Abstract

Objectives. To determine the population structure and antimicrobial sensitivity profile of carbapenem-resistant A. baumannii isolates from different hospitals of Lebanon.

Methods. Thirty-two isolates of A. baumannii were collected between Jan-June 2011 from three distant hospitals in Lebanon. Molecular identification was done by partial rpoB sequencing. The antibiotic susceptibility testing was determined by agar disc diffusion. MICs for imipenem, meropenem, colistin, tigecycline and sulbactam were also determined for imipenem resistant strains. Genetic testing was performed using MLST.

Results. Thirty isolates were identified as A. baumannii according to the molecular identification test, of these 24 exhibited imipenem resistance. Susceptibility profiles (susceptible vs. intermediate vs. resistant) of these isolates were 0% vs.12.5% vs.87.5% for meropenem, 8.3%vs.54.2%vs.37.5% for tigecycline and 8.3%vs.8.3%vs.83.4% for sulbactam, respectively. All isolates (100%) were sensitive to colistin. All isolates (except one) belonged to international clone 2.

Conclusion. High prevalence of drug resistant of A. baumannii poses a major risk for patients in Lebanon. Therefore, an effective antimicrobial treatment, strict infection control measurements and rational antibiotic use over in Lebanon are mandatory to control further aggravation of the antibiotic resistance organisms in this country.

Genetic and antibiotic susceptibility profiles of drug-resistant Acinetobacter baumannii from various parts of Lebanon

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Background

Over the past decade, the incidence of infections due to multidrug-resistant (MDR) Acinetobacter species has been on the rise worldwide [1]. Nowadays, a clear association had been established between infections caused by carbapenem-resistant Acinetobacter baumannii (A. baumannii) and increased mortality (crude mortality 26%-68%), morbidity, intensive care unit (ICU), increased stay and cost [2, 3], thus making this organism a serious threat to our patients. MDR strains of A. baumannii were being recovered in recent years. Also, occurrence of MDR Acinetobacter has been recently documented in trauma centers and ICUs in USA and Europe [4, 5,6], whereas few studies have reported on emergence of MDR Acinetobacter in Lebanon and other Arab Middle East countries [7-10]. Our study was conducted to determine the population structure and antimicrobial resistance patterns among imipenem-resistant A. baumannii clinical isolates collected from different hospitals in Lebanon.

Materials and methods

Bacterial isolates

This prospective study was extended over six months period between January 2011 and June 2011. Three hospitals from various geographic areas in Lebanon participated in the study. The study protocol included submission of non-duplicate carbapenem-resistant Acinetobacter isolates obtained from different clinical specimens (Deep tracheal aspirate, urine, wound, sputum). Each center tested its own isolates for susceptibility to imipenem by disk diffusion method according to its own practice guidelines. Only imipenem-resistant isolates were submitted to our referral laboratory and included in the study. At the time of analysis, samples were sub-cultured on blood agar base media [BioRad, France] and re-identification was carried out using Remel RapID NF Plus system [Remel, USA] according to the manufacturer’s guidelines. Molecular identification at species level was performed by partial sequencing of rpoB (RNA polymerase subunit B) gene (350 pb) [11].

Antimicrobial susceptibility testing

Confirmatory testing for imipenem resistance was done for all isolates by disc diffusion (10 μg) [Bio-Rad, France]. The susceptibility for 12 other antibiotics [BioRad, France] was also tested by disc diffusion according to the EUCAST guidelines [12]. The following antibiotics discs were used: ticarcillin (75 μg), ticarcillin/clavulanic acid (75/10 μg), piperacillin/tazobactam (100/10 μg), ceftazidime (30 μg), ciprofloxacin (5 μg), amikacin (30 μg), gentamicin (10 μg), tobramycin (10 μg), netilmicin (10 μg), cotrimoxazole (1.02-23.75 μg), colistin (50 μg), and doxycycline (30 μg). The minimal inhibitory concentrations [MICs] were determined for imipenem [Merck Sharp & Dohme], meropenem [Astra Zeneca Pharmaceuticals], tigecycline [Pfizer] and ampicillin-sulbactam [Sandoz] using the microdilution on Mueller-Hinton agar [BioRad, France] according to the protocol proposed by Courvalin et al. [13]. Whereas, colistin MIC was determined by Etest [Biomerieux France]. Only A. baumannii isolates found to be resistant to imipenem were included in the MIC study. The susceptibility to imipenem, meropenem and colistin was determined according to the EUCAST 2014 breakpoints [14]. Susceptibility to tigecycline and sulbactam was determined according to breakpoints recommended by Pachón-Ibáñez et al. [11, 14, 15] and García-Peñuela et al. [15, 16], respectively (Table 1). The American Type Culture Collection [ATCC] quality control strain Acinetobacter baumannii [ATCC 19606] was used to ensure proper performance of the disk diffusion and MIC test.
Molecular analysis

DNA extraction

After overnight growth on nutrient agar [Biolife, Milano, Italy], a loop full of the tested A. baumannii bacteria was suspended in 500 μl of sterile ultrapure water and the QIAamp DNA mini Kit [Qiagen, Hilden, Germany] was used for extraction according to the manufacturer’s guidelines.

Multi-Locus Sequence Typing

The internal fragments of seven housekeeping genes including cpn60 (60-kDa chaperonin), fusA (elongation factor EF-G), gltA (citrate synthase), pyrG (CTP synthase), recA (homologous recombination factor), rplB (50S ribosomal protein L2) and rpoB were amplified and sequenced according to the Pasteur scheme available at the following site (http://www.pasteur.fr/mlst). The different sequences were compared to the existing alleles in Pasteur MLST Database. The identification number for sequence types (ST) was given according to their allelic profiles. In order to compare our identified ST(s) to previous identified ST(s) present in MLST Database and to assign them to their corresponding clonal complexes, an eBURST analysis was used (http://eburst.mlst.net/). A clonal complex was defined as a group of similar ST sharing 6 identical loci within 7.

Results

Bacterial isolates. Re-identification of all investigated strains in our laboratory using remel technique showed that all 32 strains were belonged to A. calcoaceticus-A. baumannii complex (99% confidence). Of these, 30 were identified as A. baumannii and 2 as A. pittii strains according to the molecular identification based on partial rpoB sequencing. Only A. baumannii isolates were included for further analysis. The hospital repartition of A. baumannii isolates is shown in Table 2.

Genetic variability. MLST was performed on all 30 A. baumannii strains to discover the genetic background of A. baumannii strains infected patients in Lebanon. Two sequence types ST2 and ST458 were identified. ST2 was the major sequence type present in 29 strains, whereas ST458 was found sporadically in one strain. By eBURST analysis (Figure 1) of MLST database (last updated 07.08.2014), ST2 was the founder of the biggest and principal clonal complex (known as clonal complex 2) formed up to 33 single locus variants [SLV]. ST458 was a singleton and no similar ST has been identified yet. Those STs that can’t be linked to any other in the sample are termed singletons and appear as unlinked points.

Table 1. Susceptibility breakpoints (µg/ml) of the tested antimicrobials

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Sensitive</th>
<th>Intermediate</th>
<th>Resistance</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colistin</td>
<td>≤ 2</td>
<td>-</td>
<td>&gt; 2</td>
<td>12</td>
</tr>
<tr>
<td>Imipenem</td>
<td>≤ 2</td>
<td>[2 – 8]</td>
<td>&gt; 8</td>
<td>12</td>
</tr>
<tr>
<td>Meropenem</td>
<td>≤ 2</td>
<td>[2 – 8]</td>
<td>&gt; 8</td>
<td>12</td>
</tr>
<tr>
<td>Sulbactam</td>
<td>≤ 4</td>
<td>8</td>
<td>≥ 16</td>
<td>15</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>≤ 2</td>
<td>4</td>
<td>≥ 8</td>
<td>14</td>
</tr>
</tbody>
</table>
Table 2. Hospital repartition, sequence types and antibiotic susceptibility of A. baumannii strains

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Hospital</th>
<th>ST</th>
<th>Antibiotic</th>
<th>MICs(µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMUL 016</td>
<td></td>
<td>2</td>
<td>TIC</td>
<td>0.047</td>
</tr>
<tr>
<td>CMUL 017</td>
<td></td>
<td>2</td>
<td>TCC</td>
<td>0.032</td>
</tr>
<tr>
<td>CMUL 018</td>
<td></td>
<td>2</td>
<td>TZP</td>
<td>0.047</td>
</tr>
<tr>
<td>CMUL 019</td>
<td></td>
<td>2</td>
<td>CAZ</td>
<td>0.047</td>
</tr>
<tr>
<td>CMUL 020</td>
<td></td>
<td>2</td>
<td>IMI</td>
<td>0.032</td>
</tr>
<tr>
<td>CMUL 021</td>
<td></td>
<td>2</td>
<td>SXT</td>
<td>0.032</td>
</tr>
<tr>
<td>CMUL 022</td>
<td></td>
<td>2</td>
<td>CS</td>
<td>0.032</td>
</tr>
<tr>
<td>CMUL 023</td>
<td></td>
<td>2</td>
<td>GEN</td>
<td>0.032</td>
</tr>
<tr>
<td>CMUL 024</td>
<td></td>
<td>2</td>
<td>TM</td>
<td>0.032</td>
</tr>
<tr>
<td>CMUL 025</td>
<td></td>
<td>2</td>
<td>AMK</td>
<td>0.032</td>
</tr>
<tr>
<td>CMUL 026</td>
<td></td>
<td>2</td>
<td>NET</td>
<td>0.032</td>
</tr>
<tr>
<td>CMUL 027</td>
<td></td>
<td>2</td>
<td>CIP</td>
<td>0.032</td>
</tr>
<tr>
<td>CMUL 028</td>
<td></td>
<td>2</td>
<td>DOX</td>
<td>0.032</td>
</tr>
<tr>
<td>CMUL 029</td>
<td></td>
<td>2</td>
<td>CS</td>
<td>0.032</td>
</tr>
<tr>
<td>CMUL 030</td>
<td></td>
<td>2</td>
<td>IMI</td>
<td>0.032</td>
</tr>
<tr>
<td>CMUL 031</td>
<td></td>
<td>2</td>
<td>MER</td>
<td>0.032</td>
</tr>
<tr>
<td>CMUL 032</td>
<td></td>
<td>2</td>
<td>TIG</td>
<td>0.032</td>
</tr>
<tr>
<td>CMUL 033</td>
<td></td>
<td>2</td>
<td>SAM</td>
<td>0.032</td>
</tr>
</tbody>
</table>

MICs are expressed in µg/ml. TIC (ticarcillin), TCC (ticarcillin/clavulanic acid), TZP (piperacillin/tazobactam), CAZ (ceftazidime), IMP (imipenem), SXT (trimethoprim/sulfamethoxazole), CS (colistin), GEN (gentamicin), TM (tobramycin), AMK (amikacin), NET (netilmicin), CIP (ciprofloxacin), DOX (doxycycline), MER (meropenem), TIG (Tigecycline) and SAM (sulbactam).
Antimicrobial susceptibility

Table 2 shows the antibiotic susceptibility profile of all *A. baumannii* strains to various antibiotic classes. A total of 24 imipenem-resistant strains was detected and the rest 6 strains were imipenem-susceptible. Further susceptibility study of these 24 imipenem-resistant strains was also done by checking MIC of colistin, imipenem, meropenem, sulbactam and tigecycline (Table 2). A total of 21 *A. baumannii* isolates (87.5 %) were resistant to meropenem, and 12.5 % (3 ) showed intermediate susceptibility. For tigecycline, 2 (8.3 %) of imipenem-resistant *A. baumannii* isolates were susceptible, 54.2 % (13 ) were intermediate and 37.5 % (9) were resistant. All the resistant strains belonged to sequence type 2. They were found among the different hospitals with no specific distribution. All imipenem-resistant *A. baumannii* isolates (100 %) were susceptible to colistin, whereas 83.4 % (20) of the tested isolates were resistant to sulbactam, 8.3% (2) had intermediate susceptibility, and also 8.3 % (2) were susceptible to sulbactam.

Discussion

This study demonstrates important results due to the high resistance rates found to common used antimicrobials. All the *A. baumannii* isolates (except one) belong to international clone 2 (clonal complex 2) , while 82.8 % (24/29) isolates were imipenem-resistant. The clone notion in *A. baumannii* has been appeared for the first time in 1996 when Dijkshoorn et al. found two clones distributed in different hospitals in northwestern Europe and designated them as European clone I and II [17]. After that, these two clones and another clone (designated as European clone III) [18] have been identified throughout the world and then named international clones (I to III) [19]. The dominance of international clone 2 in our collection fits to the global situation where a shift toward this clone has been observed worldwide. It was the largest and the widely distributed clone in 5 continents [20]. The successful emergence of international clone 2 has been attributed to the frequent acquisition of antimicrobial resistance determinants [20]. Further studies using micro-epidemiological typing methods like Pulsed Field Gel Electrophoresis are required to evaluate the homogeneity or heterogeneity of the international clone 2 in Lebanon.
A. baumannii is an opportunistic pathogen with widespread antimicrobial resistance patterns to commonly used antimicrobials such as; aminoglycosides, extended-spectrum cephalosporins, beta-lactams/sulbactam, antipseudomonal penicillins/beta-lactamase inhibitors, cotrimoxazole, carbapenems, fluoroquinolones, polymixins, and tetracycline. Magiorakos et al. has categorized MDR Acinetobacter into 3 groups according to the various resistance patterns among [20]. MDR organisms are defined as strains that are resistant to at least 3 antimicrobials representing at least 3 classes. Extensively drug resistant [XDR] organisms are strains susceptible not to more than 2 classes, and Pandrug resistant organisms are strains that are resistant to all classes [21]. In addition to being resistant to carbapenems, beta-lactams/beta-lactamase inhibitor combinations, quinolones, aminoglycosides and cotrimoxazole, only 8.3 % were susceptible to tigecycline and sulbactam. This result is alarming issue since this is an evidence of emergence of XDR A. baumannii in our health care facilities, and colistin become the last resort for treatment of XDR A. baumannii in Lebanon.

It is important to note that resistance to colistin has been reported in some countries [22]. Interestingly, colistin hetero-resistance is much higher than colistin-resistance, and it is also difficult to be detected. First colistin hetero-resistance in A. baumannii had been shown by Li et al. [22]. In this study, colistin-susceptible A. baumannii strains showed early concentration-dependent killing and that sub-optimal doses of colistin induce resistance. There is two recent studies from Arab Middle East countries reported A. baumannii colistin resistance isolates, Kuwait (12 %) and Egypt (5 %) (7, 23). When investigating the dosing regimens in the corresponding Lebanese hospitals during the year preceding the study period, the doses used varied between 1MU every 12 hours and 1MU every 8 hours and rarely reached 2MU every 8 hours, and these doses were lower than that recommended by Michalopoulos and Falagas (3 million IU; 240 mg CMS) every 8 hours as the optimal dose of colistin for critically ill-patients with normal renal function [24]. Different dosing, however, was recommended by the manufacturers of European colistin products is 50,000 to 75,000 IU/kg/day of CMS in 2-3 divided doses. This study is recommending to investigate doses higher than the doses mentioned in the leaflet of manufacturers or the regimens used in Lebanon to maintain a full efficacy of colistin.

Tigecycline activity, on the other hand, is compromised by multidrug efflux pump systems such as AdeABC PUMP [ 25]. Peleg et al. showed that the gene coding for the production of this pump can be up regulated under antibiotic pressure causing a rapid rise in MIC to tigecycline upon in vitro passage [26]. Thus, the high rates of tigecycline resistance encountered in our study may be due to the poor understanding of the drug’s pharmacokinetics and pharmacodynamics in different infection sites like the bloodstream, and/or in special populations of patients like the critically ill, the obese and many others conditions [27].

Due to shortage of new antimicrobials against XDR Acinetobacter, older drugs like sulbactam are considered. Sulbactam possesses an intrinsic activity against A. baumannii independent of the accompanying beta-lactam present in commercial combinations [ 28-32]. The activity of sulbactam is mediated by binding of the drug to penicillin-binding protein 2 [PBP2] [2]. The clinical usefulness of sulbactam was proven in several studies that showed no inferiority to imipenem and better eradication rates than comparators in treating non-life threatening infections caused by imipenem- and sulbactam-sensitive A. baumannii strains [28,32]; however, all the studies had a drawback of having a small number of investigated patients.
Despite sulbactam-ampicillin being out of the market for the past 20 years, almost *Acinetobacter* strains were resistant to this combination. This can be explained by the fact that both sulbactam and beta-lactams act by binding PBP2 and mutations in the PBP2 gene, selected by antimicrobial pressure, lead to cross-resistance to both. In a recent report, the Surveillance Network in the United States showed that resistance patterns of *Acinetobacter* to sulbactam combinations vary widely with geographic distribution toward an increase in resistance rate from 10.6% in 1999 to 25% in 2010 [32], still much lower than our reported resistance rate.

**Conclusion**

With the absence of an effective therapy, the widespread of XDR *A. baumannii* in our Lebanese health care facilities poses a great danger to patients by increasing mortality, morbidity and hospital stay. Combination therapy of colistin with rifampicin or minocycline might be effective but has yet to be proven against XDR *A. baumannii* strains. Strict infection control measurements and rational antibiotic use are mandatory to control further aggravation of the antibiotic resistance in Lebanon.

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**Conflict of interest**

None to declare
References


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