Phenotypic and Genotypic Typing of Multidrug-resistant Acinetobacter Baumannii by Plasmid Profiles and Multiplex-PCR Typing

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Abstract — *Acinetobacter baumannii*, as important opportunistic pathogens, have been found to be responsible for an increasing number of Multiple-drug resistant (MDR) nosocomial infections in worldwide. In the present study, various isolates of MDR, obtained from different regions of intensive care units (ICU) from Riyadh hospitals, KSA were analyzed for antimicrobial susceptibilities, plasmid profile and carried out the molecular typing by multiplex PCR to elucidate and determine the resistance gene associated with plasmid and chromosomal DNA of *A. baumannii*. Seventy six strains of multidrug-resistant *A. baumannii* were isolated. The bacterial DNA of all isolates were subjected to the multiplex PCR to detect genes encoding oxacillinases and metallo-β-lactamases in carbapenem-resistant *Acinetobacter* spp. Multi-drug resistant isolates of *A. baumannii* obtained from different regions of intensive care units have been included in this study. These isolates showed resistance hundred percent to Ticarcillin-clavulanate, Meropenem, Aztreonam, Cefazidime, Cefoprazone, Cefazoline, Cefazidime and Carbencillin antibiotics. Some strains were also found resistant to Amikacin, Tobramycin Gentamicin with 41%, 33%, 23%. Other isolates were sensitive to some antibiotics with different percent. The plasmids from bacterial strains were found to be higher than 194 kb, harboring antibiotic resistance genes. A multiplex PCR was used to identify antibiotic resistance genes encoding the MDR phenotypes in clinical isolates of *A. baumannii*. A homogeneous pattern of multiplex PCR product revealed that MDR isolates of *A. baumannii* harbored the same resistance genes. This study will help taking effective measures for controlling disease in the Riyadh region, and the data could be used for future medical reference.

Keywords *Acinetobacter baumannii*, Carbenapem resistance, Oxa-51-like-F, plasmid, metallo-β-lactamases

Introduction:

*Acinetobacter baumannii* is a gram-negative, cocobacillus, usually of non-motilea, non-fermentative and aerobic. (1). The organisms are usually commensal. However, *Acinetobacter species* emerging as important opportunistic pathogens because they rapidly evolve toward multi-drug resistance. They are involved in various nosocomial infections such as bacteremia, urinary tract infection, are secondary meningitis, surgical site infection, and nosocomial and ventilator-associated pneumonia, especially in patients admitted to intensive care unit (2, 3). Studies on DNA-DNA relationships within the genus *Acinetobacter* have resulted in the description of 21 DNA homology groups (4). *A. baumannii* is the species most frequently isolated from patients and hospital environment (5-7). Several outbreaks of its infection or colonisation in surgical, neonatal, and burn intensive care units have been reported. The epidemiology of these infections remains unclear, because it is ubiquitous and infections may occur on either a sporadic or an epidemic basis (8-10). Extensive use of antimicrobial chemotherapy within hospitals has contributed to the emergence and increase in the number of *A. baumannii* strains resistant to a wide range of antibiotics, including broad spectrum beta-lactams, aminoglycosides, and fluoroquinolones (11-13). Due to the multiple antibiotic resistance, nosocomial infections caused by *A. baumannii*, this bacteria was found difficult to treat. These therapeutic difficulties are associated with the fact that these bacteria have a significant ability for survival in the hospital environment, thus favoring the transmission between patients, either via human reservoirs or via inanimate materials (5, 6, 11, 12). The development of molecular typing methods has given the clinical microbiology laboratory powerful tools, thus providing the means for a better knowledge of the epidemiology of bacterial infections. Many traditional and molecular typing methods such as antibiotic resistant typing, biotyping, phage typing, serotyping, cell envelope protein typing, plasmid typing, ribotyping, restriction fragment length polymorphisms and arbitrarily primed PCR (AP-PCR) typing have been employed for the epidemiological investigation of outbreaks caused by *Acinetobacter* spp. (2). Plasmid typing methods can be applied to detect polymorphism in a wide variety of organisms. These methods are very rapid and simple to generate fingerprints. This study, conduct antimicrobial susceptibility, typing profile by multiplex PCR and plasmid patterns of *Acinetobacter* strains. In addition, plasmid multiplex PCR typing profiles of the isolated *Acinetobacter* strains were compared to the antibiotic resistance profiles and the source of infection as an attempt to recognize and trace the pathogenic and genotypic background correlated with multiple-drug resistance phenomenon of *Acinetobacter* strains that associated with plasmid and chromosomal DNA. *Acinetobacter* spp. resistant to carbapenems have become common in hospitals worldwide (14). Carbapenem resistance mechanisms described in Acinetobacter spp. Include hydrolysis by b-
lactamases, alterations in outer-membrane proteins and penicillin binding proteins, and increased activity of efflux pumps (15). However, carbapenemases, such as metallo-
blactamase (MBL) or oxacillinas, cause the most concern
due to the chance of rapid dissemination (16,17,18)). Four
families of oxacillinas have been described in
Acinetobacter baumannii so far: OXA-23- like, OXA-24-like
and OXA-51-like enzymes, and OXA-51-like, a
chromosomal oxacillina enzyme present in A. baumannii
that when over expressed can be associated with
carbapenem resistance (19, 17). The aim of the present
study was therefore to conduct a multiplex PCR assay for
detecting genes encoding oxacillinas and MBLs to
evaluate the presence of carbapenemase genes among
nosocomial carbapenem-resistant Acinetobacter spp.

Materials and Methods

Bacterial isolates

A. baumannii was isolated from hospitalized patients depending of age, sex, race, underlying illness. Was
obtained by chart review based on Centers for Disease
Control guidelines (20). All A. baumannii isolates were
collected from the clinical microbiology laboratory and
stored at -70°C in brucella broth containing 20% glycerol.
A total of One hundred and eighteen A. baumannii spp.
isolates were obtained .The location of infections included
was blood, urine, wound, and respiratory tract (Table 1).
The specimens were cultured on blood agar and McConkey
agar. Isolates were identified as members of the genus
Acinetobacter by Gram negative, coccoid, oxidase negative,
non-motile, and non-fermenting bacteria. They were
identified as A. baumannii by the simplified identification
scheme including growth at 37°C, 41°C, 44°C; production of
acid from glucose under oxidative condition; gelatin
hydrolysis; and assimilation of different carbon sources
(21)clinical isolates of A. baumannii were collected and
used for this study

Antimicrobial susceptibility:

Antimicrobial susceptibility testing using Twelve
antibiotics were determined by the agar disk diffusion
method (Kirby-Bauer) (22, 23). The following antimicrobial agents (Manta Medicinal Chemicals and
Botanical Products USA) at the indicated concentrations
were tested: Ticarcillin-
clavulanate(Tc45/5µg),Meropenem(Mr10µg),
Aztreonam(Ao30 µg) ceftazidime (Ca 30 µg), cefoperazone
(Cs 65 µg), ceftizoxime (Ck 35 µg), carbenicillin (Cb 15 µg)
amikacin (Ak 30 µg), tobramycin (Tb 10 µg),Gentamicin(Gm30 µg) Polymixin B(Pb 120 µg),Colistin (C 100 µg ).(Table 2).

DNA extraction

Bacterial strains were cultured aerobically in LB broth at
37° C. Two ml of overnight culture was centrifuged at 4,000
rpm for 20 min. The pellet was resuspended in 620 ml of
lysis buffer (10 mM Tris-HCl, 50 mM EDTA, 100 mM NaCl, pH 8) containing 1% SDS and 0.4 mg/ml of proteinase K.
The mixture was incubated for 1 h at 56° C and then at 100° C for 1 h. Then, an equal volume of phenol/chloroform/isooamylalcohol was added to mixture and
centrifuged at 10,000 rpm for 10 min. The supernatant was
added to an equal volume of chloroform and after
centrifuging at 10000 rpm , the top layer was collected and
DNA was precipitated with two volumes of cold
isopropanol at -20° C for 10 min. The pellet was obtained
by centrifugation for 20 min and washed with 1.5 ml of
70% cold ethanol. Finally the pellet was resuspended in
100 ml of TE 1X buffer (10 mM Tris-Cl, 1 mM Na2 EDTA, pH 8)

Extraction of plasmid:

A colony of A. baumannii was inoculated in 5 ml of LB broth
and incubated overnight at 37°C in shaking water bath. The
cells were centrifuged at 5000 rpm at room temperature
for 10 minutes. Plasmids were then extracted by the
alkaline lysis method (24 ) Plasmid DNA was extracted
using a commercial plasmid extraction kit (Plasmid Mdi
Kit, Qiagen).

Plasmid analysis and endonuclease mapping:

Approximately 7 µl of the plasmid DNA was loaded to 1% agarose gel in TAE buffer system with Escherichia coli NCTC 50193 (CECT 678R) standard plasmid DNA as a molecular weight marker. The electrophorised gel was stained with ethidium bromide (1.5 µg/ml) and analyzed on a Bio-Rad Geldoc-2000 gel-documentation system equipped with a
Quantity 01 Software (Bio-Rad Laboratories, Richmond, California). About 1 µg of plasmid DNA was used for
digestions with restriction enzymes EcoRI, and HindIII endonucleases (invitrogen) following the indications of the manufacturer.

Multiplex PCR reaction conditions

Both multiplex PCRs were performed at the same time with
seven pairs of specific primers, one pair for each of the
seven gene families (Table 3). The DNA template was
obtained at the concentration of 20 ngµl⁻¹ using a genomic
Prep cell and tissue DNA isolation kit (Amersham Pharmacia Biotech). The PCR mixture used was as follows: 1 ml DNA template in a 49 ml mixture containing 10 mM
Tris/HCl (pH 8.8), 4 mM MgCl2, 50 mM KCl, 0.1% Triton
X-100, 200 mM each dNTP, 30 n M oxacillinase primers,
200 nM IMP primers, 100 nM VIM primers, 50 mM SIM
primers and 1 U Taq DNA polymerase. The PCR conditions
were as follows: initial denaturation at 94°C for 5 min, 33
cycles of 94°C for 25 s, 53°C for 40 s and 72° C for 50 s,
followed by a single, final, elongation step at 72°C for 6 min.

Results:

Antimicrobial susceptibility:

A total of 118 Acinetobacter spp. isolates including 76 A. baumannii and 42 non-A. baumannii strains. Table 1 shows
distribution of the isolates in terms of the sample sources. 
The strains were frequently isolated from urine samples (55%), 21, 15,8, 8 from Respiratory tract, blood and wound respectively. Analysis of twelve antibiotic were tested. Resistance patterns showed that All Acinetobacter spp.
were multi-drug resistant (100%). As shown in Table 2, all
_A. baumannii_ isolates were resistant to at least seven antibiotic. _A. baumannii_ isolates that were resistant to Ticarcillin-clavulanate, Meropenem Aztreonam, Ceftazidime, Cefoprazone, Ceftizoxime and Carbenicillin antibiotics but susceptible to Colistin (42%) , Carbenicillin (21%), Polymixin B (13%), Tobramycin (9%), Amikacin (6%), and Gentamicin (2%) that compared to Non- _A. baumannii_ isolates.

**Plasmid analysis:**

Three different groups of _A. baumannii_ isolates A were identified on the basis of antibiotic sensitivity pattern (Table 1). Representatives from each group were selected for plasmid analysis; and detection of restriction analysis profile. The analysis of plasmid in MDR isolates revealed the presence of plasmid with an approximate molecular weight over 194 kbp. almost for all the isolates. (Fig. 1).

**Molecular analysis of antimicrobial resistance gene in MDR _A. baumannii_ isolates using multiplex PCR:**

Detection of genes encoding oxacillinases and MBLs in carbenapen-resistant Acinetobacter spp. by multiplex PCR. All clinical isolates showed positively for blaOXA-51-like genes. 38% of these isolates had blaOXA-23-like genes and four (9%) had the gene blaIMP (Fig. 3). The imipenem MIC among blaOXA-23-like positive strains ranged from 16 to 128 µg mL⁻¹ and was lower compared with IMP-1 positive strains. The present study showed that all strains had blaOXA-51-like genes and 38% had blaOXA-23-like genes.

**Discussion:**

_Acinetobacter baumannii_ is an opportunistic pathogen that may be an important threat due to its increasing multidrug resistance in nosocomial isolates, mostly from intensive care units (25). _A. baumannii_ naturally produces a chromosomally encoded AmpC-type enzyme which is responsible for resistance to amino- and ureidopenicillins and narrow-spectrum cephalosporins and cephamycins and that may be overproduced (26, 27, 28). The oxacillin-hydrolyzing _β_-lactamaes (oxacillinases) belong to class D of the _β_-lactamaes (29). They usually hydrolyze oxacillin, methicillin, and cloxacillin better than benzylpenicillin, and their activity is inhibited by NaCl (29). Where as most of the oxacillinases are plasmid mediated, several natural and chromosomally encoded oxacillinases have been reported in several environmental species (27, 30, 31) and also in clinically relevant gram-negative species, such as _A. baumannii_(32). _Acinetobacter_ species are noted for their antibiotic sensitivity and for their ability to acquire genes encoding resistance determinants. Foremost among the mechanisms of resistance in this pathogens is the production of _β_-lactamaes and aminoglycoside-modifying enzymes. Additionally, diminished expression of outer membrane proteins, mutations in topoisomerases, and up-regulation of efflux pumps play an important part in antibiotic resistance. Unfortunately, the accumulation of multiple mechanisms of resistance leads to the development of multiply resistant or even “panresistant” strains. (33) Identical size plasmids were observed in isolates obtained, indicating that they were stably inherited in the population. The predominant location of the blaOXA-40 gene was large kb plasmid (34). Carbapenems are very useful for the treatment of infections caused by Gram – negative bacilli that are resistant to other _β_-lactam antibiotics.

However, Carbapenems – resistant Gram - negative bacilli are being isolated sources. _Acinetobacter_ producing IMP-type metallo- _β_-lactamaes and its other such as VIM-1 and VIM-2 that detected in _Acinetobacter pp(35)._ Multiple drug-resistant strains of _Acinetobacter_ have created therapeutic problem worldwide. This study was conducted to determine the antimicrobial susceptibility patterns and prevalence of blaOXA-type Carbapenemases among isolates of _Acinetobacter_. Here 55% of _Acinetobacter_ isolates were identified with their susceptibility to multiplex PCR targeting blaOXA genes. More than 50% of the isolates showed multidrug resistance to different antibiotics. The rate of susceptibility to imipenem Ticarcillin-clavulanate, Meropenem Aztreonam, Ceftazidime, Cefoprazone, Ceftizoxime and Carbenicillin antibiotics but susceptible to Colistin (42%) , Carbenicillin (21%), Polymixin B (13%), Tobramycin (9%), Amikacin (6%), and Gentamicin (2%) that compared to _Non- A. baumannii_ isolates. All isolates of _Acinetobacter baumannii_ possess blaOXA-51-like genes. The Co-existence of blaOXA-51-like/blaOXA-23-like and blaOXA-51-like/blaOXA-24-like was detected in the isolates. Resistance to Carbapenems is high, with the majority of isolates showing multi resistance. A wide diversity of oxa genes exist among the isolates of antibiotics. Detection of blaOXA-51-like can be used as simple and reliable methods to differentiate _Acinetobacter baumannii_.

**Table1. Distribution of the isolates by sample site.**

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Acinetobacter spp</th>
<th>Number (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><em>A. baumannii</em> (%)</td>
<td>_Non- <em>A. baumannii</em> Number (%)</td>
</tr>
<tr>
<td>Source</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>16 (21)</td>
<td>6 (14.3)</td>
</tr>
<tr>
<td>urine</td>
<td>42 (55)</td>
<td>17 (40.5)</td>
</tr>
<tr>
<td>Wound</td>
<td>6 (8)</td>
<td>10 (23.8)</td>
</tr>
<tr>
<td>Respiratory tract</td>
<td>12 (15.8)</td>
<td>9 (21.4)</td>
</tr>
<tr>
<td>Total</td>
<td>76</td>
<td>42</td>
</tr>
</tbody>
</table>
### Table 2. Antimicrobial susceptibility of 118 *Acinetobacter spp.* strains.  
*(Zone of inhibitions for different antibiotics by Kirby Bauer Disk diffusion)*

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>A. baumannii isolates</th>
<th>Non- A. baumannii isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R Number %</td>
<td>I Number %</td>
</tr>
<tr>
<td>Ticarcillin-clavulanate</td>
<td>76(100)</td>
<td>0</td>
</tr>
<tr>
<td>Meropenem</td>
<td>76(100)</td>
<td>0</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>76(100)</td>
<td>0</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>76(100)</td>
<td>0</td>
</tr>
<tr>
<td>Cefoprazone</td>
<td>76(100)</td>
<td>0</td>
</tr>
<tr>
<td>Ceftizoxime</td>
<td>76(100)</td>
<td>0</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>76(100)</td>
<td>0</td>
</tr>
<tr>
<td>Amikacin</td>
<td>41(54)</td>
<td>0</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>33(43.4)</td>
<td>4(5.3)</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>23(30.2)</td>
<td>0</td>
</tr>
<tr>
<td>Polymixin B</td>
<td>0</td>
<td>23(30.3)</td>
</tr>
<tr>
<td>Colistin</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

R: resistant, I: intermediate, S: sensitive.

### Table 3. Sequences of primers used for multiplex PCR for detection of genes encoding MBLs and oxacillinases in isolates of *Acinetobacter spp.*

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imp-F</td>
<td>5-GAATAGAATGGTTAACTCTC-3'</td>
<td>188</td>
</tr>
<tr>
<td>Imp-R</td>
<td>5-CCAAACCACCTAGTTATC-3'</td>
<td></td>
</tr>
<tr>
<td>mp-F</td>
<td>5-GTTTGTTGCGATATGCACACC-3'</td>
<td>382</td>
</tr>
<tr>
<td>Imp-R</td>
<td>5-AATGGGCGACACGAGATAG-3'</td>
<td></td>
</tr>
<tr>
<td>mp-R</td>
<td>5-GTACAAGGGATTGCGATCG-3'</td>
<td>569</td>
</tr>
<tr>
<td>imp-R</td>
<td>5-GTACAAGGGATTGCGATCG-3'</td>
<td></td>
</tr>
<tr>
<td>Oxa-51-like-F</td>
<td>5-TAATGCTTTGATGCGGCTTG-3'</td>
<td>353</td>
</tr>
<tr>
<td>Oxa-51-like-R</td>
<td>5-TGGATTGCACTTCTATTTGG-3'</td>
<td></td>
</tr>
<tr>
<td>Oxa-23-like-F</td>
<td>5-GATCGGATTGGAGAACAGA-3'</td>
<td>501</td>
</tr>
<tr>
<td>Oxa-23-like-R</td>
<td>5-ATTCTGACCGATTTCCAT-3'</td>
<td></td>
</tr>
<tr>
<td>Oxa-24-like-F</td>
<td>5-GTTGATTTGCGCCCCCTTAAA-3'</td>
<td>246</td>
</tr>
<tr>
<td>Oxa-24-like-R</td>
<td>5-AGTTGAGCAAAAGGGATT-3'</td>
<td></td>
</tr>
<tr>
<td>Oxa-58-like-F</td>
<td>5-AAGTATTTGGGCTTGTGCTG-3'</td>
<td>599</td>
</tr>
<tr>
<td>Oxa-58-like-R</td>
<td>5-CCCTCTGGCTCTACATAC-3'</td>
<td></td>
</tr>
</tbody>
</table>
Fig. 1. Plasmids of *Acinetobacter baumannii* (MDR) Lane A (low molecular size weight, standard marker); lane 1, 2, 5, and 10 antibiotic sensitive *Acinetobacter baumannii*; lane 4, 6-9, 11, 12, 13 MDR *A. baumannii* isolates

Figure 2 Different plasmid profiles obtained in the *A. baumannii* isolates. Lane 1, marker (M). Line 1-11 represent restriction analysis of isolated Plasmid with EcoRI, and HindIII restriction enzymes.
Figure 3. Detection of genes encoding oxacillinases and MBLs in carbapenem-resistant Acinetobacter spp. by multiplex PCR. Lane 9 negative control; lane 1, blaOXA-51 and blaSIM-like gene lane 2 blaOXA-24-like gene and blaOXA-51-like gene; lane 3, blaVIM-like gene; lane 4, blaMP and blaSIM-like gene; lane 5, blaOX-51 and blaSIM-like gene; lane 6, blaOXA-51 and blaSIM-like gene; lanes 7, blaOXA-51 and blaSIM-like gene lane 8, blaOXA-24 and blaOXA-51-like gene, 10 isolates carrying blaOXA-51-like gene; lanes 10, blaVIM-like gene. 9 isolates carrying blaOXA-23-like gene; lane 11, blaOXA-24 and blaOXA-51-like gene. M, Molecular mass markers (100 bp DNA ladder).

References


33. Gallego L, 2010Analysis of plasmids as genetic support of the class D Carbapenemase OXA-40 in multidrug-resistant Acinetobacter baumannii isolates Current Research technology and education topics in applied microbiology and medical biotechnology.1-9.