ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 2008, p. 1843–1845 Vol. 52, No. 5
0066-4804/08/$08.00+0 doi:10.1128/AAC.01477-07

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

Thomas R. Fritsche,[1]* Mariana Castanheira,[1] George H. Miller,[2] Ronald N. Jones,[1,3] and Eliana S. Armstrong[2]

JMI Laboratories, North Liberty, Iowa[1]; Achaogen, Inc., South San Francisco, California[2]; and Tufts University School of Medicine, Boston, Massachusetts[3]

Received 14 November 2007/Returned for modification 21 December 2007/Accepted 25 February 2008

The alteration of ribosomal targets by recently described 16S rRNA methyltransferases confers resistance to most aminoglycosides, including arbekacin. *Enterobacteriaceae* and nonfermentative bacilli acquired through global surveillance programs were screened for the presence of these enzymes on the basis of phenotypes that were resistant to nine tested aminoglycosides. Subsequent molecular studies determined that 20 of 21 (95.2%) methyltransferase-positive isolates consisted of novel species records or geographic occurrences (North America [*armA* and *rmtB*], Latin America [*rmtD*], and Europe [*armA*]; *rmtA*, *rmtC*, and *npmA* were not detected). The global emergence of high-level aminoglycoside resistance has become a rapidly changing event requiring careful monitoring.

Aminoglycosides continue to play an important role in antimicrobial therapy against both gram-negative and gram-positive pathogens, usually in combination with β-lactam agents. Resistance to the class can be 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 (14). Since 2003, methylation of 16S rRNA has emerged as a serious threat to the class through the action of plasmid-mediated methyltransferase enzymes (6, 9–11, 17). These enzymes, which are similar to those found in actinomycetes, confer intrinsic resistance to the aminoglycoside agents they produce (6, 13, 15).

Alteration of the 16S rRNA A site by these enzymes (designated ArmA, RmtA, RmtB, RmtC, RmtD, and NpmA) confers resistance to almost all aminoglycosides, including arbekacin, by limiting the binding of these agents to ribosomal target sites following methylation of specific nucleotides. *armA* (*Enterobacteriaceae*, *Acinetobacter* spp.) and *rmtB* (*Enterobacteriaceae*) appear to be the most widespread and have been detected primarily in Asia and Europe (1, 2, 9, 12, 17, 18, 20); *armA* has also been reported from a clone of *Acinetobacter baumannii* found in the United States (Pennsylvania) (5). *rmtA* and *rmtC* have been reported only from Japan (*Pseudomonas aeruginosa* and *Proteus mirabilis*, respectively) and *rmtD* from Brazil (*P. aeruginosa*) (7, 8, 15, 18, 19). A sixth plasmid-mediated methyltransferase enzyme, NpmA, was recently obtained from a strain of *Escherichia coli* recovered from a Japanese patient (16). NpmA is unique in that it produces a broad resistance phenotype to aminoglycosides including apramycin and neomycin, due to methylation of the A1408 position at the A site of 16S rRNA; all other methyltransferases described to date from clinical isolates methylate the G1405 position. The high level of resistance produced by these methyltransferases and their association with mobile genetic elements have become 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 (6, 10, 11, 17).

While the occurrence of 16S methylation by these gene products appears to be widespread globally, structured prevalence data are urgently needed. We evaluated *Enterobacteriaceae* family members and nonfermentative bacilli acquired through global surveillance programs for the presence of methyltransferases, based upon patterns of resistance to commonly used aminoglycosides.

Enterobacteriaceae (262 isolates), *P. aeruginosa* (95 isolates) and *Acinetobacter* spp. (50 isolates) were selected from recent surveillance collections (from North America, Latin America, and Europe) on the basis of their resistance to combinations of gentamicin, tobramycin, and amikacin and were tested further for susceptibility using the Clinical Laboratory Standards Institute (CLSI) broth microdilution methods against six other aminoglycosides (apramycin, arbekacin, fortimicin, kanamycin, neomycin, and netilmicin) (3, 4). High-level resistance to all aminoglycosides in the panel, except for apramycin and neomycin, has been described as the defining phenotype characteristic for ArmA, RmtA, RmtB, RmtC, and RmtD methyltransferase resistance mechanisms; additionally, resistance to apramycin and neomycin appears to be typical for the NpmA mechanism (6, 16). Strains were selected for further study based on the MIC results from this aminoglycoside screen.

Primers for diagnostic amplification of the *armA*, *rmtA* to *rmtD*, and *npmA* genes were designed from sequences deposited in the GenBank database or were selected from the literature (Table 1) (15, 19). Primers were designed to have similar annealing temperatures for simultaneous amplification on a

* Corresponding author. Mailing address: JMI Laboratories, 345 Beaver Kreek Centre, Suite A, North Liberty, IA 52317. Phone: (319) 665-3370. Fax: (319) 655-3371. E-mail: Thomas-fritsche@jmilabs.com.
▽ Published ahead of print on 17 March 2008.

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

Primer[a]	Sequence	Reference or GenBank accession no.
armA-f	TATGGGGGTCTTACTATTCTGCCTAT	EF158296
armA-r	TCTTCCATTCCCTTCTCCTTT	
rmtA-f	CTAGCGTCCATCCTTTCCTC	19
rmtA-r	TTTGCTTCCATGCCCTTGCC	
rmtB-f	TCAACGATGCCCTCACCTC	EF158300
rmtB-r	GCAGGGCAAAGGTAAAATCC	
rmtC-f	GCCAAAGTACTCACAAGTGG	AB194779
rmtC-r	CTCAGATCTGACCCAACAAG	
rmtD-f	CTGTTTGAAGCCAGCGGAACGC	DQ914960
rmtD-r	GCGCCTCCATCCATTCGGAATAG	
npmA-f	CTCAAAGGAACAAAGACGG	AB261016
npmA-r	GAAACATGGCCAGAAACTC	

[a] f, forward; r, reverse.

single thermocycler program. Templates for PCRs were prepared by suspending a single colony in 100 μl of sterile water. Suspensions were heated to 95°C for 10 min, cooled to room temperature, and centrifuged to pellet the 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 s, annealing at 58°C (45°C for those isolates found to be PCR negative at 58°C) for 30 s, and extension at 72°C for 60 s. This was followed by an additional 10-min extension at 72°C. The entire reaction mixture was loaded onto 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 (kindly provided by Patrice Courvalin) was used as a positive control for the presence of *armA* in the collection of clinical isolates. Upon our 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.

Susceptibility profiling of 407 gram-negative isolates resistant to combinations of gentamicin, tobramycin, and amikacin in the initial screen revealed 19 strains that were subsequently found to be resistant to all 4,6-di–substituted aminoglycosides (Table 2). Two additional strains were selected on the basis of apramycin susceptibility and neomycin resistance, since *aph*(*3′*)-*I* is a widespread resistance gene that reduces the potency of neomycin (14). A final strain chosen for further study displayed high-level resistance to all aminoglycosides tested (including apramycin and neomycin) and was found to be positive for *aph*(*3′*)-*I* and *aac*(*3*)-*IV* enzymes (data not shown).

Among the 22 isolates selected for further analysis, 21 (5.2% of the initial screen total) isolates were confirmed to carry methyltransferase genes (9 isolates carried *armA*, 8 carried *rmtB*, and 4 carried *rmtD*; Table 2). *armA* was detected in isolates from Poland (1 isolate of *Enterobacter cloacae*, 1 of *Escherichia coli*, 1 of *Klebsiella oxytoca*, and 3 of *Serratia marcescens*), France (1 isolate of *K. pneumoniae*), and the United States (1 isolate each of *K. pneumoniae* and *E. cloacae*), whereas *rmtB* strains were found in the United States (1 isolate of *E. coli*), Brazil (1 isolate of *E. coli* and 2 of *P. mirabilis*), and Mexico (1 isolate of *K. pneumoniae* and 3 of *E. cloacae*; Table 3). *rmtD* was observed in single isolates from Argentina (*K. pneumoniae*), Brazil (*Citrobacter freundii*) and Chile (*K. pneumoniae* and *E. cloacae*). No isolates were found to carry *rmtA*, *rmtC*, or *npmA*.

A methyltransferase gene could not be identified in one isolate (Chile [*P. mirabilis*]) that met the aminoglycoside resistance screening criteria, suggesting the presence of an additional variant enzyme or some other resistance mechanism, and is being investigated further (16). No methyltransferases were found in this collection among the *P. aeruginosa* or *Acinetobacter* sp. isolates, although an earlier pilot study identified

TABLE 2. Aminoglycoside resistance profiles among studied *Enterobacteriaceae* isolates and their detected resistance genes

Study yr	Country	Organism	MIC value (μg/ml)									Resistance gene
			Amikacin	Apramycin	Arbekacin	Fortimicin	Gentamicin	Kanamycin	Neomycin	Netilmicin	Tobramycin	
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	United States	*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	United States	*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	*rmtD*
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	United States	*K. pneumoniae*	>128	2	>128	>128	>128	>128	≤1	>128	>128	*armA*
2006	Poland	*Serratia marcescens*	>128	8	>128	>128	>128	>128	16	>128	128	*armA*
2006	Poland	*Serratia marcescens*	>128	4	>128	>128	>128	>128	2	>128	>128	*armA*
2005	Poland	*Serratia marcescens*	>128	8	>128	>128	>128	>128	8	>128	>128	*armA*
2006	Brazil	*Citrobacter 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. Geographic distribution of 22 *Enterobacteriaceae* isolates with proven or suspected 16S rRNA methyltransferase genes

Organism (no. of isolates tested)	Country from which methyltransferase genes were isolated (no. of isolates identified)			
	armA	*rmtB*	*rmtD*	Unknown
E. coli (3)	Poland (1)	Brazil (1); United States (1)		
E. cloacae (6)	Poland (1); United States (1)	Mexico (3)	Chile (1)	
K. pneumoniae (5)	France (1); United States (1)	Mexico (1)	Chile (1); Argentina (1)	
K. oxytoca (1)	Poland (1)			
P. mirabilis (3)		Brazil (2)		Chile (1)
Serratia marcescens (3)	Poland (3)			
Citrobacter freundii (1)			Brazil (1)	

armA in an *Acinetobacter* sp. isolate (Venezuela; data not shown).

In summary, the use of rigorous aminoglycoside resistance screening criteria (nine agents) identified 22 isolates with presumptive 16S rRNA methyltransferases; 21 (95.5%) were confirmed positive for recognized resistance genes (*armA* [40.9%], *rmtB* [36.4%], or *rmtD* [18.2%]). While no isolates with *rmtA*, *rmtC*, or *npmA* were detected, these enzymes have been described periodically in isolates originating from Japan; none of the isolates screened originated from Asia. With one exception (the *armA*-positive *K. pneumoniae* isolate from France) 20 of 21 (95.2%) isolates represent new geographic (Europe, United States, Latin America) and/or species occurrences, underscoring the rapid global emergence of this resistance mechanism among *Enterobacteriaceae* (previously recognized in *P. aeruginosa* and *A. baumannii*).

The extraordinary level of aminoglycoside resistance produced by methyltransferases and the association of their genes with mobile elements are troubling developments that will require enhanced laboratory capabilities for detection, appropriate infection control practices to limit continued spread, and when detected, reliance on alternative chemotherapeutic agents. Aminoglycosides continue to play a critical role in therapeutics, often for their synergistic codrug effects when paired with agents from other classes. Adjustments of existing treatment algorithms and the development of new aminoglycoside agents that bind to methylated ribosomes are critically needed to help address this latest example of emerging antimicrobial resistance.

REFERENCES

1. **Bogaerts, P., M. Galimand, C. Bauraing, A. Deplano, R. Vanhoof, R. De Mendonca, H. Rodriguez-Villalobos, M. Struelens, and Y. Glupczynski.** 2007. Emergence of ArmA and RmtB aminoglycoside resistance 16S rRNA methylases in Belgium. J. Antimicrob. Chemother. **59:**459–464.
2. **Chen, L., Z. L. Chen, J. H. Liu, Z. L. Zeng, J. Y. Ma, and H. X. Jiang.** 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. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; 7th ed. Approved standard M7-A7. Clinical and Laboratory Standards Institute, Wayne, PA.
4. **Clinical and Laboratory Standards Institute.** 2007. Performance standards for antimicrobial susceptibility testing; 17th informational supplement. CLSI/NCCLS M100-S17. Clinical and Laboratory Standards Institute, Wayne, PA.
5. **Doi, Y., J. M. Adams, K. Yamane, and D. L. Paterson.** 2007. Identification of 16S rRNA methylase-producing *Acinetobacter baumannii* clinical strains in North America. Antimicrob. Agents Chemother. **51:**4209–4210.
6. **Doi, Y., and Y. Arakawa.** 2007. 16S ribosomal RNA methylation: emerging resistance mechanism against aminoglycosides. Clin. Infect. Dis. **45:**88–94.
7. **Doi, Y., D. de Oliveira Garcia, J. Adams, and D. L. Paterson.** 2007. Coproduction of novel 16S rRNA methylase *rmtD* and metallo-β-lactamase SPM-1 in a panresistant *Pseudomonas aeruginosa* isolate from Brazil. Antimicrob. Agents Chemother. **51:**852–856.
8. **Doi, Y., A. C. Ghilardi, J. Adams, D. de Oliveira Garcia, and D. L. Paterson.** 2007. High prevalence of metallo-β-lactamase and 16S rRNA methylase coproduction among imipenem-resistant *Pseudomonas aeruginosa* isolates in Brazil. Antimicrob. Agents Chemother. **51:**3388–3390.
9. **Galimand, M., P. Courvalin, and T. Lambert.** 2003. Plasmid-mediated high-level resistance to aminoglycosides in *Enterobacteriaceae* due to 16S rRNA methylation. Antimicrob. Agents Chemother. **47:**2565–2571.
10. **Galimand, M., S. Sabtcheva, P. Courvalin, and T. Lambert.** 2005. Worldwide disseminated *armA* aminoglycoside resistance methylase gene is borne by composite transposon Tn*1548*. Antimicrob. Agents Chemother. **49:**2949–2953.
11. **Gonzalez-Zorn, B., A. Catalan, J. A. Escudero, L. Dominguez, T. Teshager, C. Porrero, and M. A. Moreno.** 2005. Genetic basis for dissemination of *armA*. J. Antimicrob. Chemother. **56:**583–585.
12. **Lee, H., D. Yong, J. H. Yum, K. H. Roh, K. Lee, K. Yamane, Y. Arakawa, and Y. Chong.** 2006. Dissemination of 16S rRNA methylase-mediated highly amikacin-resistant isolates of *Klebsiella pneumoniae* and *Acinetobacter baumannii* in Korea. Diagn. Microbiol. Infect. Dis. **56:**305–312.
13. **Liou, G. F., S. Yoshizawa, P. Courvalin, and M. Galimand.** 2006. Aminoglycoside resistance by *armA*-mediated ribosomal 16S methylation in human bacterial pathogens. J. Mol. Biol. **359:**358–364.
14. **Vakulenko, S. B., and S. Mobashery.** 2003. Versatility of aminoglycosides and prospects for their future. Clin. Microbiol. Rev. **16:**430–450.
15. **Wachino, J., K. Yamane, K. Shibayama, H. Kurokawa, N. Shibata, S. Suzuki, Y. Doi, K. Kimura, Y. Ike, and Y. Arakawa.** 2006. Novel plasmid-mediated 16S rRNA methylase, RmtC, found in a *Proteus mirabilis* isolate demonstrating extraordinary high-level resistance against various aminoglycosides. Antimicrob. Agents Chemother. **50:**178–184.
16. **Wachino, J. I., K. Shibayama, H. Kurokawa, K. Kimura, K. Yamane, S. Suzuki, N. Shibata, Y. Ike, and Y. Arakawa.** 2007. Plasmid-mediated novel m^1A1408 methyltransferase, NpmA, for 16S rRNA found in clinically isolated *Escherichia coli* resistant to structurally diverse aminoglycosides. Antimicrob. Agents Chemother. **51:**4401–4409.
17. **Yamane, K., J. Wachino, Y. Doi, H. Kurokawa, and Y. Arakawa.** 2005. Global spread of multiple aminoglycoside resistance genes. Emerg. Infect. Dis. **11:**951–953.
18. **Yamane, K., J. Wachino, S. Suzuki, H. Kato, K. Shibayama, K. Kimura, K. Kumiko, I. Satoshi, Y. Ozawa, K. Toshifumi, and Y. Arakawa.** 2007. 16S rRNA methylase-producing, Gram-negative pathogens, Japan. Emerg. Infect. Dis. **13:**642–646.
19. **Yokoyama, K., Y. Doi, K. Yamane, H. Kurokawa, N. Shibata, K. Shibayama, T. Yagi, H. Kato, and Y. Arakawa.** 2003. Acquisition of 16S rRNA methylase gene in *Pseudomonas aeruginosa*. Lancet **362:**1888–1893.
20. **Yu, Y. S., H. Zhou, Q. Yang, Y. G. Chen, and L. J. Li.** 2007. Widespread occurrence of aminoglycoside resistance due to *armA* methylase in imipenem-resistant *Acinetobacter baumannii* isolates in China. J. Antimicrob. Chemother. **60:**454–455.