

Acinetobacter baumannii Sequence Types Harboring Genes Encoding Aminoglycoside Modifying Enzymes and 16SrRNA Methylase; a Multicenter Study from Pakistan

This article was published in the following Dove Press journal:
Infection and Drug Resistance

Mohsin Khurshid^{1,2}
Muhammad Hidayat Rasool²
Usman Ali Ashfaq³
Bilal Aslam²
Muhammad Waseem²
Muhammad Akhtar Ali⁴
Ahmad Almatroudi⁵
Farhan Rasheed⁶
Muhammad Saeed²
Qinglan Guo¹
Minggui Wang¹

¹Institute of Antibiotics, Huashan Hospital, Fudan University, Shanghai 200040, People's Republic of China;

²Department of Microbiology, Government College University, Faisalabad, Pakistan; ³Department of Bioinformatics & Biotechnology, Government College University, Faisalabad, Pakistan; ⁴School of Biological Sciences, University of the Punjab, Lahore, Pakistan; ⁵Department of Medical Laboratories, College of Applied Medical Sciences, Qassim University, Buraydah, Saudi Arabia; ⁶Allama Iqbal Medical College, Jinnah Hospital Lahore, Lahore, Pakistan

Introduction: The aminoglycosides are widely used for the therapeutic management of infections caused by gram-negative bacteria, including the *Acinetobacter baumannii* strains. However, the resistance to the members of the aminoglycoside family, such as amikacin, gentamicin, and tobramycin, is increasingly being common among the clinical isolates.

Purpose: This study aimed to investigate the presence of 16SrRNA methylases and aminoglycoside modifying enzymes (AMEs) genes among aminoglycoside resistant *A. baumannii* isolates and to study the genetic diversity of the clinical population of *A. baumannii* in local hospitals.

Material and Methods: The 143 *A. baumannii* clinical strains were analyzed for antimicrobial susceptibility, genetic screening for enzymes conferring aminoglycosides resistance followed by the multilocus sequence typing.

Results: The 133/143 (93%) isolates were non-susceptible to at least one of the tested aminoglycosides, including amikacin, gentamicin, and tobramycin. The MIC distribution has shown that 87.486.7% strains were resistant to amikacin and gentamicin, respectively. The *aphA6*, *aadB*, *aacC1*, and *aphA1* were found in 74.1%, 59.4%, 16.1%, and 11.2% isolates, respectively, whereas the *armA* was found in 28% of the strains having a higher MIC value (MIC; $\geq 256\mu\text{g/mL}$). The MLST data have shown that the ST589 and ST2 were the most common STs and corresponded to 51 (35.7%) and 38 (26.6%) isolates, respectively, and few of the isolates corresponding to these STs were found to harbor the *armA* gene with a variable genotypic profile for AMEs.

Discussion: The study has reported the incidence of various enzymes conferring aminoglycoside resistance among the *A. baumannii* clones for the first time from Pakistan. The findings suggest the possibility of transmission of aminoglycoside resistance determinants through the lateral gene transfer as well as clonal dissemination.

Keywords: aminoglycosides, *armA*, MLST, gentamicin, *A. baumannii*

Introduction

The remarkable ability of *Acinetobacter baumannii* strains to develop resistance to various groups of antimicrobial agents through a mutational event or lateral acquisition of foreign genes have made this pathogen successful in healthcare settings.¹⁻⁴ The aminoglycosides along with beta-lactam drugs are used for the treatment of infections caused by Gram-negative multidrug-resistant (MDR) pathogens.⁵ The

Correspondence: Mohsin Khurshid
Department of Microbiology,
Government College University,
Faisalabad, Pakistan
Tel +923334301513
Email mohsinkhurshid@gcuf.edu.pk

combination of beta-lactams and colistin is also recommended in some studies however as far as the clinical success is concerned, the combination therapy was not found to have any clear advantage over colistin monotherapy.⁶ Despite the associated side effects, aminoglycosides particularly amikacin, tobramycin, and gentamicin are frequently prescribed in the USA for the treatment of serious infections.⁷ However, many pathogenic bacteria have developed resistance to the aminoglycosides through modification of the antimicrobial drugs, alteration of the targets, ie, ribosomes, decreased permeability, and through efflux pumps.^{8–10}

The aminoglycoside-modifying enzymes (AMEs) got substantial clinical impact because of their dissemination through transposons, plasmids, or integrons that are frequently known to harbor additional resistance determinants, including β -lactamases which helps to facilitate their dissemination. AMEs deactivate the aminoglycosides through acetylation, adenylation, and phosphorylation. Moreover, the AMEs possess a diverse resistance phenotype because of their variable spectrum of activity for particular aminoglycosides.^{7,11} The studies have reported that the phosphotransferase (APH), nucleotidyltransferase (ANT), and acetyltransferase (AAC) are mainly responsible for aminoglycoside resistance among the non-fermenters Gram-negative bacterial pathogens including the *Acinetobacter baumannii* and *Pseudomonas aeruginosa*. It is suggested that the prevalence of the genes conferring aminoglycoside resistance correlates with aminoglycoside usage and varies among different clinical settings and clinical isolates.^{12,13} The 16SrRNA methylases are increasingly being common among different gram-negative pathogens during the last decade which poses a serious threat to the clinical implications of aminoglycoside.¹⁴ The clinical isolates producing these 16SrRNA methylases were reported from various parts of the world and are associated with high-level resistance to aminoglycosides. Various 16SrRNA methylases have been identified such as *armA*, *npmA*, and *rmtA–H*.¹⁵

The study was designed to assess the occurrence of AMEs and 16SrRNA methylase among *A. baumannii* isolates obtained from multiple tertiary care centers of Pakistan for the first time. The multilocus sequence typing was performed to study the molecular epidemiology of aminoglycoside resistant *A. baumannii* clones in the local hospitals.

Materials and Methods

Bacterial Isolates

A total of 143 non-duplicate *Acinetobacter baumannii* isolates were collected from the five tertiary care medical centers of Lahore, Pakistan from January to July 2017. The isolates were cultured from clinical specimens of admitted patients including tracheal secretions (n=32), blood (n=26), sputum (n=22), urine (n=16), Pus (n=14), wound swab (n=10), CSF (n=7), Bronchial washings (n=6), endotracheal tube (n=5) catheter tip (n=3) and fluid (n=2). The clinical record of the patients from whom the specimens were recovered was reviewed retrospectively. All the isolates were originated from nosocomial infections as the strains were obtained after 48 hours of hospital admittance.

The clinical isolates were initially identified using API 20NE (bioMérieux, France) and were further verified using multiplex PCR to target the 425-bp fragment of the *recA* gene present in *Acinetobacter* spp. and the 208-bp fragment corresponding to the intergenic spacer region which is solely present in *A. baumannii* strains as described previously.^{2,4} Furthermore, the isolates were subjected to PCR to detect the intrinsic *bla*_{OXA-51-like} genes as described previously.^{2,4} The strains were stored at -80°C (40% Glycerol stocks) using brain heart infusion (BHI) broth (Oxoid, UK) and were grown on Luria-Bertani Broth (Oxoid, UK).

Antimicrobial Susceptibility Testing

The disc diffusion assays for the following antimicrobial agents were performed: amikacin (AK) 30 μg , gentamicin (CN) 10 μg , tobramycin (TOB) 10 μg , imipenem (IMP) 10 μg , meropenem (MEM) 10 μg , ceftazidime (CAZ) 30 μg , cefotaxime (CTX) 30 μg , cefepime (FEP) 30 μg , ampicillin-sulbactam (SAM) 30 μg , piperacillin-tazobactam (TZP) 110 μg , doxycycline (DO) 30 μg , ciprofloxacin (CIP) 5 μg and trimethoprim-sulfamethoxazole (SXT) 25 μg . The antimicrobial discs were obtained from Oxoid, UK. The minimal inhibitory concentrations (MICs) were determined by the agar dilution method except for colistin for which the broth micro-dilution method was used. The results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI, 2018) guidelines. To interpret tigecycline susceptibility, criteria defined by food and drug authority (FDA, USA) were used, ie, isolates with MIC $\geq 8\mu\text{g/mL}$ were considered resistant.^{2,16} *Escherichia coli* (ATCC[®] 25,922 and ATCC[®] 35,218) and *Pseudomonas aeruginosa* ATCC[®] 27,853 were used as quality control strains.

Aminoglycoside Resistant Determinants

All the *A. baumannii* isolates were examined for the presence of AMEs (*aphA1*, *aphA6*, *aacC1*, *aadA1*, and *aadB*) and 16SrRNA methylase (*armA*, *rmtA*, *rmtB*, *rmtC*, *rmtD*, *rmtE*, and *rmtF*) using specific primers. The Positive products were purified using a DNA purification kit and were sent to Shanghai Jieli Biotechnology (Shanghai, China) for sequencing. The obtained sequences were analyzed using the BLAST (NCBI) tool.

MLST

The multilocus sequence typing (MLST) was performed for all (n=143) isolates according to the Pasteur scheme as described previously.^{3,17} Briefly, the fragments of seven different housekeeping genes including the *cpn60*, *fusA*, *gltA*, *pyrG*, *recA*, *rplB*, and *rpoB* were amplified and the sequences were analyzed by the PubMLST database https://pubmlst.org/bigsub?db=pubmlst_abaumannii_seqdef.

Results

Overall 133/143 (93%) isolates were non-susceptible to at least one of the tested aminoglycosides including amikacin, gentamicin, and tobramycin (Disc diffusion method only). Among these 133 isolates, 99.2% isolates were resistant to the third and fourth generation cephalosporins including cefotaxime, ceftazidime, and cefepime as well as to the carbapenems, ie, imipenem and meropenem. The 129/133 (97%) isolates were non-susceptible to fluoroquinolones (ciprofloxacin) and 80.554.1% strains were resistant to trimethoprim-sulfamethoxazole and doxycycline, respectively. The MIC of

amikacin and gentamicin showed that 125/143 (87.4%) and 124/143 (86.7%) strains were resistant to amikacin (MIC ≥ 64 $\mu\text{g/mL}$) and gentamicin (MIC ≥ 16 $\mu\text{g/mL}$) as shown in Table 1. All the strain included in the study were found susceptible to tigecycline (MIC, ≤ 2 $\mu\text{g/mL}$) and colistin (MIC, ≤ 2 $\mu\text{g/mL}$) with MIC₉₀ as 2 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$, respectively.

The isolates that were positive for the *recA* gene and 208-bp fragment of the intergenic spacer region were also positive for *blaOXA-51* gene. All the isolates were screened for the genes encoding for aminoglycoside modifying enzyme (AMEs) and 16SrRNA methylase. Among all the investigated AMEs genes, the positive rates for *aphA6*, *aadB*, *aacC1*, and *aphA1* were 74.1%, 59.4%, 16.1%, and 11.2%, respectively, whereas the *aadA1* was not detected among the isolates. The *armA* was found in 28% of the strains (Table 2), whereas the *rmtA-E* genes were not found among any *A. baumannii* isolates.

Among the sequence types (STs), ST589 and ST2 were the most common STs and corresponded to a total of 51 (35.7%) and 38 (26.6%) isolates, respectively. The 18 (12.6%) and 8 (5.6%) isolates belong to the ST642 and ST889, respectively. Among the isolates belonging to ST 589, *armA* was found in 18 out of 51 isolates while 40 isolates belonging to ST589 were positive for *aphA6* gene.

The ST1209, New ST1, New ST2, New ST3, and New ST6 were sensitive to aminoglycosides and do not harbor any of the AMEs. The MLST types of *A. baumannii* isolates harboring the genes encoding for aminoglycoside resistance are summarized in Table 3.

Table 1 Overall Distribution of MICs of Various Antimicrobial Agents Against *A. Baumannii* (n=143) Strains

Aminoglycosides	% Resistance	MIC (mg/mL)		Number of Isolates at MIC ($\mu\text{g/mL}$) of												
		MIC ₅₀	MIC ₉₀	0.125	0.25	0.5	1	2	4	8	16	32	64	128	256	≥ 512
Amikacin	87.4	128	≥ 512	–	–	–	–	–	11	–	7	–	7	62	22	34
Gentamycin	86.7	64	≥ 512	–	–	–	9	5	5	–	13	11	44	24	7	25
Imipenem	92.3	16	32	–	–	6	5	–	–	–	71	56	5	–	–	–
Ciprofloxacin	93.7	16	64	–	–	5	4	–	1	60	16	–	53	1	3	–
Piperacillin- Tazobactam	92.3	$\geq 128/4$	$\geq 128/4$	–	–	–	1	6	–	4	–	–	–	3	129	–
Ampicillin-Sulbactam	93	$\geq 128/64$	$\geq 128/64$	–	–	–	1	6	2	1	–	3	2	15	113	–
Ceftazidime	92.3	256	256	–	–	–	–	5	1	5	–	3	–	3	126	–
Cefepime	92.3	64	256	–	–	–	5	1	–	5	–	10	65	23	34	–
Sulfamethoxazole-trimethoprim	80.5	4/76	128/2432	–	3	6	6	16	57	11	8	–	20	16	–	–
Doxycycline	54.1	16	32	–	5	11	22	14	8	6	53	11	13	–	–	–
Colistin	0.0	1	1	–	5	49	79	10	–	–	–	–	–	–	–	–
Tigecycline	0.0	1	2	–	4	18	54	67	–	–	–	–	–	–	–	–

Table 2 Combinations of Aminoglycoside Resistant Determinants and Corresponding MICs for *A. Baumannii* Strains

Genes Conferring Aminoglycoside Resistance	Aminoglycosides	Number of Isolates for Which the MIC (µg/mL) Was									Total
		1	2	4	16	32	64	128	256	≥512	
armA	Amikacin	-	-	-	-	-	-	-	6	34	40
	Gentamicin	-	-	-	-	-	8	-	7	25	40
aphA1	Amikacin	-	-	-	-	-	-	9	7	-	16
	Gentamicin	-	-	-	3	1	4	8	-	-	16
aphA6	Amikacin	-	-	-	7	-	5	51	22	21	106
	Gentamicin	-	3	5	10	10	29	22	7	20	106
aacC1	Amikacin	-	-	-	-	-	1	10	12	-	23
	Gentamicin	-	-	-	-	-	2	21	-	-	23
aadB	Amikacin	-	-	-	7	-	4	25	22	27	85
	Gentamicin	-	-	-	5	8	24	23	7	18	85
armA, aphA6	Amikacin	-	-	-	-	-	-	-	6	21	27
	Gentamicin	-	-	-	-	-	-	-	7	20	27
aphA1, aphA6	Amikacin	-	-	-	-	-	-	3	7	-	10
	Gentamicin	-	-	-	1	-	2	7	-	-	10
aphA1, aacC1	Amikacin	-	-	-	-	-	-	-	7	-	7
	Gentamicin	-	-	-	-	-	-	7	-	-	7
aphA6, aacC1	Amikacin	-	-	-	-	-	-	9	12	-	21
	Gentamicin	-	-	-	-	-	1	20	-	-	21
armA, aadB	Amikacin	-	-	-	-	-	-	-	6	27	33
	Gentamicin	-	-	-	-	-	8	-	7	18	33
aphA1, aadB	Amikacin	-	-	-	-	-	-	-	7	-	7
	Gentamicin	-	-	-	-	-	-	7	-	-	7
aphA6, aadB	Amikacin	-	-	-	7	-	2	22	22	14	67
	Gentamicin	-	-	-	4	8	13	22	7	13	67
aacC1, aadB,	Amikacin	-	-	-	-	-	1	10	12	-	23
	Gentamicin	-	-	-	-	-	2	21	-	-	23
aphA1, aphA6, aacC1,	Amikacin	-	-	-	-	-	-	-	7	-	7
	Gentamicin	-	-	-	-	-	-	7	-	-	7
armA, aphA6, aadB,	Amikacin	-	-	-	-	-	-	-	6	14	20
	Gentamicin	-	-	-	-	-	-	-	7	13	20
aphA1, aphA6, aadB,	Amikacin	-	-	-	-	-	-	-	7	-	7
	Gentamicin	-	-	-	-	-	-	7	-	-	7
aphA1, aacC1, aadB,	Amikacin	-	-	-	-	-	-	-	7	-	7
	Gentamicin	-	-	-	-	-	-	7	-	-	7
aphA6, aacC1, aadB,	Amikacin	-	-	-	-	-	-	9	12	-	21
	Gentamicin	-	-	-	-	-	1	20	-	-	21
aphA1, aphA6, aacC1, aadB,	Amikacin	-	-	-	-	-	-	-	7	-	7
	Gentamicin	-	-	-	-	-	-	7	-	-	7

Table 3 Distribution of Aminoglycoside Resistance Determinants Among Different Sequence Types (STs)

Sequence Types (STs)	Allelic Profile								Clonal Complex (CC)	No. of Isolates	Aminoglycoside Resistance Phenotypes			Hospital (No. of Isolates)	Aminoglycoside Resistance Determinants				
	cpn60	fusA	gltA	pyrG	recA	rpIB	rpoB	AK			CN	TOB	aphAI		aphA6	aacCI	aadB	armA	
ST2	2	2	2	2	2	2	2	2	38	30	37	35	A (2), B (13), C (20), D (2), E (1)	7	29	7	36	22	
ST103	7	3	2	1	7	1	4	4	4	4	4	4	B (3), C (1)	-	4	-	4	-	
ST338	8	5	5	26	13	1	2	2	6	0	0	0	C (6)	-	-	-	-	-	
ST589	1	1	2	1	9	1	1	1	51	51	51	51	A (28), B (1), C (5), D (15), E (2)	8	40	5	16	18	
ST600	2	2	2	2	2	2	1	1	4	4	4	4	A (4)	-	4	-	4	-	
ST615	41	42	13	1	9	4	14	14	5	5	0	3	D (5)	-	5	-	-	-	
ST642	1	1	1	1	9	1	1	1	18	18	17	17	A (18)	-	13	5	17	-	
ST889	5	2	4	1	30	3	4	4	8	8	8	8	B (2), C (4), D (2)	-	8	6	8	-	
ST1209	2	1	2	1	9	17	2	2	1	1	1	1	A (1)	-	-	-	-	-	
NEW ST 1	1	3	10	1	137	4	1	1	1	0	0	0	A (1)	-	-	-	-	-	
NEW ST 2	1	3	10	1	9	4	4	4	1	0	0	0	A (1)	-	-	-	-	-	
NEW ST 3	1	3	10	1	137	4	4	4	1	0	0	0	A (1)	-	-	-	-	-	
NEW ST 4	3	1	56	2	9	1	4	4	3	3	0	0	B (1), D (1), E (1)	-	3	-	-	-	
NEW ST 5	2	1	2	1	9	1	4	4	1	0	0	0	A (1)	1	-	-	-	-	
NEW ST 6	1	3	10	1	9	4	1	1	1	1	1	1	A (1)	-	-	-	-	-	
Total									143	125	124	124		16	106	23	85	40	

Discussion

The enzymes which confer resistance to aminoglycosides especially the 16SrRNA methylases possess a significant threat to public health. The treatment choices for the infections caused by the multidrug-resistant (MDR) or extensive drug resistance (XDR) bacteria have already been limited due to the increasing rate of resistance to carbapenems. In the present investigation, we have evaluated the presence of aminoglycoside resistance genes among *A. baumannii* isolates obtained from the tertiary care centers of Lahore, Pakistan for the very first time. The findings have established the fact that 93% of the isolates were non-susceptible to at least one of the three tested aminoglycosides (tobramycin, gentamicin, and amikacin).

The *A. baumannii* isolates were screened for the presence of the AME genes and 16SrRNA methylase. The AMEs, ie, *aphA6*, *aadB*, *aacC1*, and *aphA1* were found in different combinations among the aminoglycoside resistant isolates, emphasizing the foremost role of AMEs in conferring aminoglycoside resistance among *A. baumannii* strains. The *aphA6* [APH(3') VIa] was the commonest AME with an overall distribution rate of 74.1%, while its incidence was 79.7% among the isolates resistant to at least one of the tested aminoglycosides. The overall dominance of *aphA6* among the *A. baumannii* isolates has been documented in neighboring countries as well as in many other countries.^{18,19} The presence of *aphA6* in gentamicin and amikacin non-susceptible isolates in the present study is consistent with the previous studies which have shown that amikacin, gentamicin are the substrates for *aphA6* enzymes.²⁰

The aminoglycoside nucleotidyltransferase (ANT) gene, ie, *aadB* [ANT(2')-Ia] was also frequent with an overall incidence of 59.4%. The studies have widely reported the distribution of *aadB* as gene cassettes on class-I and class-II integrons, therefore, the transfer is easily mediated through the mobile genetic elements. These enzymes confer resistance to tobramycin, dibekacin, gentamicin, sisomicin, and kanamycin.²¹ The aminoglycoside acetyltransferase, ie, *aacC1* [AAC(3)-Ia] was found in 23/133 (17.3%). The studies have reported that *aacC1* confers resistance to gentamicin, tobramycin, and sisomicin although the frequency of this acetyltransferase varies among various studies.^{22,23}

The study has shown that aminoglycoside resistant strains from the hospitals corresponded to diverse sequence types. The most common STs found in the

study were the sequence type (ST) 589 belonging to clonal complex (CC) 1 and the sequence type (ST) 2 belonging to clonal complex (CC) 2 which comprised more than half of the isolates. However, the *armA* gene harboring isolates were belonging to ST589 and ST2, however, all the isolates corresponding to these two STs were not found to harbor the *armA* (16SrRNA methylase) gene. This shows heterogeneity among the STs as the isolates belonging to the same STs shown a different combination of genes. Although the studies suggest the spread of resistant bacterial strains harboring the resistant genes and plasmids through clonal dissemination, many studies proposed the transmission of the genes, as well as plasmids conferring the resistance phenotypes through the lateral gene transfer between the different strains of *A. baumannii*.²⁴⁻²⁷ The identification of same resistant determinants in the *A. baumannii* isolates with diverse STs and the isolates having the same genetic background or clones (MLST types) with the acquisition of dissimilar resistant genes in any individual clone suggests the possibility of horizontal transfer.²⁷ This transmission happens through the dissemination of mobile elements or by clonal spread possibly vary due to the diverse conditions existing in different geographical areas.

A large number of studies have shown that CC2 (Pasteur scheme)/CC92 (Oxford Scheme) is prevalent throughout Asian countries as well as the widest worldwide distribution.²⁸ The various STs belonging to CC2/CC92 (eg, ST2, and ST600) are single locus variants (SLV) to each other and have been reported from the Asian countries.^{17,29} It is suggested that *A. baumannii* clones belonging to CC2 have spread among the local hospitals and evolved, resulting in the development of different SLVs in the CC2.

The emergence of 16SrRNA methylase in addition to the multiple types of carbapenemases is evinced by the large numbers of recent studies describing the co-existence of these two mechanisms.³⁰⁻³² Before the dissemination of 16SrRNA methylase, the resistance to various aminoglycosides involves the bacterial species to acquire and gather different AMEs. Therefore, the emergence of 16SrRNA methylases is attaining a similar significance for aminoglycoside resistance that different carbapenemases have achieved for beta-lactam resistance, ie, successfully halting the therapeutic efficacy of all agents from a class of antibiotics by the acquisition of a single enzyme for example *bla*NDM.

In conclusion, the study highlighted the heterogeneity of aminoglycoside resistant *A. baumannii* mediated by the *armA* and AMEs for the first time in the Pakistani hospitals. The disparities in the frequencies of different resistance determinants among the various clones may be associated with the differential use of antibiotics in different clinical settings. The present study describes the spreading of *A. baumannii* strains belonging to CC2 (ST2 and ST600) and CC1 (ST589 and ST642) in Pakistani hospitals. The dissemination of diverse STs of *A. baumannii* in different hospitals harboring AMEs and 16SrRNA methylases, ie, *armA* genes represents the establishment of this important nosocomial pathogen in the local hospitals and highlights the necessity of taking effective control measures.

Ethics Statement

The *A. baumannii* isolates in this study were part of the routine hospital laboratory procedure; therefore, the Institutional Review Board, Government College University Faisalabad, Pakistan exempted this research for written informed consent and approved the study vide letter number GCUF/IRB/4221 dated 29-04-2016.

Funding

This work was supported by grants from the National Natural Science Foundation of China (grant # 81773785, 81603163 and 81473250) and Higher Education Commission (HEC) of Pakistan (grant # 5679/Punjab/NRPU/R&D/HEC/2016).

Disclosure

The authors report no conflicts of interest in this work.

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