Traditional and Molecular Gene Detection (blaIMP-1 and blaIMP) of Multi-drug Resistant Acinetobacter baumannii

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ABSTRACT

Acinetobacter bacteria are widely resistant to β-lactam antibiotics. The formation of carbapenemases such as metallo-β-lactamases (MBLs), which hydrolyze a variety of β-lactams including penicillin, cephalosporins, and carbapenems, is one of the primary causes of resistance in Acinetobacter baumannii. MBL-producing carbapenem-resistant strains have been detected all over the world in recent years, and at a rising pace. For this investigation, fifty-two A. baumannii isolates were chosen based on imipenem (IMP) resistance (MIC >16 g/ml). The Modified Hodge test (MHT) and the CDDT were used to detect MBL phenotypic expression (Combine Disk Diffusion Test). PCR was used to detect genotypic expressions of the blaIMP-1 and blaIMP genes in all metallo-lactamase-producing A. baumannii strains. According to the MHT test, 49 of 52 A. baumannii isolates (94.2%) produced carbapenemase, whereas the CDDT test revealed that 47 isolates (90.4%) produced MBL. Despite being negative for MBL-producer in the phenotypic technique used for control isolates, 39 (75%) of 52 putative MBL-producer isolates were positive for the blaIMP-1 gene by PCR, while fifteen A. baumannii isolates (28.8%) were positive for the blaIMP gene by PCR. In 23% (12/52) of instances, the blaIMP-1 and blaIMP genes were found together. The genotypic approach must be used to confirm isolates of A. baumannii that have been identified as MBL-producers using the MHT test and the Combine Disk Diffusion Test.

Keywords: Acinetobacter baumannii, Metallo-β-lactamase (MBL) Multidrug resistant, Imipenem resistance

INTRODUCTION

Acinetobacter baumannii is a glucose-non-fermentative, Gram-negative coccobacillus that has emerged in recent years as a main cause of nosocomial infections associated with elevated morbidity and mortality (Zarrilli et al., 2013). A. baumannii is an opportunistic infection with a high occurrence among immunocompromised people, especially those who spend a lot of time in hospitals. It has been identified as a red alert human pathogen in recent years, causing concern among medical professionals due to its wide range of antibiotic resistance (Howard et al., 2012). The most common and serious multidrug resistant (MDR) pathogens have been encompassed within the acronym ESKAPE, standing for Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, A. baumannii, Pseudomonas aeruginosa (P. aeruginosa) and Enterobacter spp. According to Centre for Disease Control (CDC) the six ESKAPE bacteria cause two third of all hospital acquired infections (Howard et al., 2012 and Ahir et al., 2012).

It is generally known that MBLs are Ambler class β metallo-enzymes that are resistant to clavulanic acid. They require zinc as a cofactor for enzymatic activity, and Ethylene Di-amine Tetra Acetic Acid (EDTA) and other metal ion chelating agents decrease their action. Pseudomonas spp. and Acinetobacter spp. are the most important nosocomial pathogens with multiple drug resistance (Corvec et al., 2003).

Carbapenems are considered the last-line drugs for treatment of infections caused by multiresistant (MR) Gram-negative bacilli (Sacha et al., 2007). Recently, the emergence of carbapenem-resistant organisms such as P. aeruginosa and A. baumannii has become a major therapeutic challenge. Carbapenem resistance due to acquired MBLs is more serious than other resistance mechanisms because MBLs can hydrolyze all β-lactam antibiotics except monobactams. In addition, MBL-encoding genes on integrons can easily be passed between strains (Yousefi et al., 2010).

So far, world widely there are five main categories of MBLs have been described, IMP hydrolyzing β-lactamase, VIM-Verona integron-encoded metallo-β-lactamases, GIM-German Imipenemase, SPM-Sao Paulo metallo-β-lactamases, and SIM-Seoul imipenemase enzymes. Recently, A novel MBL has been designated in P. aeruginosa from Australia-blaAIM-1. There are no standard guidelines by Clinical Laboratory Standards Institute’s (CLSI) for detection of these enzymes in various bacteria (Lee et al., 2011).

The aim of current research was to investigate the presence of metallo-beta-lactamase production among beta-lactam resistant A. baumannii and to compare results gathered from phenotypic and genotypic methods. Further, for molecular analysis of target genes (blaIMP and blaIMP-1 genes) encoding for metallo-beta-lactamase with specific primers by polymerase chain reaction.

MATERIALS AND METHODS

Isolate selection and Antibiotic Susceptibility

A total of 52 non-repetitive imipenem and meropenem resistant A. baumannii strains were isolated from clinical samples (endotracheal aspirates, sputum, and urine) of different patients. The isolates were identified by conventional methods (Schreckenberger et al., 2003). Identification and antibiotic susceptibility analysis of the strains were performed by VITEK 2 system (bioMérieux, Marcy l’Etoile, France) according to criteria mentioned by bioMérieux (bioMérieux 2010). Strains were stored in 20% glycerol at -80°C.

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Phenotypic Detection of MBLs

Modified Hodge Test (MHT)

A 0.5 McFarland dilution of Escherichia coli ATCC 25922 was prepared. A 1:10 dilution inoculated onto a Mueller Hinton Agar (MHA) plate (Merck, Darmstadt, Germany) and a 10 μg IMP (Imipenem) disk was placed in the plate center, while strains of A. baumannii were streaked in a straight line from the edge of the disk to plate margins. After overnight incubation, if the inoculates had carbapenemases, the test showed a cloverleaf-like indentation of E. coli growing along the test bacterium growth streak within IMP disk diffusion zone (Lee et al., 2001). Each run using MHT-Positive Klebsiella pneumoniae (ATCC BAA-1705) and MHT-Negative Klebsiella pneumoniae (ATCC BAA-1706).

Combined disc diffusion test (CDDT)

EDTA-IMP disks were prepared by adding EDTA solution (10μl of 0.1M EDTA) to 10μg-IMP (imipenem) disks. Bacterial isolates were adjusted according to McFarland 0.5 turbidity standard and were inoculated to Mueller Hinton agar. A 10-μg-imipenem disk and imipenem disc with EDTA were placed on Mueller Hinton agar. A 10μg of each of the deoxyxynucleotide triphosphate, 0.4 μM of each primer, 1 U of Thermus aquaticus DNA polymerase (Pharmacia), and 5 μl of template DNA. All tubes were transferred into thermal cycler. The PCR was started as in the following program. The initial denaturation for 5 minutes at 94°C. Thirty five cycles of: A-denaturation (94°C for 30 secs), B-annealing (at 55°C - 30 secs) for blaIMP-1 and blaIMP genes, C-extension (72°C for 45 secs) and Final extension (72°C for 7 minutes). Hold temperature (4°C for 10 minutes). The PCR product of 620 bp for blaIMP-1 and 587 bp for blaIMP was visualized by 2% agarose gel electrophoresis with Novel Juice (Novel Juice 2012).

RESULTS

Bacterial cultures, colony characterization, Gram staining, microscopic examination, and species identification by VITEK-2 were done. A total of 52 isolates were identified as Acinetobacter baumannii. Carbapenem resistance was observed in 52 A. baumannii clinical isolates by broth microdilution MIC (minimal inhibitory concentration) using the VITEK-2 automated system with VITEK card: AST-N204 (Garcia 2010).

Table (1): Antimicrobial sensitivity of MBL producing-A. baumannii

<table>
<thead>
<tr>
<th>Antibiotic used</th>
<th>Antibacterial class</th>
<th>No of isolates and represented %</th>
<th>Resistant</th>
<th>Sensitive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin/Clavulanic</td>
<td>β-lactam/inhibitor combination</td>
<td>51(98.1%)</td>
<td>1(1.9%)</td>
<td></td>
</tr>
<tr>
<td>Piperacillin/Tazobactam</td>
<td>Extended spectrum – β lactams</td>
<td>51(98.1%)</td>
<td>1(1.9%)</td>
<td></td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>Third generation Cephalosporin</td>
<td>50(96.2%)</td>
<td>2(3.8%)</td>
<td></td>
</tr>
<tr>
<td>Ceftazidine</td>
<td>Third generation Cephalosporin</td>
<td>50(96.2%)</td>
<td>2(3.8%)</td>
<td></td>
</tr>
<tr>
<td>Imipenem</td>
<td>Carbapenem</td>
<td>52(100%)</td>
<td>0(0.0%)</td>
<td></td>
</tr>
<tr>
<td>Meropenem</td>
<td>Carbapenem</td>
<td>52(100%)</td>
<td>0(0.0%)</td>
<td></td>
</tr>
<tr>
<td>Amikacin</td>
<td>Aminoglycosides</td>
<td>45(86.5%)</td>
<td>7(13.5%)</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td>Aminoglycosides</td>
<td>47(90.4%)</td>
<td>5(9.6%)</td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>Fluoroquinolone</td>
<td>51(98.1%)</td>
<td>1(1.9%)</td>
<td></td>
</tr>
<tr>
<td>Ofloxacin</td>
<td>Fluoroquinolone</td>
<td>51(98.1%)</td>
<td>1(1.9%)</td>
<td></td>
</tr>
<tr>
<td>Colistin</td>
<td>polymyxin</td>
<td>0(0.0%)</td>
<td>52(100%)</td>
<td></td>
</tr>
</tbody>
</table>

Using the VITEK-2 system method to test the susceptibility of A. baumannii isolates to different antibiotics, according to the Clinical Laboratory Standards Institute's (CLSI) guidelines (CLSI, 2017) we found that; the highest sensitivity of A. baumannii isolates was for Colistin (100% of isolates). Followed by Amikacin (13.5% of isolates) then Gentamycin by (9.6% of isolates). The lowest sensitivity of A. baumannii was for Amoxicillin/Clavulanic, Piperacillin/Tazobactam, Ciprofloxacin and Ofloxacin (1.9%) then Cefo-
taxime and Ceftazidime with 3.8% (Table 1). These carbapenem resistant *A. baumannii* isolates were tested Modified Hodge Test (MHT) for production of carbapenemase and 49 (94.2%) were carbapenemase producers (Figure 1).

![Figure 1: Modified Hodge Test (MHT)](image1)

Figure (1): Modified Hodge Test (MHT) showing positive and negative test.

The carbapenem resistant isolates were also tested for MBL production and forty-seven (90.4%) of these isolates gave positive result by CDDT. (Figure 2, A). The difference in zone diameter between IMP disc and IMP + EDTA of 4 mm was evaluated as EDTA synergy positive (the presence of an enlarged zone of inhibition was interpreted as EDTA-synergy test positive). Five isolates were MBL negative in IPM-EDTA-disk synergy test (Figure 2, B). Thirty-nine (75%) of MBL producer isolates of *A. baumannii* (out of 52 isolates) were positive for blaIMP-1 by PCR, while fifteen (28.8%) isolates were positive for blaIMP gene only. The co-appearance of blaIMP-1 and blaIMP genes in 23% (12/52) of cases (Figures 3 and 4) were reported. No blaIMP genes were found in isolates negative by the phenotypic test (CDDT).

![Figure 2: The combine disk diffusion test (CDDT) for metallo-β- lactamases (MBL) production. A, Positive CDDT with inhibition zone >4mm; B, negative CDDT.](image2)

DISCUSSION

The multidrug resistance is now a worldwide problem with the increasing of antibiotic abuse that more and more selects for resistant strains. Carbapenem resistance in *Acinetobacter baumannii* strains has been on the rise for the past decade, and it has become a major public health concern (Falagas et al., 2006 and Brusselaers et al., 2011). *A. baumannii* is an opportunistic pathogen that is a leading cause of respiratory infections, particularly nosocomial and ventilator-acquired pneumonia (VAP). Recently there is a rise in community acquired infections induced by *A. baumannii*. The *A. baumannii* occurrence among hospitalized patients depends on the hospital populations, types of performed interventions and procedures done (Giamarellou et al., 2008 and Howard et al., 2012).

Detection of MBL production poses considerable technical difficulties, including differing results due to different MHA agar brands and lack of confirmatory criteria other than genetic analysis. Many phenotypic methodologies for detecting MBL-producing isolates are available; however the CLSI Institute has not endorsed a uniform procedure for MBL screening. Currently, the technique using a disc with IMP plus 750 μg of EDTA (combined disc method) is simple to perform and highly sensitive in differentiating MBL-producing isolates (Yousefi et al., 2010 and Yong et al., 2002). MHT was used to screen the Meropenem and Imipenem resistant strains for carbap-
enemase production, and 94.2 percent of them were found to be carbapenemase producers. According to Kumar et al. (2011), the MHT detected carbapenemase producers in 71% of the isolates. This was consistent with the findings of Lee et al. (2003) in Korea, who discovered carbapenemase positive isolates in 73% of the isolates using the MHT.

The carbapenem resistant isolates were further screened for MBL production, 90.4% were positive by CDDT. Similar study conducted by Pandya et al. (2011) showed that 96.3% of strains were MBL positive by CDDT and 81.4% were positive by DDST. The findings of this study agree with those of Irfan et al. (2008), who found that 96.6 percent of carbapenem-resistant bacteria produced MBL when exposed to CDDT at Aga Khan University in Karachi. Similar findings were found with the study conducted by Noori et al. (2014), in which 86.8% of isolates were identified as MBL producers by CDDT.

PCR assay was carried out by utilizing previously published primers for amplification of genes encoding carbapenemases (blaIMP and blaIMP-1 genes). MBLs are less commonly identified in A. baumannii than the OXA-type carbapenemases but their carbapenem-hydrolyzing activities are 100–1000-fold more potent. Their presence in MDR A. baumannii isolates is in some instances difficult to detect, indicating that their contribution to the carbapenem resistance may be underestimated (Zarrilli et al., 2013).

The high percentage for blaIMP-1 gene in present study (75%), confirmed by many studies in different percentages, in a surveillance study in 2003-2004, MBLs were detected in 135 of 545 (24.8%) IMP-resistant A. baumannii isolates, the proportion of blaIMP-1 was 61% (Lee et al., 2011). In research published in 2006, blaIMP-1 was found in 15 (48.4%) of 31 carbapenem-resistant A. baumannii isolates (Sung et al., 2008). More than half of the isolates (55 percent) exhibited a positive blaIMP-1 in another investigation conducted by (Tognim et al., 2006) at a Teaching Hospital in Brazil. The proportion of blaIMP-1-producing A. baumannii isolates among carbapenem-resistant strains grew from 0% in 1993-1997 to 29% in 1998 and 100% in 1999-2001, according to the same study.

The high percentage for blaIMP-1-producing strains of A. baumannii in present study (75%), indicating that this important mechanism of antimicrobial resistance was disseminated among distinct clones. A major contributing factor in the MDR emergence strains of A. baumannii is the acquisition and transfer of antibiotic resistance via plasmids and mobile genetic elements, including transposons and integrons (Sung et al., 2008).

Fifteen (28.8%) A. baumannii out of 52 presumptive MBL producer isolates (with isolates were negative for MBL producer in control by phenotypic technique) were positive for blaIMP gene by PCR (Fig.-4). No blaIMP genes were found in isolates negative by CDDT and MHT tests.

Previous research has reported that presence of blaIMP gene in the Acinetobacter species in low percentages (5.12%) as in the study of Hwa, (2008). The prevalence of the metallo-β-lactamase genes (blaIMP gene) is generally low within A. baumannii isolates as illustrated in a study by Mendes et al., (2009) where the prevalence was 0.8% in Taiwan. Other research could not detected blaIMP genes (Aktas and Kayacan 2008; Mohamed and Raafat 2011; Ehlers et al., 2012, and Purohit et al., 2012).

The isolates which were positive MBL production by confirmatory test but negative for blaIMP amplification may have variant blaIMP or blaSIM genes (Uma et al., 2009). This was established by the current study, by the presence of the blaIMP1 gene in proportion (75%). The IMP-resistant Acinetobacter baumannii strains in present study with no phenotypic or genotypic sign of MBL production may possess other enzymes mediating carbapenem resistance, such as OXA-type lactamases (class-D) or AmpC β-lactamases and other mechanisms such as outer-membrane permeability (OMP) and efflux mechanisms (Mohamed and Raafat 2011). The mechanism of cleavage of β-lactam ring is different for MBL’s as compared to β-lactamases; however, both gene products still share a unique αββα fold in the active sites of the enzymes. The blaIMP gene is a foreign gene transferred from another bacterial species, and A. baumannii only retains it in situations where there is selective pressure in the form of IMP (Ehlers et al., 2012). The coexistence of blaIMP-1 with blaIMP genes in current research 23% (12 out of 52) of cases exemplify the extraordinary ability presented by A. baumannii to acquire multiple resistance mechanisms.

CONCLUSION

According to the findings of this study, resistance to IMP was found to be a better indicator of MBL formation. The MHT and CDDT tests appeared to be useful in separating MBL from non-metalloenzyme producers. Most A. baumannii strains were found to produce metallo-beta-lactamase using the IPMEDTA-disk synergy test and PCR for the blaIMP-1 gene (MBL). Finally, the widespread misuse, overuse, and exploitation of various antibiotics by healthcare professionals or patients may be to contribute for the growth in carbapenem resistance; hence, lowering antibiotic use aims to reduce costs and damage caused by A. baumannii.

REFERENCES


BIOMÉRIEUX. 2010. VITEK® 2 Systems Product Information. bioMérieux, Inc. USA.


SACHA, P., M. ZÓRAWSKI, T. HAUSCHILD, P. WIECZOREK, J. JAWOROWSK P JAKONIUK,
استخدام الطريقة التقليدية والجزيئية للكشف عن الجينات المقاومة لأدوية المضادات الحيوية من Acinetobacter baumannii

حازم حامد صالح، أحمد السيد قاسم

摘要

Acinetobacter baumannii 是一种不发酵的革兰氏阴性细菌，广泛分布于医院和社区环境。它对多种抗生素具有耐药性，包括碳青霉烯类药物。在过去的几年中，这种细菌的耐药性问题日益严重，尤其是利用金属β-内酰胺酶（metallo-β-lactamase, MBL）的耐药性。在某些情况下，这种细菌还可能携带多种耐药基因，导致多重耐药性。因此，确定 Acinetobacter baumannii 的耐药性以及其基因组特征对于控制其传播和治疗感染至关重要。本研究旨在通过采用传统和分子方法来检测 Acinetobacter baumannii 的耐药性基因，并分析其基因组特征。通过这种方法，我们能够更好地理解这种细菌的耐药性机制，并为临床治疗提供指导。

Acinetobacter baumannii 是一种不发酵的革兰氏阴性细菌，广泛分布于医院和社区环境。它对多种抗生素具有耐药性，包括碳青霉烯类药物。在过去的几年中，这种细菌的耐药性问题日益严重，尤其是利用金属β-内酰胺酶（metallo-β-lactamase, MBL）的耐药性。在某些情况下，这种细菌还可能携带多种耐药基因，导致多重耐药性。因此，确定 Acinetobacter baumannii 的耐药性以及其基因组特征对于控制其传播和治疗感染至关重要。本研究旨在通过采用传统和分子方法来检测 Acinetobacter baumannii 的耐药性基因，并分析其基因组特征。通过这种方法，我们能够更好地理解这种细菌的耐药性机制，并为临床治疗提供指导。