Original Article

Molecular mechanisms of antibiotic co-resistance among carbapenem resistant *Acinetobacter baumannii*

Mohsin Khurshid[1], Muhammad Hidayat Rasool[1], Muhammad Hussnain Siddique[2], Farrukh Azeem[2], Muhammad Naeem[1], Muhammad Sohail[3], Muhammad Sarfraz[3], Muhammad Saqalein[1], Zeeshan Taj[1], Muhammad Atif Nisar[1], Muhammad Usman Qamar[1], Asim Shahzad[4]

[1] Department of Microbiology, Government College University, Faisalabad, Pakistan
[2] Department of Bioinformatics and Biotechnology, Government College University, Faisalabad, Pakistan
[3] Department of Microbiology and Molecular Genetics, University of the Punjab, Lahore, Pakistan
[4] Department of Pathology, Faculty of Veterinary Sciences, University of Agriculture, Faisalabad, Pakistan

Abstract
Introduction: The spread of carbapenem-resistant *Acinetobacter baumannii* (CRAB) is difficult to control especially in the hospitals due to the successful mobilization and evolution of the genetic elements harboring the resistant determinants. The study was conducted to examine the distribution of aminoglycosides, tetracycline, and sulfonamide-resistant determinants among CRAB isolates that carry the *bla*OXA-23 gene.
Methodology: For a total of 160 CRAB strains isolated at tertiary care hospitals of Pakistan that mainly carried blaOXA-23 gene were included in the study to evaluate the assortment of antibiotic resistance genes.
Results: The susceptibility rates of CRAB for other than beta-lactam drugs were 2.5% for both ciprofloxacin and aminoglycosides and 18% and 25% for sulfonamides and tetracyclines, respectively. Polymyxin B (MIC$_{90}$, 1 g/mL) Colistin (MIC$_{90}$, 1 g/mL) and Tigecycline (MIC$_{90}$, 2 g/mL) were most active against these extensively drug-resistant CRAB isolates. The isolates were found to possess various genes mainly the *tet*B and *sul*2 for tetracycline and sulfonamide but the genes conferring resistance to aminoglycosides were varied with various combinations.
Conclusion: Despite the CRAB clones containing blaOXA-23 have been previously reported in Pakistani hospitals, the screening of genetic determinants responsible for other antimicrobial agents is crucial for developing an effective surveillance and mitigation system for infection management.

Keywords: CRAB; surveillance; aminoglycosides; tetracyclines; sulfonamides.

Introduction

Acinetobacter baumannii; an important nosocomial pathogen is rapidly developing towards pan-drug resistance. The frequency of infections among patients in various healthcare settings is also increasing mainly in intensive care units (ICUs) [1- 3]. This pathogen is generally responsible for severe hospital-acquired infections which usually involve the use of carbapenems as a drug of choice for the therapeutic management of such infections. The resistance to carbapenems, however, is increasingly being reported in *A. baumannii* isolated from the clinical settings that necessitate some novel drugs or the use of alternative therapeutic choices [4-6]. The carbapenem-resistant *A. baumannii* are increasingly reported from hospitalized patients during the past few years that are associated with high rates of mortality. The class D β-lactamases are able to confer carbapenem resistance and are the most abundant mechanism among *A. baumannii* isolates [7,8]. The data regarding the occurrence of carbapenem resistance among *A. baumannii* in Pakistan is still deficient, the fewer reports have indicated the prevalence of OXA types β-lactamases especially blaOXA-23 among carbapenem-resistant isolates from various tertiary care hospitals [3,9].

The higher rate of resistance to all clinically useful aminoglycosides have been reported among *Acinetobacter* species as compared to other pathogenic bacteria [10,11]. The aminoglycoside resistance among *A. baumannii* involves the production of various types of aminoglycosides modifying enzymes (AMEs), including acetyltransferases, nucleotidyltransferases, and phosphotransferases that vary in their antibiotic substrates and no single AME is able to modify all types of aminoglycosides [12]. The ribosomal methylation is another mechanism described during the past few years

through the production of 16S rRNA methyltransferases that reduce the affinity of almost all aminoglycosides [13,14]

The resistance to the tetracyclines is mainly ascribed to the acquisition of efflux pumps belonging to major facilitator superfamily (MFS) i.e. tetA and tetB and the resistance nodulation division family (RND) such as adeABC, adeIJK, adeFGH, adeM, adeDE in *A. baumannii* isolates. These RND efflux systems in association with the *tet*A and *tet*B genes result in the higher MIC of tetracyclines [15,16]. The sulfonamide resistance is mediated by the acquisition of dihydropteroate synthase (DHPS) such as *sul*1, *sul*2, and *sul*3 among Gram-negative pathogens. These genes are usually found on insertion elements, integrons, and conjugative plasmids that facilitate their transfer [17,18].

A. baumannii isolates from five tertiary care hospitals of Pakistan collected between (2016-2017), resistant to carbapenems and 3rd generation cephalosporins were shown to fall into seven groups based on REP-PCR typing. These isolates were found to harbor ISAba1 elements upstream to *bla*OXA51 and *bla*OXA-23 genes [3]. We have increased the collection by scrutinizing more recent *A. baumannii* isolates from a tertiary care hospital of Lahore, Pakistan. Here, we have identified the mechanism of aminoglycosides, tetracyclines and sulfonamide resistance among carbapenem-resistant *A. baumannii* clinical isolate for the very first time in Pakistan.

Methodology
Bacterial Isolates and identifications
The present study comprised 134 CRAB recovered from five different tertiary care hospitals during 2016 (April) to 2017 (March) as described previously and 26 additional carbapenem-resistant isolate. These 26 isolates were recovered during May-June 2017 from a tertiary care hospital that were added to those reported in our recent study [3]. These 26 isolates were recovered from the patients admitted to the ICUs.

The inclusion criteria included the isolates obtained from the patients with an active infection including surgical site infection, wound infection or burn, bacteremia, pneumonia, meningitis, and urinary tract infection. Exclusion criteria included the *A. baumannii* isolate from the same patient. Isolates were then identified by amplification of the *rec*A gene and a fragment of the ITS region as well as the amplification of the OXA-51-like gene which is an intrinsic beta-lactamase in *A. baumannii* using specific primers as described previously. The study got prior approval from

the institutional review board of the Government College University Faisalabad, Pakistan.

Antimicrobial Susceptibility Testing
The MICs of 9 antibiotics was reported previously for the 134 isolates [3]. All the isolates were again tested for susceptibility using the disc diffusion method and the MIC of the isolates was determined additionally for tazobactam-piperacillin, ciprofloxacin, amikacin, doxycycline, and trimethoprim-sulfamethoxazole using the broth microdilution method and interpreted consistently with the breakpoints defined by CLSI guidelines [19]. Isolates showing intermediate levels of susceptibility were classified as nonsusceptible. The *Escherichia coli* ATCC strain no. 25922 and *P. aeruginosa* ATCC strain no. 27853 were used as a control for the susceptibility testing and determination of MICs.

Screening of resistant determents
For the amplification of antibiotic resistance determinists, the PCR experiments were performed using specific pair of primers for *tet*A, *tet*B, *sul*1, *sul*2, *arm*A, *rmt*A, *rmt*B, *amt*C, *rmt*D, *rmt*E, *rmt*F, *aph*A1, *aph*A6, *aac*C1, *aad*A1, *aad*B as described previously (Table 1). The amplicons were separated by electrophoresis on 1-1.2 % (w/v) agarose gels depending on the amplicon size, stained with the dye (ethidium bromide, 3 mg/L), visualized under ultraviolet (UV) light using gel documentation system. The product sizes were assessed using GeneRuler 100-bp plus DNA ladders (Thermo Fisher Scientific, Waltham, Massachusetts, USA) as size markers.

DNA sequencing and sequence analysis
The PCR products were randomly selected and purified using the PCR product purification kit (Favorgen Biotech Corp., Pingtung County, Taiwan) and sequenced for further confirmation. The sequences were compared against the GenBank database using the BLAST tool.

Results
Of the 160 *A. baumannii* isolates included in this study, (n = 57, 35.6%) were recovered from the tracheal secretions followed by blood (n = 32; 20%) and sputum (n = 28; 17.5%). Overall, 64% of the infected patients were male and 36% were female.

Table 1. Primers used for the screening of tetracycline, sulfonamide, and aminoglycoside resistant determinants.

Genes	Primers	Sequence (5' to 3')	Aimed product (bp)	Annealing temperature	References
*tet*A	*tet*A-F	GTGAAACCCAACATACCCC	888	50	[39]
	*tet*A-F	GAAGGCAAGCAGGATGTAG			
*tet*B	*tet*B-F	CCTTATCATGCCAGTCTTGC	774	50	[39]
	*tet*B-R	ACTGCCGTTTTTTCGCC			
*sul*1	*sul*1-F	CGGCGTGGGCTACCTGAACG	433	58	[40]
	*sul*1-R	GCCGATCGCGTGAAGTTCCG			
*sul*2	*sul*2-F	GCGCTCAAGGCAGATGGCATT	293	58	[40]
	*sul*2-R	GCGTTTGATACCGGCACCCGT			
*arm*A	*arm*A-F	ATTCTGCCTATCCTAATTGG	315	56	[41]
	*arm*A-R	ACCTATACTTTATCGTCGTC			
*rmt*A	*rmt*A-F	CTAGCGTCCATCCTTTCCTC	635	56	[42]
	*rmt*A-R	TTTGCTTCCATGCCCTTGCC			
*rmt*B	*rmt*B-F	ATGAACATCAACGATGCCCT	769	56	[43]
	*rmt*B-R	CCTTCTGATTGGCTTATCCA			
*rmt*C	*rmt*C-F	CGAAGAAGTAACAGCCAAAG	711	56	[41]
	*rmt*C-F	ATCCCAACATCTCTCCCACT			
*rmt*D	*rmt*D-F	CGGCACGCGATTGGGAAGC	401	51	[43]
	*rmt*D-R	CGGAAACGATGCGACGAT			
*rmt*E	*rmt*E-F	ATGAATATTGATGAAATGGTTGC	818	46	[43]
	*rmt*E-R	TGATTGATTTCCTCCGTTTTTG			
*rmt*F	*rmt*F-F	GCGATACAGAAAACCGAAGG	589	50	[43]
	*rmt*F-R	ACCAGTCGGCATAGTGCTTT			
aacC1	*aacC1-F*	ATGGGCATCATTCGCACATGTAGG	456	60	[26]
	aacC1-R	TTAGGTGGCGGTACTTGGGTC			
aadA1	*aadA1-F*	ATGAGGGAAGCGGTGATCG	254	54	[26]
	aadA1-R	TTATTTGCCGACTACCTTGGTG			
aadB	*aadB-F*	ATGGACACAACGCAGGTCGC	524	54	[26]
	aadB-R	TTAGGCCGCATATCGCGACC			
*aph*A1	*aph*A1-F	CAACGGGAAACGTCTTGCTC	455	50	[44]
	*aph*A1-R	ATTCGTGATTGCGCCTGAG			
*aph*A6	*aphA6-F*	ATGGAATTGCCCAATATTATTC	797	50	[26]
	aphA6-R	TCAATTCAATTCATCAAGTTTTA			

Table 2. Comparative in-vitro activity of antimicrobial agents against 160 carbapenem resistant *A. baumannii*.

Antimicrobial agents	MIC μg/mL			Fully susceptible isolates (%)
	Breakpoints	50%	90%	
Imipenem	≥ 8	64	128	0
Cefotaxime	≥ 64	> 128	> 128	0
Ceftriaxone	≥ 64	> 128	> 128	0
Ceftazidime	≥ 32	> 128	> 128	0
Cefepime	≥ 32	> 128	> 128	0
Piperacillin-Tazobactam	≥ 128/4	> 128/4	> 128/4	1.9
Ampicillin-Sulbactam	≥ 32/16	64/32	> 128/64	0
Ciprofloxacin	≥ 4	64	64	2.5
Amikacin	≥ 64	128	128	2.5
Doxycycline	≥ 16	16	32	25
Trimethoprim-sulfamethoxazole	≥ 4/76	4/76	64/1216	18
Polymixin B	≥ 4	0.5	1	100
Colistin	≥ 4	0.5	1	100
Tigecycline*	≥ 8	1	2	100

*Interpreted according to the Food and Drug Administration (FDA), USA guideline; Susceptible (MIC ≤ 2 μg/mL), Resistant (MIC ≥ 8 μg/mL)

Table 3. Frequency of AMEs among amikacin resistant isolates.

MIC distribution of amikacin	Isolates n (%)	Aminoglycoside-modifying enzymes				
		*aph*A1	*aph*A6	*aac*C1	*aad*A1	*aad*B
64	12 (7.7%)	-	3	6	-	1
128	144 (92.3%)	16	143	7	-	119
Overall	156 (97.5%)	16	146	13	-	120

The rate of susceptibility among carbapenem resistance isolates to ciprofloxacin and amikacin were 2.5% while doxycycline and trimethoprim-sulfamethoxazole were 25% and 18% respectively. All these carbapenem-resistant isolates were completely resistant to cephalosporins and ampicillin-tazobactam. It was alarming to know that many of the isolates were susceptible to polymyxins and tigecycline only as shown in Table 2.

All the isolates were assessed to find the resistance determinants, including efflux pumps (*tet*A and *tet*B) sulfonamide resistance genes such as *sul*1 and *sul*2, aminoglycosides modifying enzymes and 16s methylases. The overall prevalence of *aph*A1, *aph*A6, *aac*C1 and *aad*B genes was 10%, 91.3%, 8.1% and 75% respectively. Among the 156 amikacin resistant isolates, *aph*A6 and *aad*B were mainly detected from the majority (146 and 120 respectively) of isolates as shown in Table 3. The 113/120 (94.2%) of tetracycline isolates were positive *tet*B whereas the 9 (7.5%) isolates were positive for both *tet*A and *tet*B genes and *tet*A alone was not found as shown in Table 4. The distribution of MIC for amikacin have shown that MIC_{50} and MIC_{90} for amikacin was 128 µg/mL (Breakpoints; $\geq$ 64 µg/mL). The MIC_{50} and MIC_{90} for

doxycycline were 16 µg/mL and 32 µg/mL respectively. The tetracycline-resistant isolates ranged between 16 – 64 µg/mL (Breakpoints; $\geq$ 16 µg/mL) (Table 4). The MIC_{50} and MIC_{90} for trimethoprim-sulfamethoxazole were 4/76 µg/mL and 64/1216 µg/mL respectively (Breakpoints; $\geq$ 4/76 µg/mL).

The tetracycline susceptible isolates were not found to harbor any of the tetA or tetB gene. In 131 trimethoprim-sulfamethoxazole resistant, *A. baumannii* isolates, *sul*1 and *sul*2 were detected in 129/131 isolates and 14 (10.7%) isolates were found positive for *sul*1 and 95 (72.5%) for *sul*2 gene whereas 21 (16%) isolates harbored both *sul*1 and *sul*2 as shown in Table 5.

Discussion

The CRAB isolates have been first reported in Pakistan from Karachi in 2011, and then from Rawalpindi and Lahore. These isolates mainly carried the *bla*OXA-23 gene and mostly harbored an insertion sequence ISAba1 although NDM-1 was also reported from few isolates [3,9,20]. The control of infections caused by CRAB has impelled the extensive use of antimicrobial agents such as colistin and tigecycline especially when no other antimicrobial agents are effective as seen in severe infections [21]. The suitable

Table 4. Distribution of MIC of doxycycline and resistance determinants among 120 doxycycline resistant isolates.

Resistance determinants		No. of resistant isolates for which MIC was										
		0.06	0.125	0.25	0.5	1	2	4	8	16	32	64	$\geq$ 128
*tet*A and *tet*B negative	7 (5.8%)	-	-	-	-	-	-	-	-	5	2	-	-
*tet*A positive only[a]	0	-	-	-	-	-	-	-	-	-	-	-	-
*tet*B positive only	113 (94.2%)	-	-	-	-	-	-	-	-	91	14	8	-
Both *tet*A and *tet*B positive	9 (7.5%)	-	-	-	-	-	-	-	-	-	1	8	-

[a]None of the isolates were found to have *tet*A gene alone.

Table 5. The corresponding MIC of trimethoprim-sulfamethoxazole with the presence of *sul*1 and *sul*2 genes.

Susceptibility of trimethoprim-sulfamethoxazole	MIC (µg/mL)	No. of isolates	*sul*1 alone	*sul*2 alone	*sul*1 + *sul*2
Susceptible	0.06/1.14 – 2/38	29 (18%)	0	0	0
Resistant	4/76	97	5	90	-
	8/152	7	3	4	-
	16/304	2	2	-	-
	32/608	3	-	1	3
	64/1216	22	4	-	18
	128/2432	-	-	-	-
Total		**131 (82%)**	**14 (10.7%)**	**95 (72.5%)**	**21 (16%)**

MIC; Minimum Inhibitory Concentration.

treatment and appropriate infection control procedures, however, involve the availability of local drug susceptibility patterns and the data regarding the molecular epidemiology and precise antibiotic resistance determinants. Although the few studies have reported the emergence and spread of CRAB in Pakistan, data regarding the susceptibility and resistance determinants related to other antimicrobial agents are not available.

The occurrence of 16s methylases and AME genes were investigated among amikacin resistant *A. baumannii* isolates. This is the first report regarding the distribution of aminoglycoside-resistant determinants in Pakistan. It is important to note that 97% of isolates were found non-susceptible to amikacin with an MIC50 and MIC 90 of 128 µg/mL (Table 2). The studies have reported that amikacin possesses good activity against A. baumannii strains [22]. Another important finding that the isolates were negative for 16s methylases despite the fact that *arm*A is quite frequently reported in *A. baumannii* isolates [23,24]. This might be because the presence of *armA* is associated with high-level resistance to most clinically important aminoglycosides. We found that our isolates have an amikacin MIC ≤ 128 µg/mL. A study from Iran has reported that armA methylases were found in only 30.7% isolates having MIC ≥ 256 µg/mL [25].

Among the AMEs, the *aph(3')-VIa* (aphA6) alone or in combination with other AME genes including *aph(3')-Ia* (*aph*A1) and *aac(3')-Ia* (*aac*C1) was found. The *aph(3')-VIa* was positive in 94% of amikacin resistant isolates. These AME genes are also common in A. baumannii strains although the notable differences were observed in the distribution patterns among various studies [26]. Our results are somehow similar to a study from Iran that reported the prevalence of *aph(3')-VIa* and *aph(3')-Ia* (*aph*A1) in 60.46% and 27.9% of isolates respectively [27]. The *aph(3')-VIa* was initially detected in clinical strains of *A. baumannii* and afterward in other pathogens. The whole-genome analysis has revealed that *aph(3')-VIa* is usually flanked by ISs and mostly carried by the conjugative plasmids and is responsible for conferring resistance to amikacin that is considered as the most active aminoglycoside to treat the infections caused by *Acinetobacter* species in hospital settings [28]. Additionally, *aph(3')-VIa* also confer resistance to other aminoglycosides including neomycin, kanamycin, gentamicin, paromomycin, and ribostamycin [29].

The efflux pumps genes; tetA and tetB are the most common determinants that confer resistance to tetracyclines in *A. baumannii* isolates. In our study, the 120 strains were resistant to tetracyclines and out of these, *tet*A was found in 7 and *tet*B detected from 113 strains. The 9 (7.5%) strains were found positive for both *tet*A and *tet*B gene with a high MIC as shown in Table 4. Efflux pumps confer resistance to a wide range of antimicrobials and are prevalent among Gram-negative bacteria especially in non-fermenters [30]. The *tet*A is able to confer resistance mainly to tetracycline and doxycycline whereas *tet*B can extrude minocycline in addition to the tetracycline and doxycycline, therefore, reported more among the isolates that are resistant to minocycline also [15,31]. The results are similar to the majority of published studies. A study from Iran has reported the prevalence of *tet*A as 2% and *tet*B as 87% [32]. A study from China has reported the occurrence of *tet*A and *tet*B as 26.5% and 65.3% respectively [33]. A recent study from Iran has reported that *tet*A was not found and all 35 tetracycline-resistant *A. baumannii* isolates were found to possess the *tet*B gene [34]. Most of the trimethoprim-sulfamethoxazole resistant strains in this study i.e. 95 (72.5%) were found to harbor the *sul*2 gene whereas the *sul*1 gene was present in 14 (10.7%) of isolates. The *sul*1 and *sul*2 were absent in the susceptible isolates. A previous study from South Korea reported that the *sul*1 gene was more frequent than *sul*2 in 13 trimethoprim-sulfamethoxazole resistant *A. baumannii* isolates [18]. The higher MIC of *sul*1-positive isolates was observed as compared to *sul*2-positive isolates, whereas 21 (16%) isolates that harbored both *sul*1 and *sul*2 were having the highest MIC as shown in Table 3. The previous studies have reported that that the *sul*1 gene is associated with higher MICs compared to the *sul*2 gene in pathogenic bacterial species probably due to the involvement of specific mechanisms with the *sul*1 gene [35,36]. Furthermore, various studies have reported the presence of a *sul*1 gene with the class 1 integrons still some reports do not found class 1 integron in *sul*1-positive isolates [37]. The *sul*1 and *sul*2 genes were not detected in four isolates that were resistant to trimethoprim-sulfamethoxazole. The studies have reported that the low-level resistance is not usually associated with *sul* genes and can result from different other biochemical mechanisms [37,38].

Conclusion

The study revealed the presence of multiple antibiotic-resistant determinants in multidrug-resistant *A. baumannii* strains for the first time in Pakistan. Further studies are required to analyze the sequence types and explore the function of mobile genetic elements and their role in the dissemination of these

resistant genes. Although, more comprehensive approaches must be taken to explain the specific molecular mechanisms; the present study will considerably contribute to understanding the role of various acquired antibiotic-resistant determinants in multiple drug resistance phenotype of widely dispersed *A. baumannii* strains especially in tertiary care hospitals. Moreover, this study emphasizes the significance of continuous surveillance programs to monitor the emergence and correlation of these resistance determinants among the *A. baumannii* clinical strains at a national level as well as around the world.

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Corresponding author

Dr. Mohsin Khurshid
Department of Microbiology, Liaquat Block, Government College University Faisalabad, New Campus, Jhang Road, Faisalabad-38000, Pakistan
Phone: +923334301513
Fax: +92-41-9203023
Email: mohsin.mic@gmail.com

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