Novel 16S Ribosomal RNA Methyltransferase RmtH Produced by *Klebsiella pneumoniae* Associated with War-Related Trauma

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Running title: 16S rRNA methyltransferase RmtH in *K. pneumoniae*
ABSTRACT

*Klebsiella pneumoniae* strain MRSN2404 was isolated from the chronic wound of a soldier who had been wounded in Iraq in 2006. The strain displayed very high minimum inhibitory concentrations of all aminoglycosides including arbekacin. A gene encoding a novel 16S ribosomal RNA methyltransferase, now designated RmtH, was identified. RmtH had 64% identity with RmtB1 and RmtB2. *rmtH* was bracketed by two copies of ISCR2, which may have played a role in its mobilization. (73 words)
Aminoglycosides, along with β-lactams and fluoroquinolones, remain one of the key classes of antimicrobial agents in the treatment of infections caused by gram-negative bacteria. Mechanisms of resistance to aminoglycosides include enzymatic modification of the drugs, modification of aminoglycoside-binding site, decreased permeability across the bacterial outer membranes, and augmented efflux. Among them, production of acquired 16S ribosomal RNA methyltransferase (16S-RMTase) is the most worrisome since it compromises the activity of all aminoglycosides (1). Since the initial reports in 2003, nine such enzymes have been identified (1, 2) (Bueno MFC, et al. under review with AAC for RmtG). With the exception of NpmA which methylates residue A1408 of the 16S ribosomal RNA, they methylate residue G1405 and confer high-level resistance to all aminoglycosides formulated for intravenous use, including gentamicin, tobramycin, amikacin, and arbekacin. Among the G1405 16S-RMTases, ArmA and RmtB appear to be most widely distributed worldwide, having been reported in multiple species of Enterobactericeae as well as Pseudomonas aeruginosa and Acinetobacter baumannii.

Production of 16S-RMTases is frequently accompanied by co-production of a carbapenamase or extended-spectrum β-lactamase (ESBL), which further facilitates multidrug resistance. In the United States, the most commonly encountered 16S-RMTase is ArmA produced by multidrug-resistant (MDR) and extensively drug-resistant (XDR) A. baumannii, whereas the presence of RmtB and RmtE has been reported in rare strains of Escherichia coli.

In this paper, we describe identification of RmtH, a novel 16S-RMTase in a clinical strain of Klebsiella pneumoniae. K. pneumoniae MRSN2404 was recovered from a 28 year-old male soldier who had suffered a tibial fracture from an explosion in Iraq in 2006. The strain was collected by the Multi-drug resistant organism Repository and Surveillance Network (MRSN) to enhance infection prevention, inform empiric therapy, and influence policy (3). Following
evacuation to the United States, a culture from the intramedullary wound of the right tibia grew XDR *A. baumannii*, ESBL-producing *K. pneumoniae*, and vancomycin-resistant enterococci. Subsequently, the patient had chronic draining wounds associated with the blast injury that occasionally expressed shrapnel. *K. pneumoniae* MRSN2404 was then isolated from the wound in 2009. This strain was found to have very high minimum inhibitory concentrations (>256 μg/ml) of all aminoglycosides tested, including gentamicin, tobramycin, amikacin and arbekacin when tested by the standard broth microdilution method recommended by the Clinical and Laboratory Standards Institute (4). It was also resistant to ceftriaxone, ceftazidime, cefepime, aztreonam and ciprofloxacin, but remained susceptible to ertapenem and imipenem. The strain was phenotypically confirmed as an extended-spectrum β-lactamase (ESBL) producer, and screening of β-lactamase genes with PCR and sequencing showed that it carried *bla*<sub>CTX-M-15</sub> as well as *bla*<sub>SHV-1</sub> and *bla*<sub>OXA-1</sub>. Multi-locus sequence typing (www.pasteur.fr/mlst) (5) assigned the strain to sequence type (ST) 48, an ST which has been reported in association with ESBL production worldwide (6-10).

Given the resistance phenotype consistent with 16S-RMTase production, the strain was screened for 16S-RMTase genes using PCR (2, 11, 12). However, PCR was negative for all known 16S-RMTase genes. We therefore proceeded with further experiments to identify the determinant of high-level aminoglycoside resistance. The genomic DNA of *K. pneumoniae* MRSN2404 was extracted, digested with KpnI (New England Biolabs, Ipswich, MA) and ligated with vector pBC-SK<sup>-</sup> (Agilent Technologies, Santa Clara, CA) which had been digested with the same enzyme. Electrocompetent *Escherichia coli* DH10B was transformed with this genomic library, and transformants were selected on tryptic soy agar (TSA) plates containing chloramphenicol (30 μg/ml) and gentamicin (50 μg/ml). This procedure yielded a single colony, which was cross-
resistant to other aminoglycosides as well. The recombinant plasmid harbored by this transformant (pKp2404K1) was then fully sequenced. The sequencing revealed the presence of a 3.1-kb insert, which contained an open reading frame corresponding to a 252 amino acid sequence. This open reading frame (ORF) showed 64% amino acid identity with 16S-RMTases RmtB1 and RmtB2 and 63% identity with RmtA. Identity with other 16S-RMTases was much lower, ranging from 25% with ArmA to 39% with RmtD1, RmtD2 and RmtF (Figure 1). The ORF was designated \textit{rmtH} according to the proposed nomenclature of acquired 16S-RMTases (13). We then performed PCR cloning of \textit{rmtH} using primers \textit{rmtH-XbaI-fwd} (5'-CGCTCTAGAATGACCATTGAACAGGCAGC-3') and \textit{rmtG-BamHI-rev} (5'-CGCGGATCCTCAAGCTGGGTTTGGCTGGA-3') (the restriction sites are underlined). The PCR product was digested with XbaI and BamHI and ligated with pBC-SK(-) digested with the same enzymes. Transformants were obtained using the method above. A transformant harboring a recombinant plasmid with the intact \textit{rmtH} structural gene (prmtHBX7), as confirmed by sequencing, was used for susceptibility testing. Susceptibility testing was performed using Etest (bioMérieux, Hazelwood, MO) according to the manufacturer’s instructions. As shown in Table 1, the original genomic clone as well as the PCR clone showed high-level resistance to gentamicin, tobramycin and amikacin as expected. Based on the pattern of aminoglycoside resistance and the amino acid alignment with known 16S-RMTases, RmtH likely functioned as a G1405 16S-RMTase (Figure 2). The full sequence of pKp2404K1 revealed that \textit{rmtH} was bracketed by two copies of IS\textit{CR2} in tandem. IS\textit{CR2} is an IS\textit{99}-like transposable element which is found in association with various resistance genes including those for sulfonamide, trimethoprim and florfenicol (14), and also tetracycline and cephalosporin (15, 16). It is believed to facilitate mobilization of the genetic
elements downstream. To our knowledge, this is the first instance where a 16S-RMTase gene was found in association with ISCR2. ISCR2 is usually found intact upstream of a resistance gene, while the second copy downstream is typically truncated (14). Since ISCR2 possesses a KpnI restriction site, we were not able to assess whether the two copies of ISCR2 were intact or not. Nonetheless, this unique arrangement suggested that they likely played a role in the initial mobilization of rmtH to *K. pneumoniae* MRSN2404.

Attempts to mobilize rmtH to *E. coli* were not successful by either transformation or conjugation. DNA hybridization of S1 nuclease-treated genomic DNA separated by pulsed-field gel electrophoresis (PFGE) with an rmtH-specific probe did not yield any band despite the presence of multiple plasmids on the PFGE. Finally, the rmtH probe hybridized to an approximately 500-kb band which was generated by PFGE following XbaI digestion (data not shown). These findings suggested that rmtH was likely located on the chromosome.

In summary, we reported a novel 16S-RMTase RmtH identified in an ESBL-producing *K. pneumoniae* strain which was recovered from a soldier who had been wounded during an operation in Iraq. The finding underscores the diversity of 16S-RMTases and highlights the importance of continued surveillance in identifying emerging antimicrobial resistance mechanisms.

The nucleotide sequence reported in this study has been submitted to the GenBank under accession number KC544262.

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Material has been reviewed by the Walter Reed Army Institute of Research. There is no objection to its presentation. The opinions or assertions contained herein are the private views of the authors and are not to be construed as official, or reflecting the views of the Department of the Army or the Department of Defense.

REFERENCES


Table 1. Minimum inhibitory concentrations (MICs) of aminoglycosides.

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<tr>
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<th>Gentamicin</th>
<th>Tobramycin</th>
<th>Amikacin</th>
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<td><strong>K. pneumoniae MRSN2404</strong></td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
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<tr>
<td><strong>E. coli DH10B (pKp2404K1)</strong></td>
<td>&gt;256</td>
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<tr>
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Figure legends.

Figure 1. Phylogenetic tree of G1405 16S-RMTases.
The tree was generated using the tools available at http://www.phylogeny.fr (17). GenBank references are as follows: ArmA, AY220558; RmtA, AB120321; RmtB1, AB103506; RmtB2, JN968578; RmtC, AB194779; RmtD1, DQ914960; RmtD2, HQ401565; RmtE, GU201947; RmtF, JQ808129; RmtG, JX486113.

Figure 2. Amino acid sequence alignment of G1405 16S-RMTases.
The alignment was generated using ClustalW (www.ebi.ac.uk/Tools/msa/clustalw2/).