16S rRNA Methyltransferases in Clinical Gram-negative Bacilli from a Tertiary Care Hospital in the Caribbean

G Reynolds Campbell[1], N Christian[1], A Nicholson[1], B Willey[2], T Mazzulli[2]

ABSTRACT

Objective: 16S rRNA methyltransferase enzymes or RMT confer pan-resistance to the aminoglycoside class of antibiotics. Multidrug-resistant Gram-negative bacilli with these methyltransferase genes have been identified from clinical settings in Europe, Asia and the Americas. This study reports their detection and characterization in Jamaican Multidrug-resistant Gram-negative bacilli.

Methodology: Retrospective review of clinical isolates collected between April 2009 and November 2011 at the University Hospital of the West Indies (UHWI), Kingston, Jamaica, revealed 370 multidrug-resistant Gram-negative bacilli with resistance to one or more aminoglycosides. Screening for high-level amikacin resistance [HLAR] (MIC ≥ 256 mg/L) by agar gradient MIC testing using Etest strips, identified potential RMT carrying isolates. Species level identification of HLAR isolates was confirmed by Vitek MS MALDI-TOF mass spectrometry. Conventional multiplex PCR was used for detection of *rmtA, rmtB, rmtC, rmtD, npmA* and *armA* genes with positives confirmed by sequencing.

Results: Of the 370 multidrug-resistant Gram-negative bacilli, 27 (7.3%) showed HLAR (22 *Acinetobacter baumannii*, two *Acinetobacter heamolyticus*, two *Pseudomonas aeruginosa* and a *Klebsiella pneumoniae)*. 16S rRNA methyltransferase PCR revealed 18 (67%) *armA* positive *A. baumanii* spp. and one (4%) *rmtC* positive *K. pneumoniae*.

Conclusions: The identification of 16S rRNA methyltransferase genes in Jamaican multidrug-resistant Gram-negative bacilli further confirms the widespread distribution of these genes. Identification of 16S rRNA methyltransferase genes in these multidrug-resistant organisms, indicates that adjunctive therapy of β-lactam antibiotics with aminoglycosides may no longer be a treatment option for some patients in Jamaica. Active surveillance and effective infection control will be necessary to limit its spread.

Keywords: Aminoglycoside, multidrug-resistance, 16S rRNA Methyltransferases

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From: [1]Department of Microbiology, University of the West Indies, Mona, Kingston 7, Jamaica and [2]Department of Microbiology, Mount Sinai Hospital/University Health Network, Toronto, Ontario, Canada.

Correspondence: Dr Glendee Reynolds-Campbell, Department of Microbiology, The University of the West Indies, Mona, Jamaica, West Indies**.** Email: glendee.reynoldscampbell02@uwimona.edu.jm

INTRODUCTION

Gram-negative bacilli (GNB) are a major cause of serious nosocomial infections and increasingly are multidrug-resistant [MDR] (1). The aminoglycoside class of antibiotics are commonly used as adjunctive therapy against GNB, however, resistance to these agents may be achieved through chromosomal mutations that alters cell permeability reducing drug uptake or that alters drug binding sites preventing attachment. It can also occur using efflux pumps or acquisition of any one of a wide variety of aminoglycoside modifying enzymes (AME's), the latter being the most common mechanism in Gram-negative bacilli. One of the most recently discovered mechanisms, confers pan resistance to this class of antibiotics by the acquisition of one or more of the nine known 16S rRNA methyltransferase (RMT) genes affecting humans (a*rmA, rmtA rmtD, rmtF-rmtH* or *npmA* [Table 1] (2–7). Plasmids carrying RMT genes also commonly carry other MDR genes (*eg*, *ctx-M3, spm1* or *ndm1*), nullifying the effect of combination therapy (inclusive of carbapenems) and severely limiting therapeutic options (5, 8–10). The presence of RMT genes in GNB are commonly screened for by identification of high level amikacin resistance [HLAR] (MIC $\geq$ 256 mg/L), with PCR used for identification of RMT (11). Although aminoglycosides have been heavily used for > 30 years at the University Hospital of the West Indies (UHWI) in Kingston, Jamaica, the mechanisms underlying the perceived increase in aminoglycoside resistance have not been investigated. In this study, using PCR and gene sequencing we identified GNB containing RMT genes for the first time in the Caribbean region.

Table 1: Clinical RMT-containing isolates from previous studies.

16S rRNA methylase gene	**Active site**	**Bacterial Species**	**Country/Region in which reported**	**Reference**
rmtA	G1405	*Pseudomonas aeruginosa*	Japan	4
rmtB	G1405	*Klebsiella pneumoniae, Klebsiella oxytoca, Escherichia. coli, Ciytrobacter freundii*	Japan, Taiwan, South Korea, China, Belgium	4
rmtC	G1405	*Proteus mirabilis, Salmonella enterica* serovar Virchow	Japan, Australia, UK	4
rmtD	G1405	*Pseudomonas aeruginosa*	Brazil	5
rmtF	G1405	*Klebsiella pneumoniae*	France/La Reunion Island	6
rmtG	G1405	*Klebsiella pneumoniae*	France/La Reunion Island	7
rmtH	G1405	*Klebsiella pneumoniae*	Brazil (patient returning from Iraq)	8
armA	G1405	*Citrobacter freundii, Klebsiella pneumoniae, Escherichiacoli Serratia marcesens, Enterobacter cloacae, Salmonella enterica, Shigella flexneri, Acinetobacter spp.*	Various East Asian Countries, Eastern Europe, Western Europe	3
npmA	A1408	*Escherichia coli*	Japan	9

**rmtE* as only been found in cattle reared for beef in a USA facility (15).

MATERIALS AND METHODS

The Vitek 2 platform (bioMérieux, Durham NC, USA) was used for the initial identification and susceptibility testing of GNB isolated from clinical specimen received by the microbiology laboratory at the UHWI during April 2009 to November 2011. The isolates included 370 GNB with resistance to one or more aminoglycosides, which were distributed across 10 different genera (Table 2).

Table 2: Aminoglycoside resistant organisms isolated from clinical specimens.

Organism identification (Number of isolates tested)	**Number of MDR-GNB with HLAR (MIC ≥256 mg/L)**	**RMT gene detected by multiplex PCR and confirmed by sequencing (Number of isolates with RMT gene)**	**Other resistance genes previously noted in the isolates with HLAR* (Number of isolates with the genes)**
Escherichia coli (99)	0	0	-
Klebsiella pneumoniae (51)	1	*rmtC* (1)	*ndm1,shv12, tem1, ctx-M15, oxa1, cmy6* (1)
Enterobacter spp. (14)	0	0	-
Proteus mirabilis (17)	0	0	-
Proteus spp. (5)	0	0	-
Pseudomonas aeruginosa (31)	2	0	*ges, vim* (2)
Providencia spp. (16)	0	0	-
Serratia marcescens (3)	0	0	-
Citrobacter spp. (2)	0	0	-
Acinetobacter spp. (132)	24	*armA* (18)	*oxa51, oxa 24* (11)
Total tested (370)	27	19	-

*See references (11, 12)

Extended susceptibility testing in aminoglycoside-resistant isolates was performed using the Kirby Bauer disc diffusion following Clinical Laboratory Standards Institute methods and guidelines (M100-S22 Vol 32 No. 1, M02-A11, M07-A9) to test susceptibilities to amikacin, gentamicin, kanamycin, netilmicin, and tobramycin (Oxoid, Nepean, Ontario, Canada) as well as apramycin, streptomycin and neomycin.

To identify GNB with HLAR, the E-test agar gradient MIC method (bioMérieux, Durham, NC USA) was employed. MALDI-TOF mass spectrometry using the Vitek MS Plus with IVD database 2.0 (bioMerieux, Durham NC USA) was used to confirm species identities of isolates with HLAR. Primer sequences in the polymerase chain reaction (PCR) detection of specific RMT genes in isolates with amikacin MICs > 256 mg/L were as per Fritche *et al* 2008 (12). Cycling conditions consisted of 25 cycles with 15 seconds denaturation at 94 $^{\circ}$C, 30 seconds annealing at 58 $^{\circ}$C , 60 seconds extension at 72 $^{\circ}$C , followed by a final ten-minute extension also at 72 $^{\circ}$C. Genbank accession numbers and expected amplicon sizes for *armA, rmtA, rmtB, rmtC, rmtD*, and *npmA* genes are listed in Table 3. After PCR amplification, sequencing analysis (Sanger method) was then used to confirm the RMT genes found in the isolates.

This study was submitted to and approved by the University Hospital of the West Indies/University of the West Indies/ Faculty of Medical Sciences, Ethics Committee located at the UWI Mona Campus, Jamaica (ECP 55, 2011/2012). The study did not require informed consent.

Table 3: Primers used for RMT detection.

Primer	Target /Gene	Sequence (5' to 3')	GenBank accession no. (reference)	Amplicon Size (bp)
npmA-f	npmA	CTCAAAGGAACAAAGACGG	AB261016 (https://www.ncbi.nlm.nih.gov/nuccore/AB261016)	640
npmA-r		GAAACATGGCCAGAAACTC		
rmtB-f	rmtB	TCAACGATGCCCTCACCTC	EF158300 (https://www.ncbi.nlm.nih.gov/nuccore/EF158300)	420
rmtB-r		GCAGGGCAAAGGTAAAATCC		
rmtC-f	rmtC	GCCAAAGTACTCACAAGTGG	AB194779 (https://www.ncbi.nlm.nih.gov/nuccore/AB194779)	712
rmtC-r		CTCAGATCTGACCCAACAAG		
armA-f	armA	TATGGGGGTCTTACTATTCTGCCTAT	EF158296 (https://www.ncbi.nlm.nih.gov/nuccore/EF158296)	468
armA-r		TCTTCCATTCCCTTCTCCTTT		
rmtA-f	rmtA	CTAGCGTCCATCCTTTCCTC	**(16)**	635
rmtA-r		TTTGCTTCCATGCCCTTGCC		
rmtD-f	rmtD	CTGTTTGAAGCCAGCGGAACGC	DQ914960 (https://www.ncbi.nlm.nih.gov/nuccore/DQ914960)	~350
rmtD-r		GCGCCTCCATCCATTCGGAATAG		

RESULTS

The 370 GNB isolates identified by Vitek 2 (bioMérieux, Durham NC USA) with aminoglycoside resistance were distributed over a total of 10 genera including *Acinetobacter* spp, (n = 132), *Escherichia. coli*, (n = 99), *Pseudomonas aeruginosa*, (n = 31), *Enterobacter* spp, (n =14), and *Klebsiella pneumoniae*, [n = 51] (Table 3). Kirby Bauer disc diffusion susceptibility testing for the 27 isolates showed zone sizes of 6mm (*ie* resistant) for all aminoglycosides tested and all 27 had the E-test amikacin MIC values of > 256 mg/L *ie*: *Acinetobacter baumannii* complex (n = 22), *Acinetobacter haemolyticus* (n = 2), *Pseudomonas aeruginosa*, (n = 2) and *Klebsiella pneumoniae* [n = 1] (Table 2). All 27 HLAR isolates were subjected to multiplex RMT PCR amplification which identified 17 *A. baumannii* complex isolates and one *A. haemolyticus* isolate as *armA* positive, while a single *K. pneumoniae* isolate was found to be *rmtC* positive. Neither of the *P. aeruginosa* isolates tested positive for RMT genes. After PCR amplification, sequencing using the Sanger technique was used to confirm the RMT genes found in the isolates. Previous sequencing analysis of these isolates showed that 13 of the HLAR *A. baumannii* complex isolates were carrying the *blaOXA-24* and *blaOXA-51* carbapenemase genes (9–10). The single *rmtC* positive isolate of *K. pneumoniae* was also previously found to carry the *blaNDM-1, blaSHV-12, blaTEM-1, blaCTX-m-15, blaOXA-1*, and *blaCMY-6* genes. This isolate was from a urine sample collected from an Indian immigrant to Jamaica and was designated *K. pneumoniae* N11-02395 (9–10).

DISCUSSION

This data indicates that the RMT-containing MDR-GNB which have been identified in South and North America, Asia and Europe, are also now present in the Caribbean (3, 8, 11–14). Of the GNB tested in this study, the *Acinetobacter baumanii* complex were the single group with the highest amikacin resistance. Consistent with previous studies, we identified *Acinetobacter spp*. containing the *armA* gene, but we did not identify *rmtB*-containing *Acinetobacter spp*. (15). Six *Acinetobacter baumannii complex* and two *P. aeruginosa* isolates with amikacin MIC > 256 mg/L were negative by PCR for any of the RMT genes tested. At the time of the study, the *Rmt E-H* genes were unknown. It is possible that these RMT genes or others yet to be identified could explain the discrepant results of HLAR isolates that were negative for the RMT examined. These isolates may have also possessed other combined mechanisms of aminoglycoside resistance such as multiple aminoglycoside modifying enzymes and genes associated with the impermeability of these isolates to the aminoglycoside antibiotics (16–17).

The *armA* positive *Acinetobacter baumannii* complex isolates were detected in samples from a myriad of infected sites including wounds, sputum, blood and urine of patients from several different wards of the hospital which do not share staff such as the emergency room, medical, surgical, intensive care and paediatric wards and not a single identifiable source. It is however, not uncommon for these species to be found across the hospital environment, and the one *armA* positive *A. haemolyticus* strain may have had sporadic plasmid transfer of this and other resistance genes from GNB within the hospital environment (18). The combination of RMT genes with other resistance genes, such as the *blaNDM* gene in these multi-drug resistant organisms, further limits treatment options. The potential spread of the *blaNDM* positive *K. pneumoniae* that tested positive for the *rmtC* gene was thwarted by the fact that the child was held in quarantine while in hospital

due to presentation with respiratory symptoms and a previous history of *Mycobacterium tuberculosis* infection (9–10). Active surveillance in Jamaica and the Caribbean will be necessary to detect and control the spread of these genes to other clinically important pathogens as aminoglycoside resistance is often overlooked and other antimicrobial drug options are limited.

The presence of RMT in Jamaica causes major concern as the aminoglycosides are generally heavily relied upon, especially in Gram-negative sepsis (19). If these genes continue to spread in this region, reliance on older agents such as polymixin B for extremely drug-resistant organisms will likely result in its overuse, eventually causing selection pressure for bacteria resistant to possibly the only currently available drug active against these strains, as the few newer antimicrobials are usually cost prohibitive in this setting (1, 20).

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Author contributions

This study was conceived, by G Reynolds-Campbell and N Christian, who both also did the study design, data collection and analysis, wrote and approved the final version. B Willey, A Nicholson and T Mazzulli participated in study design, as well as critically revised and approved the final version. B Wiley also oversaw data interpretation. All authors declare they have no conflicts of interest.

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