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ORIGINAL ARTICLE

Prevalence and Resistance Profile of Clinical Isolates of Acinetobacter Species from Karachi, Pakistan

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ABSTRACT

Acinetobacter baumannii causes a variety of infections including pneumonia, urinary tract infection, bacteremia, peritonitis etc. This organism is developing resistance to a number of antibiotics due to various intrinsic and acquired antibiotic resistance genes. The aim of the present study was to determine the prevalence of antibiotic-resistant *Acinetobacter* species from Karachi, Pakistan. A total of 111 strains of *Acinetobacter baumannii* and 8 strains of non-*baumannii Acinetobacter* were isolated from various hospitals of Karachi from September 2013 to December 2014. Identification of the isolates was based on the standard biochemical tests and detection of OXA-51 and OXA-23. Antibiotic resistance profile of the isolates was determined by Kirby-Bauer disc diffusion method and Minimum Inhibitory Concentration (MIC) was also determined by broth macro-dilution method. Among 111 *Acinetobacter baumannii* isolates, 8 were pan-drug resistant (PDR) and 103 isolates were multidrug resistant (MDR) while all non-*baumannii Acinetobacter* were MDR. The effective antibiotics against *A. baumannii* were colistin, gentamicin, trimethoprim/sulfamethoxazole and ciprofloxacin with MIC_{50} value 1, 256, 256, 256µg/ml, respectively. These findings strongly suggest the proper detection and reporting of PDR/MDR *Acinetobacter* from clinical samples and also the judicious use of broad-spectrum antibiotics is necessary to prevent the further spread of resistant strains of *Acinetobacter*.

Keywords
Acinetobacter baumannii, OXA-51, minimum inhibitory concentration, pan-drug resistant, multidrug resistant, broad-spectrum antibiotics.

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INTRODUCTION

Acinetobacter spp. are gram-negative, aerobic, emerging opportunistic pathogens with an exceptional ability to develop resistance to different groups of antibiotics and associated with a wide range of iatrogenic infections including pneumonia, meningitis, bacteremia, and urinary tract infections[1]. The taxonomy of genus *Acinetobacter* has been revised extensively and the species level identification by phenotypic characterization is difficult[2].

Among the *Acinetobacter* spp., *A. baumannii* has become one of the top seven pathogens threatening the health care settings, particularly the intensive care setting (ICUs). Because of its remarkable capability to colonize patients in the hospital environment, it causes hospital outbreaks due to cross-transmission between patients[3]. It is also increasingly exhibiting multiple antibiotic resistance and several prevalent strains are resistant to nearly all antibiotics currently in use. Excessive use of antimicrobials in the clinical environment has contributed to the emergence and spread of nosocomial infections. *Acinetobacter baumannii* presents an array of antibiotic resistance mechanisms which result in limited treatment options for clinicians. *Acinetobacter baumannii* exhibits resistance by both natural and acquired drug resistance mechanisms. Multidrug-resistant *Acinetobacter baumannii* (MDRAB) are often associated with co-infection by other pathogenic organisms which make it difficult to determine

its attributable mortality[4]. In Pakistan, *Acinetobacter baumannii* has emerged as one of the most common nosocomial pathogen[5] and there is very limited data regarding the persistence of this notorious organism in developing countries like Pakistan. Due to the higher incidence of nosocomial infections caused by MDRAB, there is a need to pay attention to the detection of this organism within the hospital environment and also in the general population. The present study was designed to determine the prevalence of drug-resistant *Acinetobacter baumannii* isolates in the clinical settings in Karachi, Pakistan.

MATERIALS AND METHODS

Bacterial Isolation and Identification

Along with 111 strains of *Acinetobacter baumannii* and 8 strains of non-*baumannii Acinetobacter* species were obtained from various hospitals and diagnostic laboratories of Karachi from September 2013 to December 2014. For this study, strains were collected and inoculated on McConkey's agar, and Gram staining was performed. The pure cultures were maintained on Trypticase soy agar (TSA), stored at 4˚C and can be available for routine testing.

The isolates were further identified on the basis of the standard biochemical tests including oxidase test, catalase test, temperature growth test (44˚C), glucose fermentation, hemolysis on blood agar, citrate utilization test and gelatin liquefaction[6]. For additional confirmation, OXA-23 and OXA-51 genes were detected by PCR[7,8] using specific primers (Table **1**).

DNA Preparation and PCR Conditions for the Detection of OXA-23 and OXA-51 Genes

The boiling method was used for the DNA preparation, by adding 200 µl of endonuclease free water in 1.5 ml Eppendorf tube (Cornell). Take 2-3 colonies of bacteria from Nutrient agar plate to make a suspension in Endonuclease free water. Heat this suspension in a water bath at 90˚C for 10 minutes. Then cool to ambient temperature[24]. The reaction mixture contains twelve and half microliter Master mix (2x) (Merck), 0.5 µl of reverse primer, 0.5 µl of forward primer (IDT, USA) and 9 µl of Endonuclease free water were mixed in PCR tubes (Cornell) two and half microliter of DNA template was added in this 22.5 µl of reaction mixture (Total volume of reaction mixture in each PCR tube was 25 µl) and subjected to thermocycler and set to perform 30 to 35 cycles.

The isolates possessing these genes were referred to as *Acinetobacter baumannii*, while only OXA-23 positive strains were categorized as non-*baumannii Acinetobacter*[8].

Antibiotic Susceptibility Testing

The antimicrobial susceptibility profile was determined using the Kirby-Bauer disk diffusion technique according to the protocol of Clinical and Laboratory Standards Institute (CLSI)[9]. Sensitivity to colistin was interpreted according to the criteria defined by Galani and co-workers[10]. A total of 12 antibiotics belonging to seven classes of antibiotics were used in this study including cefepime (30µg), ceftriaxone (30µg), ceftazidime (30µg),

Table 1. Primers and PCR conditions for OXA-51 and OXA-23 genes.

Target gene	Sequence	PCR conditions				Amplicon size (bp)	Reference
		Denaturation	Annealing	Extension	Number of cycles		
blaOXA-51	TAATGCTTTGATCGGCCTTG TGGATTGCACTTCATCTTGG	94˚C for 1 minute	50˚C for 1 minute	72˚C for 90 seconds	30	353	8
*bla*OXA-23	CTTGCTATGTGGTTGCTTCTC ATCCATTGCCCAACCAGTC	94˚C for 1 minute	50˚C for 1 minute	72˚C for 90 seconds	30	650	7

cefotaxime (30µg), trimethoprim/ sulfamethoxazole (25µg), gentamicin (10µg), amikacin (10µg), imipenem (10µg), meropenem (10µg), ciprofloxacin(5µg), piperacillin/tazobactam (100µg) and colistin (10µg) [Oxoid].

Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) of 100 *A. baumannii* and eight non-*baumannii Acinetobacter* isolates was determined by broth macro-dilution method using at least one antibiotic from each class including cefotaxime, trimethoprim/ sulfamethoxazole, gentamicin, meropenem, ciprofloxacin, piperacillin/tazobactam, and colistin following the guidelines provided by CLSI[9]. Test concentrations of antibiotics used are mentioned in Table **6**. The lowest antibiotic concentration which inhibited growth was considered as the Minimum inhibitory concentration (MIC)[2].

RESULTS

In total, 119 *Acinetobacter* strains were isolated from 160 clinical specimens. Of these, 111 strains were identified as *Acinetobacter baumannii* and eight were non-*baumannii Acinetobacter* with the prevalence rate of 69% and 5% respectively. Species identification was confirmed after the successful PCR amplification of OXA-23 and OXA-51 genes. The highest number of isolates were obtained from tracheal aspirates (62%) followed by sputum (14%), pus (9%), wounds (5%) and single isolate were recovered from urine, blood, central venous catheter (CVC) tip, endotracheal tubes (ETT) tip, bronchoalveolar lavage (BAL) and peritoneal fluid (Table **2**).

A higher frequency of *Acinetobacter* species was isolated from males (68/111). High-risk age groups ranged from neonates to teenagers whereas patients with the age above fifty also had a higher frequency (Figure **1** and **2**). After successful PCR amplification of OXA-23 and OXA-51 genes, isolate were easily identified and categorized up to the specie level.

The presence of other organisms in the study samples was also detected and *A. baumannii* was found co-existing with other pathogenic organisms in 30 samples whereas non-*baumannii Acinetobacter* spp. we're not associated with other pathogens (Table **3**).

Table 2. Frequency of *Acinetobacter baumannii* and non-*baumannii Acinetobacter* species from various clinical samples.

Specimens	No. of isolates (%)	
	Acinetobacter baumannii (n= 111)	Non-*baumannii Acinetobacter* spp. (n =8)
Tracheal aspirate	69 (62)	5 (62)
Sputum	16 (14)	1 (12)
Pus	9 (8)	0 (0)
Wounds	6 (5)	1 (12)
Urine	2 (1)	0 (0)
Blood	2 (1)	1 (12)
CVC tip	2 (1)	0 (0)
ETT tip	2 (1)	0 (0)
BAL	2 (1)	0 (0)
Peritoneal fluid	1 (0.9)	0 (0)

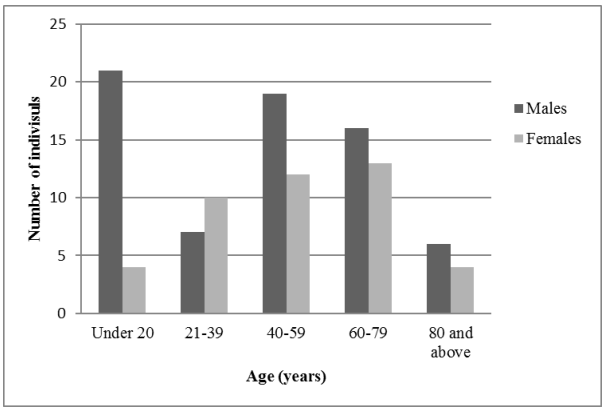

Figure 1. Distribution of age and gender of study population w.r.t. isolation of *Acinetobacter baumannii*.

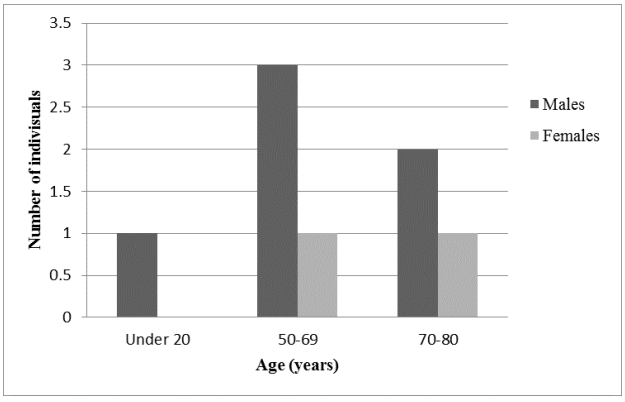

Figure 2. Distribution of age and gender of study population w.r.t isolation of non-*baumannii Acinetobacter*

Table 3. Co-infecting organisms in the hospitalized patients.

Organisms	No.	%
Methicillin sensitive *S. aureus*	2	6
Methicillin resistant *S. aureus*	5	16
Pseudomonas aeruginosa	7	23
Escherichia coli	3	10
Klebsiella pneumoniae	6	20
Streptococcus fecalis	1	3
Burkholderia cepacia	1	3
Enterobacter sp.	3	10
Enterococcus sp.	1	3
Citrobacter freundii	1	3

Table 4. Antibiotic resistance profile of *Acinetobacter baumannii* by Kirby- Bauer disc diffusion method (N=111).

Antibiotic Groups	Name of antibiotic	Symbols (potency)	S	R
B-latams/β-lactamase inhibitor combinations	Tazobactam/pipercillin	TZP (110µg)	0	111
Cephems	Ceftazidine	CAZ (30 µg)	0	111
	Cefepime	FEP (30 µg)	0	111
	Cefotaxime	CTX (30 µg)	0	111
	Ceftriaxone	CRO (30 µg)	0	111
Carbapenems	Imipenem	IMP (10 µg)	0	111
	Meropenem	MEM (10 µg)	0	111
Lipopeptides	Colistin	CT (10 µg)	109	2
Aminoglycosides	Gentamicin	CN (10 µg)	1	110
	Amikacin	AK (10 µg)	0	111
Fluoroquinolones	Ciprofloxacin	CIP (5 µg)	0	111
Folate pathway inhibitor	Trimethoprim/ Sulfamethoxazole	SXT (25 µg)	10	101

Table 5. Antibiotic resistance profile of non-*baumannii Acinetobacter* (N=8).

Antibiotic groups	Name of antibiotic	Symbols (potency)	S	I	R
B-latams/β-lactamase inhibitor combinations	Tazobactam/ pipercillin	TZP (110µg)	1	0	7
Cephems	Ceftazidine	CAZ (30 µg)	1	0	7
	Cefepime	FEP (30 µg)	1	0	7
	Cefotaxime	CTX (30 µg)	0	1	7
	Ceftriaxone	CRO (30 µg)	0	1	7
Carbapenems	Imipenem	IMP (10 µg)	2	0	6
	Meropenem	MEM (10 µg)	3	0	5
Lipopeptides	Colistin	CT (10 µg)	8	0	0
Aminoglycosides	Gentamicin	CN (10 µg)	1	0	7
	Amikacin	AK (10 µg)	1	0	7
Fluoroquinolones	Ciprofloxacin	CIP (5 µg)	2	1	5
Folate pathway inhibitor	Trimethoprim/ Sulfamethoxazole	SXT (25 µg)	1	1	6

Table 6. Minimum inhibitory concentration of *Acinetobacter baumannii by* broth macro-dilution method (N=100).

Antibiotics	Range (µg/ml)	MIC	MIC50	MIC90	MBC (µg/ml)
CTX	0.5-1024	>1024	512	>1024	-
TZP	0.5-1024	>1024	512	>1024	-
CT	0.625-16	2	0.5	1	4
CN	0.5-512	>512	256	>512	-
SXT	0.5-512	>512	256	>512	-
CIP	0.25-512	>512	256	>512	-
MEM	0.25-512	>512	512	>512	-

Table 7. Minimum inhibitory concentration of non- *baumannii Acinetobacter* spp. by broth macro-dilution method (N=8).

Antibiotics	Range (µg/ml)	MIC	MIC50	MIC90	MBC (µg/ml)
CTX	0.5-512	512	128	512	-
TZP	0.5-512-	512	128	512	-
CT	0.625-16	1	0.25	0.5	2
CN	0.5-512	>128	32	>128	-
SXT	0.5-512	64	32	64	-
CIP	0.25-512	64	32	64	-
MEM	0.25-512	64	32	64	-

All *A. baumannii* strains were resistant to 9 of the 12 antibiotics tested i.e., piperacillin/ tazobactam, ceftazidime, cefepime, cefotaxime, ceftriaxone, imipenem, meropenem, amikacin, and ciprofloxacin while 90% strains were found resistant to trimethoprim/ sulfamethoxazole and 99% to gentamicin. Importantly in this study, we observed that two strains were resistant to colistin (Table **4**). In case of non-*baumannii Acinetobacter* all strains were found to be sensitive against colistin, while 75% were found resistant to trimethoprim/ sulfamethoxazole, 72% to imipenem, 62% to meropenem and ciprofloxacin, while 87.5% to piperacillin/ tazobactam, cefepime, ceftriaxone, ceftazidime, cefotaxime, gentamicin and amikacin (Table **5**).

MIC results showed very high level of resistance among *A. baumannii* against the tested antibiotics except colistin which showed promising results (Table **6**). The MIC 50 values for CTX, TZP, CT, CN, SXT, CIP, and MEM were 512 µg/ml, 512 µg/ml, 0.5 µg/ml, 256 µg/ml, 256 µg/ml, 256 µg/ml, and 512 µg/ml respectively, and MIC 90 values were >1024 µg/ml, >1024 µg/ml, 1 µg/ml, >512 µg/ml, >512 µg/ml, >512 µg/ml, >512 µg/ml respectively. Whereas, in case of non-*baumannii Acinetobacter* MIC 50 values for CTX, TZP, CT, CN, SXT, CIP, and MEM were 128 µg/ml, 128 µg/ml, 0.25 µg/ml, 32 µg/ml, 32 µg/ml, 32 µg/ml, and 32 µg/ml respectively, while MIC 90 values were 512 µg/ml, 512 µg/ml, 0.5 µg/ml, >128 µg/ml, 64 µg/ml, 64 µg/ml, 64 µg/ml respectively (Table **7**).

DISCUSSION

Due to the increasing reports of the involvement of *A. baumannii* in human infections, it is the most extensively studied among the *Acinetobacter* species. In recent years, it has become a significant pathogen causing infections with higher morbidity and mortality rate[4]. Other than *Acinetobacter baumannii, Stenotrophomonas maltophilia, Pseudomonas aeruginosa,* and *Burkholderia cepacia* complex are the most important clinical aerobic, non-fermenting and Gram-negative rods[11]. The emergence of resistance against major classes of antibiotics has been reported globally. The definition of MDRAB and PDRAB for *Acinetobacter* varies in the literature. Generally, an isolate is considered MDRAB if it shows resistance to ≥ 3 classes of antibiotics while PDRAB describes *Acinetobacter* strains that show resistance to all standard antimicrobial agents (except colistin)[12]. Review of the literature reveals that *A. baumannii* is mostly involved in nosocomial infections, especially the immunocompromised, chronically ill or debilitated individuals or the patients with underlying medical problems such as diabetes and cancer are at higher risk[11]. The local surveillance of drug-resistant organisms in clinical samples enables us to monitor the emergence of opportunistic pathogens and their antimicrobial susceptibility patterns provide the most suitable treatment options. Molecular methods were useful to identify the genus *Acinetobacter* up to the species level. As OXA-51 gene is intrinsic to *A. baumannii*[8], plays the key role in the identification. In this study, OXA-51 was also detected for this purpose.

In this study, 7.2% *Acinetobacter baumannii* strains were multidrug resistant and 92.7% pan-drug resistant, moreover two strains were found to be colistin resistant indicating the emergence of colistin resistance, while all strains of non-*baumannii Acinetobacter* were MDR. The antibiogram of the isolates shows the high resistance profile against almost all antibiotics. In our report, *A. bauammii* were more resistant to antibiotics than non-*baumannii Acinetobacter spp.* We observed high MIC values against all tested antibiotics particularly against carbapenems which are considered to be a good choice for *Acinetobacter* infections. However, in our study high resistance against meropenem (>512µg/ml) was observed.

Based on other similar studies from Pakistan, it is evident that antibiotic resistance has been increasing among *A. baumannii* strains in our region. Saleem and co-workers reported 21 MDR and 87 PDR strains of *Acinetobacter* species[3]. A study by Kaleem *et al*. reported 27 (84.3%) Metallo-β lactamase (MBL) producing *A. baumannii*[13]. In another study by Begum *et al*., 100% resistance was observed against cephalosporins, fluoroquinolones, carbapenems, and β lactam drugs, but minocycline and tigecycline were found to be active against MDR *A. baumannii*[14]. In Pakistan (2010) Hasan and colleagues reported 87 MDR isolates, 26 XDR isolates, and 19 PDR *A. baumannii* isolates from hospitals of Islamabad and Lahore[5].

Pan-drug resistant *A. baumannii* outbreaks have also been reported from other regions of the world. Fallah *et*

al. found a high degree of resistance in *A. baumannii* isolates against various groups of antibiotics including colistin with 1.8% resistance[15]. Kou *et al*. reported 100% resistance against carbapenems, cephalosporins, fluoroquinolones, and β lactam drugs while no resistance against colistin[16]. In a study from China, Wang and co-workers (2018) found that 34 isolates were non-susceptible to both imipenem and meropenem but a single isolate was resistant to meropenem only. Resistance against other antibiotics was detected as 58.2% to ceftazidime, 52.2% to sulbactam, ciprofloxacin 64.2%, and 70.1% resistance were observed against cotrimoxazole. Whereas, no resistance was found against polymixin and rifampicin, but one isolate was non-susceptible to minocycline[23]. Büyük *et al* (2017) reported 84 MDR *Acinetobacter* strains and these isolates showed resistance against amikacin (50%), imipenem (58.33%), moxifloxacin (22.62%), ciprofloxacin (90.47%) and rifampicin (47.62%). While no resistance was found against Colistin and Tigecycline[22].

In 2013, an Iranian study reported resistance against 21 antibiotics including colistin resistance in 11% isolates, which is higher than our isolates[17]. Indian research by Badave and Dhananjay (2015) reported, >84% *A. baumannii* strains resistant against six antibiotics (ampicillin-sulbactam, piperacillin, amikacin, ciprofloxacin, ceftazidime, and imipenem)[18]. In 2016, Chinese scientists reported multidrug or extensive drug resistance in 72.4% of the isolates[19]. A study by Solomon reported a high degree of resistance among *A. baumannii* where >80% of the isolates were resistant to cefepime, sulfamethoxazole, ciprofloxacin, and ceftriaxone[20]. Our current findings are in accordance with these international reports. Colistin and tigecycline are considered as the last resort drugs against MDRAB. There are increasing reports of colistin-resistant *A. baumannii* world-wide which is a growing concern among the medical community as this could lead to treatment failure[21].

CONCLUSION

Pan-drug resistant *Acinetobacter baumannii* infections are life-threatening for neonates and elderly patients. This study shows that clinical isolates of *Acinetobacter baumannii* are highly resistant to most of the currently used antibiotics. Colistin is the last resort drug for treating MDRAB infections. Gentamicin and trimethoprim/sulphamethoxazole can be used in combination with colistin. Early detection of *Acinetobacter* spp. in hospital care settings requires adequate monitoring of the outbreaks using the modern molecular biology, strict infection control that is the most cost-effective preventive measure and also to control the indiscriminate use of broad-spectrum antibiotics without any identification of organism and susceptibility testing. However, the lack of standardized laboratory resources makes this an underreported pathogen in developing countries.

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