C2-2053

Detection of Methyltransferases Conferring High-level Resistance to Aminoglycosides in Enterobacteriaceae from Europe, North America and Latin America

TR FRITSCHE, GH MILLER, RN JONES, ES ARMSTRONG
JMI Laboratories, North Liberty, IA, USA; Achaogen, Inc., South San Francisco, CA, USA

ICAAC 2007
JMI Laboratories
North Liberty, IA, USA
www.jmilabs.com
319.665.3370
fax 319.665.3371
thomas-fritsche@jmilabs.com

ABSTRACT

Background: Alteration of ribosomal targets by methyltransferases (MT; ArmA, Rmt A, RmtB, RmtC and RmtD) that confer resistance (R) to most aminoglycosides (AG), including arbekacin, has recently been reported. *armA* and *rmtB* are most widespread and found exclusively in Asia and Europe. *rmtA* and *rmtC* have been reported from Japan (*P. aeruginosa* [PSA] and *P. mirabilis* [PM], respectively) and *rmtD* from Brazil (PSA). We evaluated Enterobacteriaceae (ENT) and non-fermentative bacilli acquired through global surveillance programs for the presence of MT based upon R patterns to marketed AG.

Methods: ENT (262 isolates), PSA (95) and *Acinetobacter* spp. (ASP; 50) were selected on the basis of R to combinations of gentamicin, tobramycin and amikacin and further susceptibility (S) tested using CLSI microdilution methods against six other AG. PCR using primer sets designed from the published gene sequences of *armA*, *rmtA*, *rmtB*, *rmtC* and *rmtD* was performed on AG-R isolates with products visualized by gel electrophoresis; rmtB and rmtD bands were confirmed by sequencing.

Results: S testing revealed 22 strains that were R to all 4,6-disubstituted AG, 20 of which (4.9% of total) were confirmed to have MT genes (9 *armA*, 8 *rmtB* and 3 *rmtD*). *armA* was detected in isolates from Poland (*E. cloacae* [ECL], *E. coli* [EC], *K. oxytoca* and *S. marcescens*), France (*K. pneumoniae* [KPN]) and the USA (KPN and ECL) whereas *rmtB* strains were found in the USA (EC), Brazil (EC and PM) and Mexico (KPN and ECL). *rmtD* was observed in Brazil (*C. freundii*) and Chile (KPN and ECL). No MT were found in this collection of PSA or ASP, although an earlier pilot study identified *armA* in ASP (Venezuela).

Conclusions: New geographic and species results reported here demonstrate global occurrence of MT among AG-R ENT. Recent emergence of high-level AG-R is a worrisome development requiring continued monitoring. New AG agents that are active against methylated ribosomes are critically needed.

INTRODUCTION

Aminoglycosides continue to play an important role in antimicrobial therapy of both Gram-negative and -positive pathogens, usually in combination with ß-lactam agents. Resistance to the class is widespread and has primarily been the result of aminoglycoside inactivation through the chemical processes of acetylation, phosphorylation and/or adenylation, with varying effects depending upon the particular agent. Since 2003, methylation of 16S rRNA has emerged as a serious threat to the class through the action of plasmid-mediated methyltransferase enzymes. These enzymes are similar to those found in actinomycetes, that confer intrinsic resistance to the aminoglycoside agents they produce.

Alteration of the 16S rRNA A-site by these enzymes (designated ArmA, RmtA, RmtB, RmtC and RmtD) confers resistance to most all aminoglycosides, including arbekacin, by limiting binding of these agents due to methylation of specific nucleotides. *armA* and *rmtB* are most widespread and have been found exclusively in Asia and Europe. *rmtA* and *rmtC* have been reported only from Japan (*Pseudomonas aeruginosa* and *Proteus mirabilis*, respectively) and *rmtD* only from Brazil (*P. aeruginosa*). The high level of resistance produced by these methyltransferases, and their association with mobile genetic elements, is a worrisome development that will require enhanced laboratory capabilities for detection, appropriate infection control practices to limit continued spread and, when detected, reliance on alternative chemotherapeutic agents.

While the occurrence of 16S methylation by these gene products appears to be widespread globally, actual prevalence data remains limited. We evaluated Enterobacteriaceae and non-fermentative bacilli acquired through global surveillance programs for the presence of methyltransferases based upon resistance patterns to marketed aminoglycosides.

MATERIALS AND METHODS

Strain Collection: Enterobacteriaceae (262 isolates), *P. aeruginosa* (95) and *Acinetobacter* spp. (50) were selected on the basis of resistance to combinations of gentamicin, tobramycin and amikacin and further susceptibility tested using CLSI microdilution methods (M7-A7 [2006]) against six other aminoglycosides (apramycin, arbekacin, fortimicin, kanamycin, neomycin, and netilmicin). Strains were selected for further study based on the MIC results from this aminoglycoside screen.

High-level resistance to all aminoglycosides in the panel except apramycin and neomycin is a defining characteristic of the methylase resistance mechanism (Galimand M, Courvalin P, and Lambert T. *Antimicrob Agents Chemother* 47: 2565-71, 2003). Applying these selection criteria allowed us to detect 19 potential methyltransferase-positive strains. Two additional strains were selected on the basis of apramycin susceptibility alone, since *aph(3')-I* is a widespread resistance gene that reduces potency of neomycin (Wright GD, Berghuis AM, Mobashery S. *Adv Exp Med Biol* 456:27-69, 1998). A final strain displayed high-level resistance to all aminoglycosides tested, and was positive for *aph(3')-I* and *aac(3)-IV* enzymes (data not shown). Including this strain a total of 22 were selected for further analysis.

Molecular studies: Primers for diagnostic amplification of the *armA* and *rmtA-D* genes were designed from sequences deposited in the Genbank database or were selected from the literature (Table 1). All primers were designed to have similar annealing temperatures for simultaneous amplification on a single thermocycler program. Templates for PCR reactions were prepared by suspending a single colony in 100 µL of sterile water. Suspensions were heated to 95°C for 10 minutes, cooled to room temperature and centrifuged to pellet cell debris. The addition of 2 µL of the template supernatant to reaction mixtures containing the appropriate primer sets was used to initiate reactions.

Amplification was performed in an Eppendorf Mastercycler (Eppendorf, Westbury, NY). The PCR method consisted of 25 cycles of denaturation at 94°C for 15 seconds, annealing at 58°C for 30 seconds and extension at 72°C for 60 seconds. This was followed by an additional 10 minute extension at 72°C. The entire reaction mixture was loaded on an E-Gel (Invitrogen, Carlsbad, CA) for rapid gel electrophoresis. Visualization of bands by UV light confirmed the presence of the target methylase gene. A plasmid containing the *armA* gene (gift of Patrice Courvalin) was used as a positive control for the presence of *armA* in the collection of clinical isolates. Upon initial identification of *rmtB* and *rmtD*, these PCR products were submitted for DNA sequencing to confirm their identity by comparison to previously reported sequences. These confirmed strains were subsequently employed as positive controls for further screening.

RESULTS

- Susceptibility profiling of 407 Gram-negative isolates resistant to combinations of gentamicin, tobramycin and amikacin revealed 22 strains that were resistant to all 4,6-disubstituted aminoglycosides, 20 of which (4.9% of total) were confirmed to have methyltransferase genes (9 *armA*, 8 *rmtB* and 3 *rmtD*; Table 2).

Table 1. PCR primer sets utilized in the detection of aminoglycoside methyltransferase resistance genes.

Primer	Sequence
armA-f	TATGGGGGTCTTACTATTCTGCCTAT
armA-r	TCTTCCATTCCCTTCTCCTTT
rmtA-f	CTAGCGTCCATCCTTTCCTC[a]
rmtA-r	TTTGCTTCCATGCCCTTGCC[a]
rmtB-f	TCAACGATGCCCTCACCTC
rmtB-r	GCAGGGCAAAGGTAAAATCC
rmtC-f	CGCGGATCCAGTGTATGAAAAATGTCTGG[b]
rmtC-r	CGGGGTACCGGTGTGTTAGAATTTGCCTT[b]
rmtD-f	CTGTTTGAAGCCAGCGGAACGC
rmtD-r	GCGCCTCCATCCATTCGGAATAG

a. Yokoyama et al. *Lancet* 362:1888-93, 2003
b. Washino et al. *Antimicrob Agents Chemother* 50:178-84, 2006.

- *armA* was detected in isolates from Poland (*Enterobacter cloacae* [1 isolate], *Escherichia coli* [1], *Klebsiella oxytoca* [1] and *Serratia marcescens* [3]), France (*K. pneumoniae* [1]) and the USA (*K. pneumoniae* and *E. cloacae* [1 each]) whereas *rmtB* strains were found in the USA (*E. coli* [1]), Brazil (*E. coli* [1] and *P. mirabilis* [2]) and Mexico (*K. pneumoniae* [1] and *E. cloacae* [3]; Table 3).

- *rmtD* was observed in single isolates from Brazil (*Citrobacter freundii*) and Chile (*K. pneumoniae* and *E. cloacae*).

- *rmtA* and *rmtC* genes have only been reported previously from *P. aeruginosa* and *P. mirabilis* (both from Japan) and were not identified here (Table 3).

Table 2. Aminoglycoside resistance profiles among studied Enterobacteriaceae and their detected resistance genes.

Study Year	Country	Organism	Amikacin	Apramycin	Arbekacin	Fortimicin	Gentamicin	Kanamycin	Neomycin	Netilmicin	Tobramycin	Resistance gene
2005	Brazil	*E. coli*	>128	>128	>128	>128	>128	>128	>128	>128	>128	*rmtB*
2005	Poland	*E. coli*	>128	8	>128	>128	>128	>128	2	>128	>128	*armA*
2005	USA	*E. coli*	>128	8	>128	>128	>128	>128	2	>128	>128	*rmtB*
2006	Chile	*E. cloacae*	>128	4	>128	>128	>128	>128	16	>128	>128	*rmtD*
2005	Mexico	*E. cloacae*	>128	4	>128	>128	>128	>128	4	>128	>128	*rmtB*
2005	Mexico	*E. cloacae*	>128	2	>128	>128	>128	>128	128	>128	>128	*rmtB*
2006	Mexico	*E. cloacae*	>128	4	>128	>128	>128	>128	4	>128	>128	*rmtB*
2006	Poland	*E. cloacae*	>128	4	>128	>128	>128	>128	≤1	>128	>128	*armA*
2005	USA	*E. cloacae*	>128	4	>128	>128	>128	>128	>128	>128	>128	*armA*
2005	Poland	*K. oxytoca*	>128	4	>128	>128	>128	>128	≤1	>128	>128	*armA*
2005	Argentina	*K. pneumoniae*	>128	4	>128	>128	>128	>128	8	>128	>128	Unknown
2005	Chile	*K. pneumoniae*	>128	2	>128	>128	>128	>128	8	>128	>128	*rmtD*
2005	Mexico	*K. pneumoniae*	>128	2	>128	>128	>128	>128	≤1	>128	>128	*rmtB*
2005	France	*K. pneumoniae*	>128	2	>128	>128	>128	>128	≤1	>128	>128	*armA*
2005	USA	*K. pneumoniae*	>128	2	>128	>128	>128	>128	≤1	>128	>128	*armA*
2006	Poland	*S. marcescens*	>128	8	>128	>128	>128	>128	16	>128	128	*armA*
2006	Poland	*S. marcescens*	>128	4	>128	>128	>128	>128	2	>128	>128	*armA*
2005	Poland	*S. marcescens*	>128	8	>128	>128	>128	>128	8	>128	>128	*armA*
2006	Brazil	*C. freundii*	>128	4	128	128	>128	>128	16	>128	>128	*rmtD*
2006	Brazil	*P. mirabilis*	>128	8	>128	>128	128	>128	4	>128	>128	*rmtB*
2006	Brazil	*P. mirabilis*	128	8	>128	128	64	>128	8	>128	>128	*rmtB*
2005	Chile	*P. mirabilis*	>128	4	>128	128	>128	>128	64	>128	128	Unknown

Table 3. Prevalence and distribution of 22 Enterobacteriaceae isolates with proven or suspected 16S rRNA methyltransferase genes.

	Methyltransferase gene (no. identified and country of origin[a])					
Organism (no. tested)	*armA*	*rmtA*	*rmtB*	*rmtC*	*rmtD*	unknown
E. coli (3)	1 **Poland**	-	1 **Brazil** 1 **USA**	-	-	-
E. cloacae (6)	1 **Poland** 1 **USA**		3 **Mexico**	-	1 **Chile**	-
K. pneumonia (5)	1 France 1 **USA**	-	1 **Mexico**	-	1 **Chile**	1 Argentina
K. oxytoca (1)	1 **Poland**	-	-	-	-	-
P. mirabilis (3)	-	-	2 **Brazil**	-	-	1 Chile
S. marcescens (3)	3 **Poland**	-	-	-	-	-
C. freundii (1)	-	-	-	-	1 **Brazil**	-

a. Highlighted country indicates new country/species record.

- Methyltransferase genes could not be identified in two isolates (*K. pneumoniae* [Argentina] and *P. mirabilis* [Chile]) meeting the aminoglycoside resistance screening criteria, suggesting the presence of additional variant enzymes or other resistance mechanisms.

- No methyltransferases were found in this collection of *P. aeruginosa* or *Acinetobacter* spp., although an earlier pilot study identified *armA* in an *Acinetobacter* spp. isolate (Venezuela; data not shown).

CONCLUSIONS

- Use of rigorous aminoglycoside resistance screening criteria (9 agents) identified 22 isolates with presumptive 16S rRNA methyltransferases; 20 (91%) were found positive for recognized resistance genes (*armA* [41%], *rmtB* [36%] or *rmtD* [14%].

- Among this collection, 19 of 20 (95%) represent new geographic (Europe, USA, Latin America) and/or species occurrences, further demonstrating the rapid global emergence of this resistance mechanism among Enterobacteriaceae (previously recognized in *P. aeruginosa*).

- The extraordinary level of resistance produced by methyltransferases and the association of their genes with mobile genetic elements, is a worrisome development that will require enhanced laboratory capabilities for detection, appropriate infection control practices to limit continued spread and, when detected, reliance on alternative chemotherapeutic agents.

- New aminoglycoside agents that are active against methylated ribosomes are critically needed.

SELECTED REFERENCES

1. Bogaerts P, Galimand M, Bauraing C, Deplano A, Vanhoof R, De Mendonca R, Rodriguez-Villalobos H, Struelens M, Glupczynski Y (2007). Emergence of ArmA and RmtB aminoglycoside resistance 16S rRNA methylases in Belgium. *J Antimicrob Chemother* 59: 459-464.
2. Chen L, Chen ZL, Liu JH, Zeng ZL, Ma JY, Jiang HX (2007). Emergence of *rmtB* methylase-producing *Escherichia coli* and *Enterobacter cloacae* isolates from pigs in China. *J Antimicrob Chemother* 59: 880-885.
3. Clinical and Laboratory Standards Institute. (2006). *M7-A7, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard - seventh edition.* Wayne, PA: CLSI.
4. Clinical and Laboratory Standards Institute. (2007). *M100-S17, Performance standards for antimicrobial susceptibility testing, 17th informational supplement*. Wayne, PA: CLSI.
5. Doi Y, Arakawa Y (2007). 16S ribosomal RNA methylation: Emerging resistance mechanism against aminoglycosides. *Clin Infect Dis* 45: 88-94.
6. Doi Y, de Oliveira Garcia D, Adams J, Paterson DL (2007). Coproduction of novel 16S rRNA methylase *rmtD* and metallo-ß-lactamase SPM-1 in a panresistant *Pseudomonas aeruginosa* isolate from Brazil. *Antimicrob Agents Chemother* 51: 852-856.
7. Doi Y, Ghilardi AC, Adams J, de Oliveira Garcia D, Paterson DL (2007). High prevalence of metallo-ß-lactamase and 16S rRNA methylase coproduction among imipenem-resistant *Pseudomonas aeruginosa* isolates in Brazil. *Antimicrob Agents Chemother* 51: 3388-3390.
8. Galimand M, Courvalin P, Lambert T (2003). Plasmid-mediated high-level resistance to aminoglycosides in Enterobacteriaceae due to 16S rRNA methylation. *Antimicrob Agents Chemother* 47: 2565-2571.
9. Galimand M, Sabtcheva S, Courvalin P, Lambert T (2005). Worldwide disseminated *armA* aminoglycoside resistance methylase gene is borne by composite transposon Tn1548. *Antimicrob Agents Chemother* 49: 2949-2953.
10. Gonzalez-Zorn B, Catalan A, Escudero JA, Dominguez L, Teshager T, Porrero C, Moreno MA (2005). Genetic basis for dissemination of *armA*. *J Antimicrob Chemother* 56: 583-585.
11. Liou GF, Yoshizawa S, Courvalin P, Galimand M (2006). Aminoglycoside resistance by *armA*-mediated ribosomal 16S methylation in human bacterial pathogens. *J Mol Biol* 359: 358-364.
12. Yamane K, Wachino J, Doi Y, Kurokawa H, Arakawa Y (2005). Global spread of multiple aminoglycoside resistance genes. *Emerg Infect Dis* 11: 951-953.
13. Yamane K, Wachino J, Suzuki S, Kato H, Shibayama K, Kimura K, Kumiko K, Satoshi I, Ozawa Y, Toshifumi K, Arakawa Y (2007). 16S rRNA methylase-producing, Gram-negative pathogens, Japan. *Emerg Infect Dis* 13: 642-646.
14. Yokoyama K, Doi Y, Yamane K, Kurokawa H, Shibata N, Shibayama K, Yagi T, Kato H, Arakawa Y (2003). Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*. *Lancet* 362: 1888-1893.
15. Yu YS, Zhou H, Yang Q, Chen YG, Li LJ (2007). Widespread occurrence of aminoglycoside resistance due to *armA* methylase in imipenem-resistant *Acinetobacter baumannii* isolates in China. *J Antimicrob Chemother* 60: 454-455.