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Coproduction of 16S rRNA Methyltransferase RmtD or RmtG with KPC-2 and CTX-M Group Extended-Spectrum β-Lactamases in *Klebsiella pneumoniae*

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Eight *Klebsiella pneumoniae* clinical strains with high-level aminoglycoside resistance were collected from eight hospitals in São Paulo State, Brazil, in 2010 and 2011. Three of them produced an RmtD group 16S rRNA methyltransferase, RmtD1 or RmtD2. Five strains were found to produce a novel 16S rRNA methyltransferase, designated RmtG, which shared 57 to 58% amino acid identity with RmtD1 and RmtD2. Seven strains coproduced KPC-2 with or without various CTX-M group extended-spectrum β-lactamases, while the remaining strain coproduced CTX-M-2.

The production of 16S rRNA methyltransferases (16S-RMTases) has emerged as a mechanism of high-level aminoglycoside resistance among Gram-negative pathogens in the last decade (1). Eight groups of such enzymes have been reported to date. Seven of them (ArmA and RmtA through RmtF) confer high-level resistance to 4,6-disubstituted deoxystreptamine (DOS) aminoglycosides, including gentamicin, tobramycin, and amikacin, by post-transcriptional methylation of position N7 at residue G1405 of 16S rRNA (1–3). N7 G1405 16S-RMTases have a global distribution and are often coproduced with carbapenamases or extended-spectrum β-lactamases (ESBLs). The other 16S-RMTase, NpmA, confers high-level resistance to 4,6-disubstituted DOS aminoglycosides, as well as 4,5-disubstituted DOS aminoglycosides, such as neomycin. NpmA has been shown to methylate position N1 at residue A1408 and has only been found in a single *Escherichia coli* strain in Japan (4).

Worldwide, ArmA and RmtB are the most commonly encountered 16S-RMTases, having been identified in *Enterobacteriaceae*, as well as *Pseudomonas aeruginosa* and *Acinetobacter baumannii* (1). The epidemiology appears to be distinct in South America, however, where RmtD, which includes the two closely related enzymes RmtD1 and RmtD2, predominates. RmtD1 was initially identified in *P. aeruginosa* clinical strains which were collected from hospitals in São Paulo, Brazil, in 2005 (5). The majority of these strains coproduced SPM-1 metallo-β-lactamase (6). RmtD1 was then identified in multiple species of *Enterobacteriaceae* from Brazil, Argentina, and Chile (7). Subsequently, RmtD2 was reported in *Enterobacter* and *Citrobacter* spp. from Argentina as the first variant of RmtD, differing from RmtD1 by nine amino acids (8).

The present study was conducted to investigate the 16S-RMTase contents among aminoglycoside-resistant *Klebsiella pneumoniae* clinical strains collected at Instituto Adolfo Lutz (IAL) from hospitals across the state of São Paulo. IAL serves as a state reference laboratory and receives multidrug-resistant Gram-negative pathogens on an ongoing basis. In 2010 and 2011, eight *K. pneumoniae* strains with high-level resistance to amikacin, gentamicin, and tobramycin (MIC of >256 μg/ml) were identified, all collected from different hospitals in São Paulo State. The sources of the strains included tracheal aspirate (2), rectal swab (2), bone (1), catheter tip (1), urine (1), and unknown (1) samples (Table 1).

We first screened for known 16S-RMTase genes as described previously (9). Three strains were positive for *rmtD*. Sequencing of the full structural genes identified them as *rmtD1* (1 strain) or *rmtD2* (2 strains). The other strains were negative for any of the previously reported genes. One of these five strains without a known 16S-RMTase gene, *K. pneumoniae* 350/10, was selected for further investigation. The genomic DNA of *K. pneumoniae* 350/10 was extracted, digested with HindIII (New England BioLabs, Ipswich, MA), and ligated with vector pBC-SK(−) (Agilent Technologies, Santa Clara, CA). 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 several colonies, all of which grew readily on TSA plates containing 100 μg/ml of arbekacin, a phenotype suggestive of 16S-RMTase production (9). The recombinant plasmid harbored by one of these transformants (pKp350/10H3) was then fully sequenced. The sequencing revealed the presence of a 1.6-kb insert, which contained two overlapping open reading frames. The first open reading frame was partial and corresponded to a 252-amino-acid sequence showing 76% identity with a putative tRNA ribosyltransferase reported upstream from *rmtD1* and *rmtD2* (8, 10). The second open reading frame overlapped the first one by eight nucleotides and corresponded to a 264-amino-acid sequence, which showed 58% and 57% identity with RmtD1 and RmtD2, respectively, 36% with RmtA, RmtB2, and RmtF, 35% with RmtB1, 29% with RmtE, 23% with RmtC, and 22% with ArmA. This open reading frame encoded a novel 16S-RMTase, which was designated RmtG (Fig. 1 and 2). Given the relative

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TABLE 1 Aminoglycoside susceptibilities of the *K. pneumoniae* clinical strains and *E. coli* experimental strains

Strain	Origin	MIC (μg/ml) of[a]:											16S-RMTase	β-Lactamase	ST	Inc type[b]
		GEN	TOB	AMK	ABK	NEO	APR	CAZ	CTX	FEP	ETP	MEM				
K. pneumoniae 64/11	Tracheal aspirate	>256	>256	>256	>256	>256	8	128	>256	128	>32	>32	*rmtD1*	KPC-2, CTX-M-15	437	N, A/C
K. pneumoniae 368/10	Unknown	>256	>256	>256	>256	128	8	12	64	>256	>32	>32	*rmtD2*	KPC-2	11	A/C
K. pneumoniae 253/11	Rectal swab	>256	>256	>256	>256	32	8	8	256	>256	>32	>32	*rmtD2*	KPC-2	11	A/C
K. pneumoniae 145/11	Rectal swab	>256	>256	>256	>256	16	4	32	32	12	>32	32	*rmtG*	KPC-2, CTX-M-59, TEM-1	1046	N, L/M
K. pneumoniae 1194/11	Catheter tip	>256	>256	>256	>256	16	4	192	>256	48	8	4	*rmtG*	KPC-2, CTX-M-15, TEM-1	340	ND
K. pneumoniae 350/10	Bone	>256	>256	>256	>256	4	8	16	256	96	4	4	*rmtG*	CTX-M-2, TEM-1	442	ND
K. pneumoniae 84/11	Tracheal aspirate	>256	>256	>256	>256	16	2	>256	>256	32	>32	>32	*rmtG*	KPC-2, CTX-M-59, TEM-1	442	N, L/M
K. pneumoniae 922/11	Urine	>256	>256	>256	>256	16	8	>256	>256	>256	>32	>32	*rmtG*	KPC-2, CTX-M-2, TEM-1	442	N, A/C
E. coli DH10B(pKp350/10H3)		>256	>256	>256	>256	2	4						*rmtG*			
E. coli DH10B(prmtG)		>256	>256	>256	>256	2	4						*rmtG*			
E. coli DH10B(pKp84/11)		>256	>256	>256	>256	2	8						*rmtG*	CTX-M-59		N
E. coli DH10B[pBC-SK(−)]		1	0.5	2	1	2	8									
E. coli DH10B		1	1	2	1	4	4									

[a] The MICs of aminoglycosides were determined by the agar dilution method. The MICs of cephalosporins and carbapenems were determined by Etest (bioMérieux, Hazelwood, MO). GEN, gentamicin; TOB, tobramycin; AMK, amikacin; ABK, arbekacin; NEO, neomycin; APR, apramycin; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; ETP, ertapenem; MEM, meropenem.

[b] ND, not determined.

sequence similarity between RmtG and the RmtD proteins, as well as an analogous alignment observed with a putative tRNA ribosyltransferase gene located upstream from *rmtD1* and *rmtD2*, it appears likely that these 16S-RMTases originated from closely related but as-yet-unidentified nonpathogenic species. The G+C content of *rmtG* (60%) was also similar to that of *rmtD1* and *rmtD2* (59%).

We then amplified the *rmtG* structural gene by PCR using primers rmtG-F-XbaI (5′-GCTCTAGAATGCGTGATCCGTTG TTT-3′) and rmtG-R-BamHI (5′-GCGGATCCTCATTCAGATT CCCGATG-3′) (the restriction sites are underlined). The product was digested with XbaI and BamHI, ligated with pBC-SK(−), and used to transform *E. coli* DH10B. The recombinant plasmid from a colony which grew on a TSA plate containing chloramphenicol and gentamicin (prmtG) was found to contain *rmtG*, which was confirmed to be intact by sequencing. *E. coli* DH10B(prmtG) displayed high-level resistance to 4,6-disubstituted DOS aminoglycosides but not 4,5-disubstituted DOS ones (Table 1). We therefore speculate that RmtG is an N7 G1405 16S-RMTase (1).

We then designed detection primers rmtG-F (5′-AAATACCG

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GCGGCAATGCCGATGGCGTGCGCCGCGCGGACGGGAAATTCTATAAATTCCATTACGCGATGGACGATAAGCACGCGCGG
                    -35                    -10
GACGGCCGCCCCGTGGATGAAACCTGCGATTGCGAATTGTGCCGCAATCATTCGCGCGCCTATTTGCAGCATCTGTTTCG

GGTGAACGATCCGAACGCCATGCGATTGGCGACGGCGCACAACCTGCGCTTCTACGGGCGGCTGATGGAGTATCTGCGCG
                                                                 RBS
RmtG            MRDPLFEKLAASKKYRDVCPDTIARILTECRAKYRREKEIDKAAREKLHGITAAFMTDAE 60
RmtD1           -MSELKEKLLASKKYRDVCPDTIERIWRECSAKFKKEKDVDKAAREALHGVTGAFMTERE 59
RmtD2           -MSELKEKLLASKKYRDVCPDTIERIWRECSAKFKKEKDADKAAREALHGVTGAFMTERE 59
                  . * *** ************* **  ** **:::**: ****** ***:*.****: *

RmtG            YRRAMEIAVRGGELAELMECHASTRERLPLEETDAVYARLLGAPDESA--LDLACGLNPA 118
RmtD1           YKRAMEMAAAR-DWEALLGMHASTRERLPVESMDRVFDQLFEASGTPARILDLACGLNPV 118
RmtD2           YKRAMELAATR-DWEALLGMHASTRERLPVESMDRVFDQLFEAIGTPARILDLACGLNPV 118
                *:****:*.   :   *:  *********:*. * *: :*: * . .*  *********.

RmtG            YLQNRYPEMRVTGIDISGQCVRVLRALG-VDARLGDLLAENAIPRARYSVALLFKILPLL 177
RmtD1           YLAHRLPNAAITGVDISGQCVNVIRAFGGAEARLGDLLCE--IPEDEANAALLFKVLPLL 176
RmtD2           YLAHRLPNAAIAGVDISGQCVNVIRAFGGAEARLGDLLCE--IPEDEADAALMFKVLPLL 176
                ** :* *:  ::*:*******.*:**:* .:*******.*  **. . ..**:**:****

RmtG            DRQSAGAARRILEAVNADALICSFPTRSLSGRNVGMAVHYAAWMRDQLPEKWRIERTVET 237
RmtD1           ERQRAGAAMDALMRVNAEWIVASFPTRSLGGRNVGMEKHYSEWMEAHVPENRAIAARLTG 236
RmtD2           ERQRTGAAMEALMRVNAEWIVASFPTRSLGGRNVGMEKHYSEWMEAHVPENRAIAARLTG 236
                :** :***   *  ***: ::.*******.******  **: **. ::**:  *   :

RmtG            DNELYYVLKEKQDGEAVRGGDSHRESE 264
RmtD1           ENELFYVLKRK---------------- 247
RmtD2           ENELFYVLKRK---------------- 247
                :***:****.*
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FIG 1 Amino acid alignment of RmtG with RmtD1 and RmtD2, the 16S-RMTase group with the highest similarity with RmtG (produced with Clustal W [http://www.ebi.ac.uk/Tools/msa/clustalw2/]). Part of the nucleotide sequence preceding *rmtG* is also shown, with the −10 and −35 regions of the putative promoter and potential ribosomal binding site (RBS) underlined.

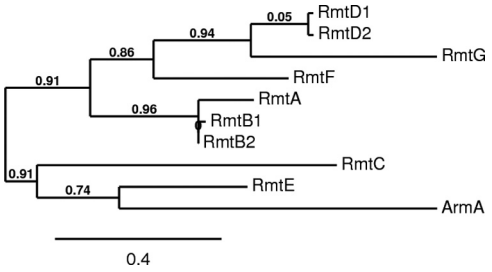

FIG 2 Dendrogram of confirmed and putative acquired N7 G1405 16S-RMTases. The dendrogram was generated using the tools available at http://www.phylogeny.fr (27). GenBank protein sequence accession numbers are as follows: ArmA, AAP50754.1; RmtA, BAD12551.1; RmtB1, BAC81971.1; RmtB2, AFC75738.1; RmtC, BAE48305.1; RmtD1, ABJ53409.1; RmtD2, ADW66527.1; RmtE, ADA63498.1; RmtF, AFJ11385.1. The numbers represent branch support values. The scale bar shows length in proportional difference.

CGATGTGTGTCC-3′) and rmtG-R (5′-ACACGGCATCTGTTT CTTCC-3′) to screen the four remaining *K. pneumoniae* strains, which were negative for known 16S-RMTase genes. The PCR conditions were the following: initial denaturation at 95°C for 2 min; 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s; and final incubation for 7 min at 72°C. The results showed that they were all positive for the presence of *rmtG*. Sequencing of the entire genes confirmed them as identical to the originally identified *rmtG* from *K. pneumoniae* 350/10. Furthermore, the upstream sequence of *rmtG* was identical for all five strains for the 0.7-kb region captured in pKp350/10H3. Of the five RmtG-producing *K. pneumoniae* strains, transfer of *rmtG* by transformation to *E. coli* DH10B was successful only for *K. pneumoniae* 84/11(pKp84/11), suggesting a plasmidic location of *rmtG* for this strain. *rmtG* was cotransferred with $bla_{\text{CTX-M-59}}$ but not $bla_{\text{KPC-2}}$. Transfer of *rmtG* to *E. coli* J53 by broth mating was not successful for any of the five strains despite repeated attempts. We therefore conducted pulsed-field gel electrophoresis (PFGE) of S1 nuclease-treated genomic DNA (11). This was followed by DNA hybridization using an *rmtG*-specific probe and methodology described previously (12). As shown in Fig. 3, the size of pKp84/11 was estimated to be approximately 200 kb. While they did not transfer to *E. coli*, the *rmtG* genes in the other four *K. pneumoniae* strains also appeared to be carried on plasmids, which ranged in size between 200 and 400 kb. Replicon typing using a previously described method (13) revealed pKp84/11 to be an IncN plasmid (Table 1). However, two of the *rmtG*-harboring *K. pneumoniae* strains were negative for any replicon, including IncN, based on this protocol.

K. pneumoniae strains producing various ESBLs and, more recently, KPCs (*Klebsiella pneumoniae* Carbapenemases), are reported from Brazil (14–19). We screened the eight 16S-RMTase-producing strains for KPC, CTX-M, SHV, and TEM group β-lactamases by PCR and sequencing as described previously (20). All but one strain were found to harbor $bla_{\text{KPC-2}}$. In addition, six strains carried $bla_{\text{CTX-M}}$ ($bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-59}}$, or $bla_{\text{CTX-M-15}}$) (Table 1). KPC-producing *K. pneumoniae* strains in Brazil are predominantly clonal complex 258 (CC258), which includes sequence types (STs) such as ST11, ST258, and ST437 (16, 21). We determined the STs of the eight clinical strains using the standard protocol (22). The strains producing RmtD1 and RmtD2 belonged to ST11 or ST437 (Table 1). One of the RmtG-producing strains belonged to ST340, which is also part of CC258. The other four strains belonged to ST442 or ST1046. ST442 was reported in a clinical strain which was recovered from blood in the state of Goiás in Brazil in 2009 (21). ST1046 is a double-locus variant of ST961, which was recently registered as an environmental strain from Portugal. Therefore, the *rmtD* alleles were likely acquired by global epidemic strains from other *Enterobacteraceae* species or *P. aeruginosa*, whereas the strains carrying *rmtG* appeared to be of a more-local origin. Coproduction of KPC and 16S-RMTase has been reported for ArmA and RmtB in *K. pneumoniae* and *Enterobacter cloacae* (23–25). RmtG is thus the third 16S-RMTase to be described in KPC-producing *Enterobacteriaceae*. The production of 16S-RMTase by KPC-producing *K. pneumoniae* could further limit the treatment options for infection caused by this organism, the majority of which otherwise remain susceptible to one or more aminoglycosides, gentamicin in particular (26).

Nucleotide sequence accession number. The sequence reported in this work has been deposited to the GenBank under accession number JX486113.

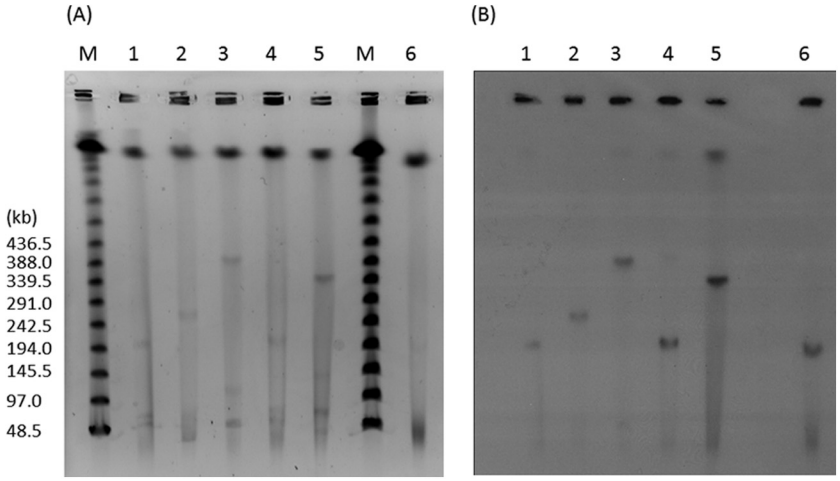

FIG 3 (A) PFGE of S1 nuclease-digested plasmids. (B) DNA hybridization with *rmtG*-specific probe. Lanes M, marker; lanes 1, strain 145/11; lanes 2, strain 1194/11; lanes 3, strain 350/10; lanes 4, strain 84/11; lanes 5, strain 922/11; lanes 6, *E. coli* DH10B transformant of strain 84/11.

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