Characterization of Two New Variants of 16S rRNA Methylase Encoding Genes, *rmtB2* and *rmtB3*

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Amended Abstract

Objectives: To characterize two G1405 16S rRNA methylase encoding genes showing 3-4 amino acid changes compared to *rmtB* from Enterobacteriaceae isolates collected in 2005 and 2006.

Methods: Genes encoding RmtB-like were sequenced on both strands. Clinical strains carrying these genes were analyzed. Primers comprising the open reading frame of the *rmtB*-like genes were used to amplify the entire gene and amplicons were cloned into PCRScript/XL1 Blue *E. coli* kan^R. *E. coli* DH5α was used as a secondary host and transformation plated onto selective media containing 30 mg/L of chloramphenicol. Plasmid preparations of clinical strains were transformed into *E. coli* DH5α by electroporation and selected in media containing 4 mg/L of kanamycin. Susceptibility testing was performed according to CLSI reference broth microdilution methods using extended MIC dilution ranges for amikacin, tobramycin, gentamicin, arbekacin, apramycin, kanamycin, neomycin and streptomycin.

Results: *rmtB* was sequenced in nine strains initially positive by PCR using primers targeting this gene. Five strains carried variants of the *rmtB* gene: *rmtB2* showing three aminoacid changes A41T, I124V and I132V and *rmtB3* showing one additional alteration at position 82 (A→V). *rmtB2* was detected in 2 isolates from Mexico (2 *E. cloacae* strains; 2 hospitals). *rmtB3* was detected among 3 strains from USA (Texas; *E. coli*) and Mexico (one *E. cloacae* and one *K. pneumoniae*). Susceptibility testing demonstrated that isolates carrying *rmtB1*, *rmtB2* and *rmtB3* had elevated MIC values for amikacin (32-256 mg/L), tobramycin (16-64 mg/L), gentamicin (4-64 mg/L), arbekacin (16-64 mg/L) and kanamycin (64-256 mg/L) when compared to the *E. coli* host carrying PCRScript plasmid without insert. RmtB-variant produced MIC values for apramycin, neomycin and streptomycin no different from *rmtB* (0.25-2 mg/L) expressed in the same genetic background. Plasmids from three of the five clinical strains were transferred to *E. coli* and MICs were elevated for aminoglycosides (8-256-fold) that are susceptible to G1405 methylation.

Conclusions: Two G1405 16S rRNA methylase genes similar to *rmtB* were detected among several Enterobacteriaceae isolates collected during 2005-2006 from different countries in Latin and North America, suggesting that these variants could be widespread in this geographic region.

Introduction

Aminoglycoside resistance among Gram-negative bacilli is a matter of concern since these antimicrobial agents are often used in combination to β-lactam agents, including cephalosporins and carbapenems for the treatment of serious infections caused by Enterobacteriaceae and non-fermentative Gram-negative bacilli. Distinct aminoglycoside resistance mechanisms have been detected in Gram-negative organisms; however, aminoglycoside-modifying enzymes and 16S rRNA methylases are threatening effective chemotherapy due to their association with β-lactamase-carrying genetic elements that often encode resistance to multiple antimicrobial classes. The modifying enzymes do not affect all aminoglycosides equally, but in contrast 16S rRNA methylases confer high-level resistance to virtually all agents within this class, including commonly used amikacin, tobramycin and gentamicin and resistance levels cannot be overcome by dose adjustments.

Seven different 16S rRNA methylase encoding genes have been described and homology of these genes vary from 10 to 81%: *armA* and *rmtA-E* encodes for proteins that methylates residue G1405 and the *npmA* product methylates residue A1408. A limited number of variants of these genes have been identified. In this report, we describe two novel *rmtB* variants detected among Enterobacteriaceae species collected from different countries (Mexico and southern USA).

Materials and Methods

Bacterial isolates. Isolates initially screened for *armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD* and *npmA* using custom primers that yielded positive *rmtB* amplification were further evaluated. Amplicons were sequenced on both strands and nucleotide sequences obtained were analyzed using Lasergene® software package (DNAStar; Madison, Wisconsin, USA) and compared to available sequences via NCBI BLAST search (http://www.ncbi.nlm.nih.gov/blast/).

Cloning of *rmtB*-variants. Amplicons containing the complete sequence of *rmtB* (here in named rmtB1), *rmtB2* and *rmtB3* were cloned into pPCRScriptCam SK+ (Stratagene, California, USA). The colonies obtained after transformation in XL10-Gold® Kan ultracompetent *Escherichia coli* were selected on plates containing 30 mg/L chloramphenicol. The presence and orientation of inserts was confirmed by PCR and sequencing. Plasmid extractions were performed by alkaline lysis and the vectors containing *rmtB*-variants were transferred by electroporation to *E. coli* DH5α and selected in 4 mg/L of kanamycin. The MIC for aminoglycoside antimicrobial agents was determined from recombinant and clinical strains by reference broth microdilution method according to Clinical and Laboratory Standards Institute (CLSI) guidelines (M07-A9; 2012). Extended MIC dilution ranges were tested for amikacin, tobramycin, gentamicin, arbekacin, apramycin, kanamycin, neomycin and streptomycin.

Methods-continued

Plasmid transfer. Plasmid preparations (QIAprep Spin Mini prep kit, Qiagen, Hilden, Germany) were electroporated into *E. coli* DH5α. Electroporation parameters were 2.5 kV, 25 μF and 400 Ω using the Bio-Rad Gene Pulser apparatus (BioRad, Richmond, California, USA). Selection was performed in agar plates containing 4 mg/L of kanamycin and confirmation was carried out by PCR. Susceptibility testing was performed as described above.

Gene location analysis. Total cellular DNA embedded in 1% agarose plugs was subjected to partial digestion with S1 nuclease. Plasmids were resolved by electrophoresis performed on the CHEF-DR II (BioRad, Richmond, California, USA), with the following conditions: 0.5 x TBE, 1% agarose, 13°C, 200V, for 6 hours with switch time ramping from 5 to 25 seconds and 14 hours with the switch time from 30 - 45 seconds. ICeuI digested genomic DNA was also resolved on PFGE as described previously. DNA gels were transferred to nylon membranes by southern blotting and hybridized with a digoxigenin labeled (Roche Diagnostics GmbH, Mannheim, Germany) *rmtB* probe.

Nucleotide sequence accession number. The nucleotide sequence of *rmtB2* and *rmtB3* has been submitted to GenBank nucleotide database and assigned accession number JN968578 and JN968579, respectively.

Results

- Five strains carrying novel *rmtB*-variants were evaluated. These strains belonged to three bacterial species: *Enterobacter cloacae* (four strains) and one of each for *E. coli* and *Klebsiella pneumoniae* and were recovered from hospitalized patients in Mexico (four strains) and USA (one). One *E. coli* from Brazil carrying *rmtB1* was included for comparison purposes. These samples were recovered from blood cultures, invasive pulmonary specimens and urine (Table 1).
- Three isolates from Mexico (two *E. cloacae* from distinct cities/hospitals) and one *E. coli* (*rmtB1*) from Brazil carried *rmtB2*, whereas two strains from Mexico (one *E. cloacae* and one *K. pneumoniae*) and one from the USA (Texas; *E. coli*) harboured *rmtB3*. Isolates from the same bacterial species were genetically distinct by PFGE (Table 1).
- The two variants named *rmtB2* and *rmtB3* encode proteins displaying three aminoacid changes (A41T, I124V and I132V) and one additional alteration was noted on RmtB3 (A82V) when compared to RmtB1 (Figure 1).
- Isolates carrying *rmtB1*, *rmtB2* and *rmtB3* had elevated MIC values for amikacin (32-256 mg/L), tobramycin (16-64 mg/L), gentamicin (4-64 mg/L), arbekacin (16-64 mg/L) and kanamycin (64-256 mg/L) when compared to the *E. coli* host carrying PCRScript plasmid without insert (Table 1). Apramycin, neomycin and streptomycin MIC results were lower and similar to the recombinant plasmid with no insert.
- *E. coli* DH5α strains harbouring recombinant plasmids carrying *rmtB2* and *rmtB3* displayed MIC results four-fold higher to gentamicin and tobramycin and eight-fold higher to amikacin, arbekacin and kanamycin when compared to the *rmtB1* construct carried in the same genetic background (Table 1).
- All strains analyzed carried *rmtB* variants in plasmids of different sizes ranging from 95 to 150-Kb (Table 1 and Figure 1). When transferred to the same background, the natural plasmids carrying *rmtB2* and *rmtB3* produced elevated MIC values to amikacin, arbekacin, kanamycin, gentamicin, and tobramycin; however, similarly to the constructs carrying these genes, apramycin, neomycin and streptomycin MIC values were unchanged.

Figure 1. Alignment of the protein sequences of *rmtB1* and two new variants (*rmtB2* and *rmtB3*). Identical amino acids are not displayed.

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rmtB1.pro   MNI NDALTSI LASKKYRALCPDTVRRI LTEEWGRHKSPKQTVEAARTRLHGI CGAYVTPESLKAA  65
rmtB2.pro   . . . . . A. . . . . .  65
rmtB3.pro   . . . . . A. . . . . .  65

rmtB1.pro   AAALSAGDVKKALSLHASTKERLAELDTLYDFI FSAETPRRVLDI ACGLNPLALYERGI ASVWGC 130
rmtB2.pro   . . . . . V. . . . . .  130
rmtB3.pro   . . . . . V. . . . . .  130

rmtB1.pro   DI HQGLGDVI TPFAREKDWDFTFALQDVLCAPPAEAGDLALI FKLLPLLEREQAGSAMALLQSLN 195
rmtB2.pro   . V. . . . . T. . . . . I . 195
rmtB3.pro   . V. . . . . T. . . . . I . 195

rmtB1.pro   TPRMAVSFPTRSLGGRGKGMEANYAAWFEGGLPAEFEI EDKKTI GTELI YLI KKNG  251
rmtB2.pro   . . . . . T. . . . . .  252
rmtB3.pro   . . . . . T. . . . . .  252
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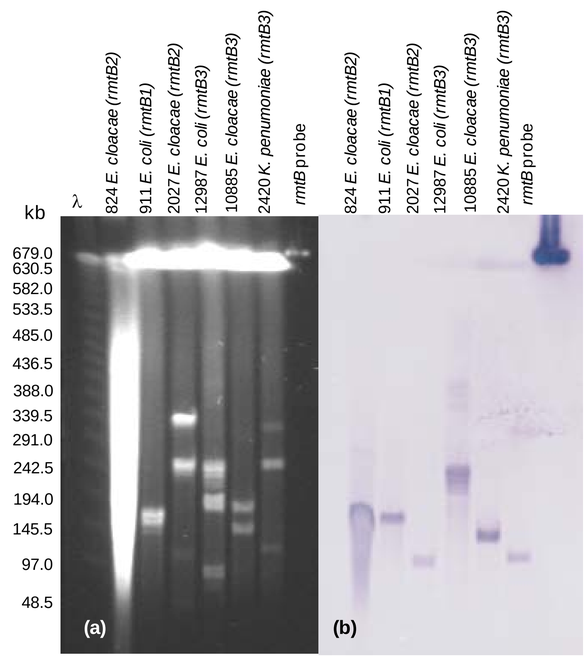
Figure 2. (a) S1 digested DNA profiles in agarose gel electrophoresis of *rmtB2*- and *rmtB3*-carrying strains and (b) hybridization with digoxigenin labeled *rmtB* probe. Lambda ladder (λ) was used as molecular weight marker and negative control. Amplicons used to prepare the *rmtB*-probe were used as positive control.



Conclusions

- A limited number of variants of 16S rRNA methylase genes have been described. Here we describe two new *rmtB*-like genes that have been identified in clinical strains collected in Mexico and the USA among three different bacterial species.
- These RmtB variants encoded for higher resistance levels to aminoglycoside agents susceptible to G1405 methylation when compared to the original RmtB variant previously described.
- These new *rmtB*-variants were detected in clinical isolates collected in 2005 and 2006 and their current prevalence is unknown. Further studies should be performed to evaluate the spread of these genes in North America.

References

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Table 1. Demographic information and antimicrobial profile of *rmtB2*- and *rmtB3*-carrying isolates and *rmtB1*-carrier used as control.

Bacterial species/isolate	*rmtB* variant	Year Isolated	Country	Specimen Type[a]	PFGE	Plasmid molecular weight (Kb)	MIC (mg/L)							
							Amikacin	Apramycin	Arbekacin	Gentamicin	Kanamycin	Neomycin	Streptomycin	Tobramycin
E. cloacae														
824	*rmtB2*	2006	Mexico	BC	ECL115A	125	>1024	4	>1024	>1024	>1024	4	32	>1024
2027	*rmtB2*	2005	Mexico	IR	ECL126A	95	>1024	4	>1024	>1024	>1024	256	512	>1024
10885	*rmtB3*	2005	Mexico	BC	ECL115B	100	>1024	8	>1024	>1024	>1024	8	32	>1024
E. coli														
911 (Control)	*rmtB1*	2005	Brazil	IR	EC46A	112	>1024	>1024	>1024	>1024	>1024	>1024	1024	>1024
12987	*rmtB3*	2005	USA	BC	EC25B	150	>1024	16	>1024	>1024	>1024	4	16	>1024
K. pneumoniae														
2420	*rmtB3*	2005	Mexico	UR	KPN115A	96	>1024	4	>1024	1024	>1024	1	4	512
E. coli DH5α														
Transconjugants														
(p824)	*rmtB2*	NA[b]	NA	NA	NA	NT[c]	256	2	64	32	512	0.5	1	64
(p2027)	*rmtB2*	NA	NA	NA	NA	NT	128	2	64	32	256	0.5	2	64
(p12987)	*rmtB3*	NA	NA	NA	NA	NT	256	2	64	32	512	0.5	1	64
Recombinants														
PCRScript(*rmtB1*)	*rmtB1*	NA	NA	NA	NA	NT	32	2	16	4	64	0.25	2	16
PCRScript(*rmtB2*)	*rmtB2*	NA	NA	NA	NA	NT	256	2	64	32	512	0.5	1	64
PCRScript(*rmtB3*)	*rmtB3*	NA	NA	NA	NA	NT	256	2	64	32	512	0.5	1	64
PCRScript(no insert)	NA	NA	NA	NA	NA	NT	0.5	2	0.25	0.25	1	0.5	2	0.25

a. BC= bloodculture, IR= invasive respiratory, UR= urinary.
b. NA= Not available.
c. NT= Not tested.