Identification and Characteristics of *Acinetobacter baumannii* Isolates Co-harboring OXA-Carbapenemases and 16S rRNA Methylase Gene in a Chinese Hospital

**ABSTRACT**

Multiple drug-resistant strains of *Acinetobacter* have become common in hospitals worldwide. In this study, the occurrence and co-existence of OXA-type carbapenemases and 16S rRNA methylase genes among clinical isolates of nosocomial *Acinetobacter baumannii* in a Chinese hospital was determined. A total of 32 *A. baumannii* isolates were included in the study between January, 2012 and December, 2013. Susceptibility to antibiotics was determined by the standard microdilution method and phenotypic testing used to detect the presence of carbapenemases. PCR was performed using specific primers for detection of class D carbapenemase genes and 16S rRNA methylase gene. Nucleotide sequencing and plasmid conjugation were performed respectively. Our results indicated that, all imipinem resistant *A. baumannii* isolates were resistant to all antibiotics tested except polymycin E. 62.5% of the isolates harbored OXA-51, 37.5% OXA-23, 18.8% OXA-58 and 3.1% OXA-24 and 59. 4% harbored 16S rRNA methylase gene. Of the 19 isolates harboring 16S rRNA methylase gene, 47.4% coexisted with at least one oxacillinase gene while 40.6% of the 32 *A. baumanii* isolates harbored more than one different oxacillinase gene. OXA-genes and 16S rRNA genes resistance phenotypes were expressed in the recipient transformants. The current study shows co-existence of resistance determinant genes and existence of hidden genes responsible for multiple resistances in *A. baumanii* isolates. The coexistence poses a threat of limiting future therapeutic options.

**Key words:** *A. baumannii*, metallo-β-lactamases, nosocomial infection.

**INTRODUCTION**

*Acinetobacter baumannii* is a glucose non-fermentative Gram-negative bacillus classified as an opportunistic pathogen usually involved in infectious outbreaks originating in intensive care units (Peleg et al., 2008). The infections caused by *A. baumannii* include bloodstream infections and ventilator-associated pneumonia as well as, urinary tract infection. The extensive dissemination of carbapenene-resistant *A. baumannii* clinical strains causing episodes of bacteremia and/or sepsis mostly result in transmission through multiple contaminated surfaces, objects and hands of transiently colonized health care workers’ (Perez et al., 2007). The beta-lactam antibiotics are an important group of antibiotics used to treat infections caused by various micro-organisms, including *A. baumannii*, due to their efficacy and safety and because their activity can be increased by chemical modification (Livermore and Woodford, 2006). This was until 1970s when some isolates of *A. baumannii* were found to be resistant to a wide range of antibiotics including broad spectrum beta-lactams, aminoglycosides and
flouroquinolones (Renu et al., 2010). However, decreased susceptibility to carbapenems was reported worldwide (Uvil et al., 2011; Trecarichi et al., 2011). This fact is associated with high mortality and morbidity rates, prolonged hospital stays and increased treatment-related costs.

The emergence of carbapenem resistance in A. baumannii has become a global concern since these β-lactams are often the only effective treatment left against many multidrug-resistant strains. The major mechanism of carbapenem resistance in A. baumannii is the production of OXA-type genes (OXA-23, OXA-24, OXA-51 and OXA-58) and/or metallo-β-lactamase (VIM, IMP and NDM) (MBL) enzymes belonging to Ambler classes D carbapenemases or Class B Metallo-β-lactamase (MBL) respectively (Poirel et al., 2008). A recent development has been the discovery of a novel group of narrow-spectrum OXA β-lactamases in carbapenem-resistant strains, some of which acquired the ability to hydrolyze the carbapenems (Opazo et al., 2012). These enzymes belong to three unrelated groups of clavulanic acid-resistant β-lactamases, represented by OXA-23, OXA-24 and OXA-58, that can be either plasmid- or chromosomally-encoded. A. baumannii also possesses an intrinsic carbapenem-hydrolysing oxacillinase, the expression of which may vary and play a role in carbapenem resistance (Lee et al., 2006).

These OXA-type cabarpanemas (OTC) were described in different provinces of China. Wang et al. (2008) indicated that OXA-58-like a carbapenem-resistance determinant located on transferable plasmids and spread by clonal dissemination in most cases is an emerging threat in China. OXA-51 and OXA-23 in China were reported to be carried by almost all A. baumannii strains isolated due to their chromosomal location (Dijkshoorn et al., 1998). The 16S rRNA methylase gene is often responsible for the phenotype of Gram-negative pathogens that show high MICs to most aminoglycosides partly due to its enzymatic modification and methylation. 16S rRNA methylase gene was isolated in A. baumannii strains from Korea, Japan, China and other parts of the world with the first gene reported in a strain of Klebsiella pneumoniae in France (Wang and Chen, 2005).

The 16S rRNA methylase genes are mostly located on transposons within transferable plasmids, which provide them with the potential to horizontally spread. Some of the A. baumannii strains were found to coproduce extended-spectrum β-lactamases or metallo-β-lactamases, contributing to their multidrug-resistant phenotypes. In addition, studies revealed that A. baumannii strains harboring more than one OXA-encoding gene are emerging.

Identification of OTC carrying isolates has been a challenge due to emergency of hidden carbapenem susceptible oxacillinases which may be missing daily in laboratory practice. These carbapenem susceptible organisms with hidden oxacillinase genes can spread unnoticed in hospitals. Furthermore, these resistance determinants are located on transferable plasmids consequently easily spread.

In China, many documents described that carbapenem-resistant A. baumannii isolates harboring the blaoXA-23, blaoXA-51 in addition to blaoXA-58 determinants were disseminated widely across different cities (Wang et al., 2015).

The objective of this study was to characterize A. baumannii isolates carrying Oxa-type carbapenemase (OTC) from clinical specimens collected from patients in a Jilin hospital in order to clarify the phenotypes and genotypes. The study emphasized on determination of the occurrence and co-existence of OXA-type carbapenemases with 16S rRNA methylase gene.

MATERIALS AND METHODS

Bacteria strains

A total of 32 non-repetitive imipenem-resistant isolates of A. baumannii were recovered from sputum of hospitalized patients (n=137) in the Intensive Care Unit ward of a 2,000 bed tertiary care hospital from January, 2012 to December, 2013. All isolates were identified to the Acinetobacter calcoaceticus-A. baumannii complex using the a Vitek GN+ card system (bioMerieux) and the species of A. baumannii identification was confirmed by sequence analysis of the 16S-23S rRNA gene integenic spacer region as previously described (Wang et al., 2015). Formal ethical approval was obtained to collect the clinical samples (formal ethical approval number: Protocol Number 2012-01-1, approved by Ethical Committees of Beihua University).

Kirby Bauer disc diffusion

Antibiotic susceptibility testing was done on all isolates using commercially available discs by the Kirby Bauer disc diffusion method and interpreted as recommended by Clinical Laboratory Standards Unit (CLSI, 2010). The turbidity of the culture suspension was diluted to match 0.5 McFarland standards according to CLSI. The following antimicrobial discs were used: ampicillin (10 µg), piperacillin (160 µg), cephebthin (30 µg), tetracycline (30 µg), aztreonam (30 µg), chloramphenicol (30 µg), polymycin B (10 µg), cefotaxime (30 µg), ceftaxime (30 µg) and ceftriaxone (30 µg). Reference strains included as internal standards in all tests were Escherichia coli ATCC 25922, Staphylococcus aureus ATCC 25923 and Pseudomonas aeruginosa ATCC 27853.

Determination of MIC

In all Acinetobacter spp. isolates, susceptibility to antibiotics was determined by a standard micro-dilution
method according to the Clinical Laboratory Standards Unit (CLSI, 2010).

**Phenotypic determination of carbapenemase production**

Carbapenemase production in *Acinetobacter spp.* isolates with a MIC for meropenem of >2 mg/L was phenotypically determined by the Modified Hodge Test (MHT) for carbapenemases production (Amjad et al., 2011).

**PCR detection of OXA- genes and 16S rRNA genes**

The boiling method was used to extract DNA from the bacteria as described by Vaneechoutte et al. (1995). Briefly, one colony of a pure culture grown on TSI slanting agar was re-suspended in 50 μl of LB broth and incubated at 37°C in an orbital shaker for 14 h. 50 μl of the growth was centrifuged and resultant pellet re-suspended in 50 μl of distilled water and heated at 100°C for 10 min. After centrifugation in a micro-centrifuge, at 6000 × g for 3 min, the supernatant was kept at 0-20°C for further use.

PCR was carried out in a 25 μl reaction volumes with 1 μl of extracted DNA, 12.5 μM of the PCR master Mix containing 1.25 U of Taq polymerase, 1× PCR buffer containing 0.1 mM MgCl2 and 240 μM of each dNTP. Table 1 presents specific primers used. PCR conditions were as follows: 94°C for 5 min and then for 35 cycles, 94°C for 30 s, 57°C for 45 s, 72°C for 30 s and then final extension at 72°C for 10 min. Amplified products from the isolates were analyzed by electrophoresis on 1% (w/v) agarose gel stained with Gel Red.

Competent cells were prepared from protocol given by Joe Graber (Graber J). The DNA band of interest was excised, purified with a QIAquick PCR purification kit, ligated to pGEM-T Easy vector (Promega), transformed into *E. coli* DH5α competent cells and the white colonies successfully transformed and selected were screened.

Nucleotide sequencing was performed directly on cloned fragments using an ABI Prism 377 DNA sequencer as a control and to rule out non specificity in the case of novel gene sequences. Sequence similarity searcher was carried out with the BLAST program available at the website of the National Center of Biotechnology Information (www.ncbi.nlm.nih.gov).

**Plasmid conjugation**

Conjugal transfer of all *A. baumannii* isolates was assayed in MH agar plates using *E. coli* C600 as recipient, in order to detect a putative transfer of OXA- genes and 16S rRNA genes and resistance properties as previously described (Mingcheng et al., 2011). Transconjugant *cbms* were selected in MH agar containing rifampicin (400 μg/ml) plus sulfamethoxazole (200 μg/ml). Presumptive transconjugants were confirmed by using the API 20E test kit.

**RESULTS**

**Antimicrobial susceptibility determined by Kirby Bauer disc diffusion**

During the 2-year period, a total of 32 imipenem resistant *A. baumannii* isolates were recovered from patients with respiratory tract infections in the surgical ICU wards. Phenotypically, all *A. baumannii* strains were found to be extended-spectrum β-lactamase-producing (ESBL) and all isolates were imipenem resistant (data not shown). In imipenem-resistant *A. baumannii* isolates, phenotyping showed resistance to chloramphenicol (100%), gentamicin (100%), amikacin (100%), ciprofloxacin (100%) and levofloxacin (100%) as well as β-lactams and aztreonam (100%). However, all isolates (100%) were susceptible to polymyxin E.

**MICs**

The MICs of the 32 *A. baumannii* isolates were determined.

**Table 1: Multiplex PCR primers for detecting genes encoding oxa carbapenemase.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequences</th>
<th>Expected size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OXA-23 F</td>
<td>GATGTGTACATGTATACGTCGTT</td>
<td>501</td>
</tr>
<tr>
<td>OXA-23 R</td>
<td>TCACAAACTAAACGACTGT</td>
<td>-</td>
</tr>
<tr>
<td>OXA-51 F</td>
<td>TAATGCCGTGCTGGCTTGG</td>
<td>353</td>
</tr>
<tr>
<td>OXA-51 R</td>
<td>TCGATTGACCTTCATTTCGG</td>
<td>-</td>
</tr>
<tr>
<td>OXA-24 F</td>
<td>ATGAAAAATTATCCCTCTATACG</td>
<td>246</td>
</tr>
<tr>
<td>OXA-24 R</td>
<td>TTTAGATTCCAGATTTTCTAGC</td>
<td>-</td>
</tr>
<tr>
<td>OXA-2B F</td>
<td>AAGATATTGGGCTTGTGCTG</td>
<td>599</td>
</tr>
<tr>
<td>OXA-2B R</td>
<td>CCCCTCTGCGCTTACCAAT</td>
<td>-</td>
</tr>
<tr>
<td>16SrRNA F</td>
<td>GTCGAAAACAGTGGTGTCG</td>
<td>1450</td>
</tr>
<tr>
<td>16SrRNA R</td>
<td>GGGTTTCCGCTGGAAAT</td>
<td>-</td>
</tr>
</tbody>
</table>

*A. baumannii: Acinetobacter baumannii; OXA: Oxacillinase.*
Table 2: Distribution of 16SrRNA methylase gene among MBL and non MBL producing A. baumannii.

<table>
<thead>
<tr>
<th>Types</th>
<th>MBL Producers</th>
<th>No MBL Producers</th>
<th>Total (n=32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16S rRNA gene</td>
<td>11(61.1%)</td>
<td>7(38.9%)</td>
<td>18(56.25%)</td>
</tr>
<tr>
<td>No 16S rRNA gene</td>
<td>8(57.14%)</td>
<td>6(42.86%)</td>
<td>14(43.75%)</td>
</tr>
</tbody>
</table>

A. baumannii: Acinetobacter baumannii; MBL: Metallo beta lactamase.

All isolates (100%) were resistant to all drugs tested, that is, Cephalosporines (Cefetamet, Cefazidime, Cefotaxime, Cefuroxime and Ceftriaxone) as their MICs were >2 mg/L. These isolates were resistant to three or more classes of antibiotics. According to the definition given by Magiorakos et al (2011), these isolates were described as MDR.

Phenotypic determination of carbapenemase production in A. baumannii

Imipenem-resistance by disc diffusion method was found in all 32 isolates of Acinetobacter spp. Of the 32 imipenem-resistant Acinetobacter spp. 18 (56.3%) were carbapenemase producers when tested by MHT. Whereas, remaining 14 (43.8%) isolates did not show evidence of carbapenemase production.

16 SrRNA gene

Thirty two Imipinem resistant A. baumannii strains were tested for 16S rRNA methylase genes. Of the 32 A. baumannii isolates, 19 (59.4%) harbored 16S rRNA methylase gene of which 11 (61.1%) tested positive for carbapenemase and also 8 (57.1%) out of 14 of the non-carbapenemase producing isolates harbored 16S rRNA methylase gene (Table 2). We also found that 47.4% of the isolates harboring 16S rRNA methylase gene co-existed at least with one oxacillinase gene (Figure 1).

Carbapenem-resistant genes

Thirty two imipinem resistant A. baumannii strains were studied to determine presence of oxacillinase genes. Twenty out of 32 (62.5%) A. baumannii isolates harbored OXA-51 gene with 75% of these isolates being carbapenemase producers and the remaining 25% non carbapenemase producers. Of the 32 A. baumannii strains, 37.5% isolates harbored OXA-23 gene and 75% of them were carbapenemase producers. Six out of 32 (18.6%) A. baumannii isolates harbored OXA-58 gene and 50% were non-carbapenemase producers. Only one isolate contained OXA-24 gene.

Table 3 shows the co-existence of different blaoxa genes among the 32 isolates of A. baumannii. None of the isolates carried blaoxa-24 associated with blaoxa-23 or blaoxa-58 genes. A big percentage (72.7%) of A. baumannii isolates containing blaoxa-51-like gene coexisted with all other OXA-like genes, because this gene is intrinsic to A. baumannii isolates (Figure 2). Cloning and sequencing
confirmed that the PCR products were 100% identical to the OXA-51 genes (GenBank accession nos. AY795964 and AY949204).

Transferability of resistance properties

Conjugation experiments with E. coli 600 as the recipient confirmed that all plasmids were conjugative and transferred from the donor bacteria to E. coli 600 at a frequency of $10^{-3}$ to $10^{-5}$ per recipient. Again, OXA- genes and 16S rRNA genes resistance phenotypes were expressed in E. coli 600 transformants.

DISCUSSION

Carbapenem resistance in A. baumannii was increasingly detected in Asian countries, which is the same case with China (Zong et al., 2008; Wang and Chen, 2005). In China, national resistance surveillance data from intensive care units (ICU) at 19 teaching hospitals (1996 to 2002) showed that 5% of Acinetobacter isolates were resistant to imipenem (Wang and Chen, 2005). However, another national surveillance program involving 10 geographically disparate hospitals found that resistance to carbapenems increased from 4.5% in 2003 to 18.2% in 2004 (Wang and Chen, 2005).

Carbapenems (for example, imipenem and meropenem) and aminoglycosides are the drugs of choice in the treatment of Acinetobacter infections in almost all medical centers, but are being compromised by the emergence of carbapenem-hydrolyzing-lactamase (carbapenemase) of molecular classes D, especially, OXA-type and methylasation of 16S rRNA methylase gene respectively. Furthermore, several studies indicated that imipenem may be hydrolyzed by the extended-spectrum-lactamases of Gram-negative bacteria.

In this study, A. baumannii isolates were described as Multi-Drug Resistant (MDR) strains as the isolates were resistant to all antibiotics tested with the exception of polymyxin E. This was based on the definition of MDR as given by Magiorakos et al. (2011). These findings also agreed with Muthusamy and Boppe’s claim that strains of A. baumannii emerged as MDR (Muthusamy and Boppe, 2012). These results are in line with the findings of Dalin et al. (2015) who observed that, all the isolates of A. baumannii isolated in an affiliated hospital of Jilin, China were resistant to all antibiotics.

Using the modified Hodge test as a screening test for carbapenemase production (Amjad et al., 2011), it was observed that 19 (56.25%) of 32 A. baumannii isolates were carpanemase producing isolates. This agrees with the observation of Irfan et al. (2008) suggesting the presence of OTC enzymes since they are considered to be the mechanism of resistance in these organisms. This finding is also consistent with reports from other tertiary care hospitals around the world and even China (Zong et al., 2008; Wang and Chen, 2005). The resistance of A. baumannii to antimicrobial agents is mediated by almost all resistance mechanisms found in bacteria. Methylation of 16S rRNA methylase gene emerged as a mechanism of high-level resistance to aminoglycosides among Gram-negative bacteria. In this study, the 16S rRNA methylase genes were amplified and detected in 19 (59.38%) of the isolates and 61.1% were carbapenemase.
producers. These findings are consistent with other published reports suggesting that *A. baumannii* carries 16S rRNA methylase gene (Lee et al., 2011). As 16S rRNA methylase gene confer high-level resistance to aminoglycosides, its occurrence in *A. baumannii* limits the clinical use of aminoglycosides.

Our study revealed that 50% of the of 32 *A. baumannii* isolates studied harboring 16S rRNA methylase gene co-existed with at least one oxacillinase genes. This finding agrees with that of Tanya et al (2012) were they showed that *A. baumannii* isolates producing 16S rRNA methylase gene harbored both *bbl*OXA-23-like and *bbl*OXA-51-like genes. This is as a result of these genes being linked with mobile genetic elements and usually confirmed located on large conjugative plasmids, or integrins allowing potential spread among bacterial populations. In fact, the observation that multi-resistant phenotypes were transferred suggests that these resistance genes were present on the plasmids co-transferred. The potential role of the mobile genetic elements in dissemination of multi-resistance in *A. baumannii* isolates is well-established.

In addition, this co-existence with other genes encoding resistance to clinically relevant antimicrobials such as β-lactams (*bla*SHV, *bla*CTX-M, plasmid-mediated AmpC), carbapenems (*bla*KPC, *bla*TEM, *bla*VIM) and fluoroquinolones (plasmid-mediated *qepA, aac(6′)-Ib-cr* and *qnr* family) allows potential co-selection and maintenance of resistance by use of other antimicrobial agents. The coexistence poses a threat of limiting future therapeutic options.

β-lactamases are the most prevalent group of enzymes responsible for resistance in *A. baumannii* and more than 50 different types were identified in this strain. Several reports from around the world indicated a large increase in the rates of carbapenem-resistant *A. baumannii* from 8% in 2003 to 52 and 74% in 2005 and finally to 96% in 2007 (Stoeva et al., 2009; Qi et al., 2008) due to these group of hydrolytic enzymes.

As shown in our study, 20 (62.5%), 13 (40.63%), 6 (18.75%) and 1 (3.13%) of the 32 multidrug-resistant strains studied carried OXA-51, OXA-23, OXA-58 and OXA-24 genes respectively. Moreover, 10 (31.25%) isolates were positive for both OXA-51 and OXA-23 genes. Our data support those of other studies that demonstrated that OXA-51 may be used as a marker to identify *A. baumannii* (Zhao et al., 2015). The OXA-23 genes were documented in strains associated with outbreaks of carbapenem resistant *A. baumannii* in Asia, Europe and South America (Merkier and Centrón, 2006). Four strains were OXA-58 positive and only 1 (3.4%) strain was positive for OXA-24. Our results also indicated that the main drug-resistant genes of *A. baumannii* among the OXA-carbapenems are OXA-51 and OXA-23 which is consistent with other studies (Zhao et al., 2015; Amudhan et al., 2011). This study also reveals that 9 (28.1%) out of 32 *A. baumannii* isolates carried at least one oxacillinase gene though they were negative for carbapenemase production. This suggests the existence of hidden carbapenemase genes. These hidden genes can easily be missed in daily laboratory practice hence, posing as a diagnostic and therapeutic challenge and can easily spread unnoticed in the hospital. We also observed that 13 (40.63%) out of 32 isolates carried more than two oxacillinase genes and were MDR.

The main limitation of this study is that it was confined to a single centre and it would be valuable to extend the origin of the strains. Another limitation is the small sample size which led to lack of power in determining the individual effects of each broad spectrum antibiotics.

In conclusion, the emergence of broad-spectrum antibiotic resistance profiles in *A. baumannii* clinical isolates is perturbs the hospital. The current study shows co-existence of resistance determinant genes and existence of hidden genes responsible for resistance. Multiple mechanisms are likely to work in synergism to produce this phenotype. The co-existence and the multiple mechanisms working in synergy led us to an insight of defining *A. baumannii* strains in this study as extensive drug resistant *A. baumannii* (XDRAB), as there was still hope with the use of the highly toxic polymyxin E. Our results drew attention to enhanced surveillance and health policies for the detection and control of these MDR pathogens urgently needed to avoid the emergence and spread of such organism.

**REFERENCES**


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