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 MICso 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. The taxonomy of genus *Acinetobacter* has been revised extensively and the species level identification by phenotypic characterization is difficult.

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. 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. In Pakistan, *Acinetobacter baumannii* has emerged as one of the most common nosocomial pathogen 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. For additional confirmation, OXA-23 and OXA-51 genes were detected by PCR using specific primers (Table 1).

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

| Target gene | Sequence | PCR conditions | Amplicon size (bp) | Reference |
|-------------|----------|----------------|--------------------|-----------|
| blaOXA-51   | TAATGCCTTGTACGCGCCTTG<br>TGGATGCTCCTTCATCTTGG | 94°C for 1 minute, 50°C for 1 minute, 72°C for 90 seconds<br>30 cycles | 353 | 8 |
| blaOXA-23   | CTTCATGATGGTGGTCTCTC<br>ATCCATTGCCAACCAGTC | 94°C for 1 minute, 50°C for 1 minute, 72°C for 90 seconds<br>30 cycles | 650 | 7 |

**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. 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*.

**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). Sensitivity to colistin was interpreted according to the criteria defined by Galani and coworkers. A total of 12 antibiotics belonging to seven classes of antibiotics were used in this study including cefepime (30μg), ceftriaxone (30μg), ceftazidime (30μg),...
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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 CLSI9. 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 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          | Acinetobacter baumannii (n = 111) | Non-baumannii Acinetobacter (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-latsms/β-lactamase inhibitor combinations** | Tazobactam/pipercillin | TZP (110μg)       | 0  | 111 |
| Cepheems                         | 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-\textit{baumannii} \textit{Acinetobacter} (N=8).

| Antibiotic groups                    | Name of antibiotic | Symbols (potency) | S | I | R |
|--------------------------------------|--------------------|-------------------|---|---|---|
| B-latams/\beta\text{-}lactamase inhibitor combinations | Tazobactam/\text{piperocillin} | TZP (110µg) | 1 | 0 | 7 |
| Cepheams                             | Ceftazidime        | 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/\text{Sulfamethoxazole} | SXT (25 µg) | 1 | 1 | 6 |

Table 6. Minimum inhibitory concentration of \textit{Acinetobacter baumannii} \textit{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-\textit{baumannii} \textit{Acinetobacter} \textit{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, and 512 μg/ml respectively, and MIC 90 values were $>1024 \mu g/ml$, $>1024 \mu g/ml$, 1 μg/ml, $>512 \mu g/ml$, $>512 \mu g/ml$, $>512 \mu g/ml$, 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, $>128 \mu g/ml$, 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. Other than *Acinetobacter baumannii*, *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa*, and *Burkholderia cepacia* complex are the most important clinical aerobic, non-fermenting and Gram-negative rods. 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). 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. 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*, 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. baumannii* 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 ($>512 \mu g/ml$) 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. A study by Kaleem et al. reported 27 (84.3%) Metallo-β lactamase (MBL) producing *A. baumannii*. 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*. 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.

Pan-drug resistant *A. baumannii* outbreaks have also been reported from other regions of the world.
al. found a high degree of resistance in A.baumannii isolates against various groups of antibiotics including colistin with 1.8% resistance18. Kou et al. reported 100% resistance against carbapenems, cephalosporins, fluoroquinolones, and β-lactam drugs while no resistance against colistin18. 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 minocycline23. 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 Tigecycline23.

In 2013, an Iranian study reported resistance against 21 antibiotics including colistin resistance in 11% isolates, which is higher than our isolates17. 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 isolates19. A study by Solomon reported a high degree of resistance among A. baumannii where >80% of the isolates were resistant to cephepine, sulfamethoxazole, ciprofloxacin, and ceftriaxone20. 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 failure21.

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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