PCR may be performed for armA, rmtB, and rmtC in strains belonging to the family *Enterobacteriaceae* and *Acinetobacter* species and for rmtA and rmtD in *P. aeruginosa* (figure 4). Recommended primers and thermal cycling conditions are listed in table 3. Of note, some other glucose nonfermentative organisms, such as *Stenotrophomonas maltophilia* and *Burkholderia cepacia*, may also present with panaminoglycoside-resistant phenotype, but the mechanism of this resistance has not been elucidated.

### CONCLUSIONS

Methylation of the 16S rRNA in the 30S ribosomal subunit confers high-level resistance to most clinically useful aminoglycosides by inhibiting their access to the site of action. Gram-negative pathogens possessing this mechanism were first reported in 2003 and are increasingly reported worldwide. The organisms that produce 16S rRNA methylase are often multidrug resistant, especially against broad-spectrum β-lactams via production of ESBLs or metallo-β-lactamas, a process that is likely to be facilitated by the association of 16S rRNA methylase genes with genetic recombination systems. Although the clinical outcome for patients infected with these organisms is still unknown, early identification of the resistance mechanisms will be helpful in optimizing antimicrobial therapy and infection-control measures. A 2-tiered approach, consisting of disk diffusion tests followed by PCR confirmation, is recommended for detection of 16S rRNA methylase-mediated resistance.
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