PREVALENCE OF METALLO-B-LACTAMASE GENES IN MULTIDRUG RESISTANT GRAM NEGATIVE BACILLI

ABSTRACT:
In this study 107 Gram negative bacilli resistant to ceftazidime were collected from a clinical microbiology laboratory of a public hospital in Cairo, Egypt. Isolates were screened for their ability to produce metallo-β-lactamase (MBL) using the double disc synergy test (DDST). This test was conducted using imipenem (IPM) and ceftazidime discs (CAZ) as substrates and ethylene diamine tetra-acetic acid (EDTA) and 2-mercapto propionic acid (MPA) as inhibitors. Fourteen isolates that were inhibited by both tests and not with EDTA were selected for further investigations. Biochemical characterization using Api20E strips resulted in; Pseudomonas fluorescence (4), Klebsiella pneumoniae (3), Pseudomonas aeruginosa (2), Pseudomonas spp, Proteus mirabilis, Enterobacter cloacae, Escherichia coli and CDC group. The minimum inhibitory concentration of (CAZ) and (IPM) ranged from 8 to 64 µg/ml and 2 to 32 µg/ml, respectively. Polymerase chain reaction (PCR) was used to study the occurrence of the most common blaMBL-genes (blaIMP, blaVIM, blaSPM, blaSIM, blaSIM and blaIM), resulted in the detection of the examined bla-genes in 13 isolates. The number of detected genes in each isolate was varied and ranged between 1 to 4. The prevalence of genes in the isolates was; 69.2, 61.5, 38.55, 30.8 and 15.4% for blaSPM, blaVIM, blaSIM, blaSIM and blaDM, respectively. Moreover, blaIMP allele was not detected in any of the studied isolates. To the best of our knowledge this is the first study detect the six most common MBL genes in Egypt.

KEY WORDS:
Gram-negative, ceftazidime, imipenem, (EDTA), metallo-β-lactamasases and blaMBL-genes

INTRODUCTION:
Members of the family Enterobacteriaceae and Pseudomonas spp. are among the most important bacterial human pathogens accounting for the majority of bacteria isolated from clinical samples. These Gram negative bacilli (GNB) are rapidly acquiring resistance to one or more antimicrobial agents traditionally used for treatment (Eisenstein and Zaleznik, 2000). Some (GNB) demonstrated consistent resistance to a variety of broad-spectrum β-lactams (Jacoby and Medeiros, 1991). Senda et al. (1996) reported that the numbers of carbenem-resistant clinical isolates which belong to the family Enterobacteriaceae or Pseudomonads have been increasing worldwide and some of them were found to produce a new type of β-lactamase called metallo-β-lactamase (MBL). Resistance mediated by metallo-β-lactamase (MBL) is not conquered by conventional β-lactamase inhibitors (tazobactam, clavulanic acid and subactam). Metallo-β-lactamasases (MBLs) are an Ambler class B enzyme, characterized by their ability to hydrolyze a very wide range of broad-spectrum β-lactams. MBLs are considered to be more serious than other resistance mechanisms, thus MBL-producing Gram-negative bacteria have been recognized.

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to be among the most important nosocomial pathogens, further proliferation of these isolates in clinical settings will pose a serious global problem in the future. Their monitoring has become an important issue in clinical microbiology (Wang et al., 2014). For this reason, careful surveillance of MBL producers with respect to the classification of the genetic determinant for MBLs is extremely important.

The first Metallo-β-lactamase identified was found in non-pathogenic strain of Bacillus cereus. For long time MBLs were considered interesting but clinically unimportant (Sabath and Abraham, 1966). This view changed when the MBL producing Pseudomonas aeruginosa was first reported from Japan in 1991 (Senda et al., 1996) and since then there has been a dramatic increase in the detection and spread of acquired and transferable families of these metallo-β-enzymes with multiple variants as (Imipenemase (IMP), Verona Imipenemase (Vim), Sao-Paul Imipenemase (SPM), German Imipenemase (GIM), Seoul Imipenemase (SIM) and New-Delhi metallo-β-lactamase (NDM)). The genes for all these MBLs may be carried on mobile genetic elements or may become chromosomally integrated, this facilitate horizontal gene transfer within and between species and genera (Ellington et al., 2007). The resistance may spread rapidly to various species of Gram negative bacilli, as the MBL genes reside in mobile gene cassettes inserted in integrons.

Two major groups of plasmid-mediated MBLs the IMP, VIM, types have been recognized worldwide and their genetic determinants are often associated with integrons. Acquired MBLs have been reported mainly in clinical isolates of Pseudomonas aeruginosa and Acinetobacter spp. as well as in members of the Enterobacteriaceae with less extent (Yong et al., 2002; Lee et al., 2003; Franklin et al., 2006; Irfan et al., 2008; Yousefia et al., 2010).

This study was carried out to describe the commonness of the 6 most known MBL bla genes in the Egyptian isolates. The rapid detection of MBL- positive Gram-negative bacilli is necessary to aid infection control and to prevent their dissemination.

### MATERIAL AND METHODS:

All chemicals used in this study were purchased from Sigma Chemicals, St. Louis, MO., while media and antibiotic discs were from Oxoid-England, otherwise it will be stated.

### Bacterial isolates:

Gram negative bacilli (107) were collected from a clinical microbiology laboratory of a public hospital in Cairo, Egypt, that initially showed resistance to CAZ by disc diffusion method according to CLSI (2002).

### Screening of metallo-β-lactamase producers:

The 107 (GNB) isolates were screened for their ability to produce MBLs using the double disc synergy test in which two MBL inhibitors; ethylene diamine tetra-acetic acid (EDTA) and 2-mercapto propionic acid (MPA) along with imipenem (IPM) or ceftazidime (CAZ) were used (Arakawa et al., 2000). Overnight broth cultures (200 µl) of the tested isolates (adjusted to 0.5 Mcfarland) were used to inoculate Muller- Hinton agar plates. Two blank filter paper discs impregnated with 3 µl of (MPA, 97%) (Johnson Matthey Company) and 5 µl of EDTA (0.5 M) separately were placed on pre-inoculated Muller-Hinton agar plates. Imipenem (10 µg) disc was placed 20 mm away from the MPA discs and 10 mm away from the EDTA disc. The same was done for ceftazidime (30 µg). Two filter paper discs impregnated with 3 µl of MPA and 5 µl of EDTA were used as a control. After overnight incubation at 35°C, any enlargement of the inhibition zone between the two discs (antibiotic and EDTA or MPA) was considered as positive for the presence MBL enzyme (Franklin et al., 2006).

### Susceptibility test:

Isolates were examined for their resistance pattern by the disc agar diffusion method according to the guidelines of CLSI (2002) using 21 antibiotics belonging to different groups: Antibiotics inhibiting the cell wall synthesis including; Piperacillin (PRL 100 µg), Cephadrine (CE 30 µg), Cefpodoxime (CPD 30 µg), Ceftazidime (CAZ 30 µg), Cefotaxime (CTX 30 µg), Cefepime (FEP 30 µg), Imipenem (IPM 30 µg), Meropenem (MEM 30 µg) and Aztreonam (ATM 30 µg). Antibiotics inhibiting protein synthesis including; Amikacin (AK 30 µg), Gentamycin (CN 120 µg), Tetracycline (TE 30 µg), and Azithromycin (AZM 15 µg). Antibiotics inhibiting the nucleic acid synthesis, including; Levofloxacin (LEV 5 µg) and Ciprofloxacin (CIP 5 µg). Antibiotics inhibiting the cell membrane synthesis; Colistin sulphate (CT 25 µg). Antibiotics act as competitive inhibitors; Sulphamethoxazole Trimethoprim (SXT 25 µg). Antibiotics in combination with β-lactamaase inhibitor; Piperacillin/Tazobactam (TZP 100 µg), Amoxycillin/Clavulanic acid (AMC 30 µg), Ampicillin/Sulbactam (SAM 20 µg) and Cefoperazone/Sulbactam (SCF 105 µg).

### Determination of minimum inhibitory concentrations (MIC) for imipenem and ceftazidime:

The MICs of selected isolates that showed positive for the presence MBL enzyme were determined using commercial IPM powder (Tinam 1 g from Merk sharp) and CAZ (Fortum 1 g from GlaxoSmithKline),
according to (Miles and Amyes, 1996) and (Parekh and Chanda, 2006).

**Detection of metallo-β-lactamase coding genes:**

Polymerase Chain Reaction (PCR) analysis was performed for *blaSM*, *blaIM*, *blaVIM*, *blaSIM*, *blaIMP* and *blaNDM* to study the prevalence of these types in Egyptian isolates. Colony PCR reactions were carried out using Applied Biosystems 2720 Thermal Cycler, Singapore. A loopful of overnight grown cells was transferred to 25 µl of MQ-water and boiled for 5 min. Then, 1 µl of cell suspension was used as DNA template for PCR reaction. PCR was performed using Fermentus Dream Taq Mastermix according to instruction manual. The universal primers and references for PCR conditions for the amplification of MBL-β-lactamases were shown in table 1.

<table>
<thead>
<tr>
<th>Oligonucleotide primers</th>
<th>PCR products</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>blaSIM</em> -F 5'-CTACCGCCACAGACTCTTGG-3'</td>
<td>587 bp</td>
<td>Senda et al., 1996</td>
</tr>
<tr>
<td><em>blaIMP</em> -R 5'-AACACATTTGGCGCCACATC-3'</td>
<td>387 bp</td>
<td>Miriajou et al., 2003</td>
</tr>
<tr>
<td><em>blaVIM</em> -F 5'-AGT GTGTGATATCGACAG-3'</td>
<td>271 bp</td>
<td>Ellington et al., 2007</td>
</tr>
<tr>
<td><em>blaSIM</em> -R 5'-AGT AAG TGC GGT GGA-3'</td>
<td>477 bp</td>
<td>Ong et al., 2011</td>
</tr>
<tr>
<td><em>blaVIM</em> -R 5'-TGG GTA CGC AAA CG-3'</td>
<td>570 bp</td>
<td></td>
</tr>
<tr>
<td><em>blaIMP</em> -R 5'-AAC TCCCAA CTT GGC CAT GC-3'</td>
<td>127 bp</td>
<td></td>
</tr>
<tr>
<td><em>blaNDM</em> -F 5'-TAC AAG GGA TTC GGCATC G-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>blaSIM</em> -R 5'-TAA TGG CCT GGT CCA GC CAT GC-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>blaVIM</em> -R 5'-GAA GCT GAG CAC CGC ATT AG-3'</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>blaIMP</em> -R 5'-GGG CCG TAT GAG TGA TTG C-3'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The PCR products were analyzed by electrophoresis with 1.0% agarose gel in TAE buffer. The gels were stained with ethidium bromide, and visualized with GelDoc. Ingenius 3.

**Identification of some selected isolates of MBL producer:**

Fourteen multidrug resistant Gram negative pathogenic isolates, positive to MBL screening test were selected and identified using Api20E strips tests (bioMe'rieux, Marcy-‘Etoile, France).

**RESULTS:**

Sensitivity test for CAZ was carried out for all isolates supplied from the clinical microbiology laboratory. One hundred and seven Gram negative bacilli showed resistance to CAZ. All these isolates were subjected to DDST to detect the MBL producers. Positive isolates with both substrate-inhibitor combinations on DDST were considered MBL producers. Accordingly, among the 107 isolates, 14 bacterial isolates showed production of MBL enzymes and were chosen for further investigation, taking into consideration that, they were not inhibited by EDTA disc alone to avoid misleading results.

All isolates were identified using Api20E strips. The isolates were *Pseudomonas fluorescens* (No. 83, 88, 101, and 102), *Klebsiella pneumoniae* (No. 65, 78, and 84) *Pseudomonas aeruginosa* (No. 5 and 92), *Proteus mirabilis* (No. 1), *Escherichia coli* (No. 17), CDC group (No. 18), *Enterobacter cloacae* (No. 63) and *Pseudomonas* spp. (No. 10).

All MBL-producers were found to be completely resistant (100%) to PRL, TZP, AMC, CE, CPD, CAZ, CTX, TE, AZM and SXT in addition, MBL-producers showed variable resistance towards the rest of the antibiotics used. ATM did not inhibit any of the tested MBL-producers except isolate No. 92. However, CT was able to inhibit the growth of only four isolates No. 17, 63, 65, and 84 as shown in figure 1.
64 and 2-32 µg/ml respectively. Isolate No. 5 showed the highest MIC with CAZ (64 µg/ml), while isolates No. 83, 88, and 101 exhibited the highest MICs with IPM (32 µg/ml). Table 2. Isolate no, source of isolation, age, gender, minimum inhibitory concentrations of (CAZ) and (IPM) and type of MBL-β-lactam genes present in each selected MBL-producers.

<table>
<thead>
<tr>
<th>Isolate (Isolate no.)</th>
<th>Source of isolate</th>
<th>Detected MBL-β-lactam genes with PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudomonas fluorescence 83</td>
<td>Throat catheter</td>
<td>blaIM, blaSPM, blaGIM, blaSIM</td>
</tr>
<tr>
<td>Pseudomonas fluorescence 88</td>
<td>Sputum specimens</td>
<td>blaIM, blaSPM</td>
</tr>
<tr>
<td>Pseudomonas fluorescence 101</td>
<td>Sputum specimens</td>
<td>blaIM, blaSPM</td>
</tr>
<tr>
<td>Pseudomonas fluorescence 102</td>
<td>Sputum specimens</td>
<td>blaIM, blaSPM</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa 5</td>
<td>Urine sample</td>
<td>blaIM, blaSIM</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa 92</td>
<td>Wound/pus swab</td>
<td>blaIM, blaSPM, blaSIM</td>
</tr>
<tr>
<td>Pseudomonas spp.10</td>
<td>Urine sample</td>
<td>blaSPM, blaSIM</td>
</tr>
<tr>
<td>Klebsiella pneumoniae 65</td>
<td>Sputum specimens</td>
<td>blaIM, blaSIM</td>
</tr>
<tr>
<td>Klebsiella pneumoniae 78</td>
<td>Sputum specimens</td>
<td>blaSPM, blaSIM</td>
</tr>
<tr>
<td>Klebsiella pneumoniae 84</td>
<td>Blood sample</td>
<td>N.D</td>
</tr>
<tr>
<td>Proteus mirabilis 1</td>
<td>Throat swab</td>
<td>blaGIM</td>
</tr>
<tr>
<td>Enterobacter cloacae 63</td>
<td>Wound/pus swab</td>
<td>blaSPM, blaSIM</td>
</tr>
</tbody>
</table>


PCR was used to detect the presence of the 6 most common MBL-β-lactam genes. All isolates were found to harbour one or more MBL-β-lactam genes except Klebsiella pneumoniae 84 which did not show any presence of the 6 tested β-lactam genes. Four β-lactam genes (blaIM, blaSPM, blaGIM, blaSIM) were detected in only one isolate; Pseudomonas fluorescence 83. Klebsiella pneumoniae 65, 78, and Pseudomonas aeruginosa 92 were found to have 3 different MBL-β-lactam genes. Meanwhile, blaIM and blaSPM were present in Pseudomonas fluorescence 88, 101, and 102, Pseudomonas aeruginosa 5 and Enterobacter cloacae. Pseudomonas spp. harboured blaSPM and blaSIM. The remaining 3 isolates showed the presence of only one type of blaMBl-genes (Fig. 2 & Table 2). blaSPM was the most predominant gene, detected in 9 isolates and blaIM was detected in 8 out 14 isolates. On the other hand, blaGIM gene was not detected in any of the tested isolates.

It was noticed that, the MBL-producing isolates were more frequent in female than male patients. Moreover, MBL-producing isolates were recovered mainly from the respiratory tract infections followed by wound infection (Table 2).

**DISCUSSION:**

Gram negative bacilli (GNB) are pathogens that cause a wide range of opportunistic infections and nosocomial outbreaks. Their high intrinsic resistance to antibiotics and ability to develop multidrug resistance pose serious therapeutic problems. Broad-spectrum β-lactams, such as carbapenems, are potential drugs for treatment infections caused by multi-drug resistant GNB. However, the increasing use of these compounds has resulted in the emergence of carbapenem-resistance among GNB, thus limiting their treatment options. During the last few years GNB including Enterobacteriaceae and Pseudomonas spp.
has been increasingly isolated from severely ill patients and they were usually showed cross-resistance to most available antimicrobial drugs, including carbapenems (Wang et al., 2014).

One of the major causes behind carbapenem resistance is the production of Metallo-β-lactamase enzyme (MBL). Metallo-β-lactamase producing Gram negative organisms have been reported in many geographic regions (Walsh et al., 2005). Due to their ability to disseminate rapidly, early laboratory detection is of great clinical importance (Franklin et al., 2006). MBL producing organisms are characterized by their ability to hydrolyze most commonly used antibiotics including extended spectrum cephalosporins (3rd and 4th generation of cephalosporins), quinolones, aminoglycosides and antibiotics combined with β-lactamase inhibitor; SAM, AMC, TZP, and SCF.

Results of this study indicated that 14 isolates that that showed production of MBL out of 107 GNB were resistant to CAZ, as well as high resistance to other antibiotics used in this study (Lee et al., 2008; Galani et al., 2008). Franco et al. (2010), and Chen and Wang (2013) indicated that aztreonam (ATM) and colistin sulphate (CT) could be the drugs of choice in case of MBL-producing organisms since ATM disc produced inhibition zone >30mm in 63% of MBL-carrying isolates. However, in this study, all isolates showed 100% and >70% resistance to ATM and CT, respectively. As a result, this poses further challenge to prescribe such antibiotics treatment infections caused by MBLs producers.

Primary screening for MBLs production indicated by the resistance to CAZ (Nishio et al., 2004) and confirmed by addition of metal chelating agents as inhibitors such as EDTA or MPA to CAZ or IPM (Lee et al., 2003; Franklin et al., 2006; Giske et al., 2008; Samulesen et al., 2008). In this study, phenotypic detection of MBLs was detected by DDST technique because it is simple to perform making it highly applicable to routine clinical laboratories (Franco et al., 2010).

In this work, identification of potentially metallobeta lactamase producers 14 isolates (13.08%) revealed that; 4 isolates Pseudomonas fluorescence, 3 isolates Klebsiella pneumoniae, 2 isolates of Pseudomonas aeruginosa, one isolate of each of the following Proteus mirabilis, Enterobacter cloacae, Pseudomonas spp. Escherichia coli and CDC group. Metallo-β-lactamase production was thought to be common only in Pseudomonas aeruginosa and Acinetobacter spp (Yong et al., 2002; Lee et al., 2003; Irfan et al., 2008; Yousefia et al., 2010). Recently MBLs are increasingly detected in members of Enterobacteriaceae (Franklin et al., 2006) as demonstrated in this work.

Previous work indicates the possible correlation between the high MIC and the presence of MBL genes (Senda et al., 1996; Galani et al., 2008). Such correlation was not obvious in the present study, the MIC of IPM was relatively low and varied from 2 to 32 µg/ml however bla<sub>MBL</sub> genes were detected in 92.8% of tested isolates. Similar observations were reported by (Nishio et al., 2004; Galani et al., 2008; Lee et al., 2008).

PCR detection assay continue to be the golden method to confirm the presence of MBL genes. To date, nine MBL types, namely, IMP-like, VIM-like, SPM-1, SIM-1, SIM-1, NDM-1, DIM-1, TMB-1 and KHM-1 are identified in Gram negative bacilli worldwide. The IMP and VIM types are the most commonly detected MBLs in P. aeruginosa, while, VIM-types MBLs are predominant in the Mediterranean region (Avlami et al., 2010). The results of this study contradict with those findings. IMP was not detected in any of the examined MBL producers. SPM was the most predominant bla gene in this study (69.23%) unlike the finding of Toleman et al. (2002) who reported that SPM-1 gene is not widely detected worldwide. Moreover, the newly discovered blaNDM-1 which was detected in New Delhi-India in 2010, was found in two of studied local Egyptian isolates (14.28%).

Previous studies by Lee et al. (2008) indicated possible existence of more than one bla<sub>MBL</sub> gene in the same bacteria, they reported the presence bla<sub>VIM</sub> and bla<sub>IMP</sub> in S. marcescens. In this study four genes were detected in Pseudomonas fluorescens 83 (bla<sub>VIM</sub>, bla<sub>SPM</sub>, bla<sub>SIM</sub>, and bla<sub>NDM</sub>). Three isolates; Klebsiella pneumonia 65, 78, and Pseudomonas aeruginosae 92 contained three different genes. Klebsiella pneumonia 65, 78, and Pseudomonas aeruginosae 92 carried bla<sub>VIM</sub>, bla<sub>SIM</sub>, and bla<sub>SPM</sub>, bla<sub>SIM</sub>, and bla<sub>NDM</sub>, respectively. Six isolates carried two genes, while three isolated carried one gene.

The distribution of MBL genes in different organisms suggested possible high genetic transfer, many authors reported that MBLs are carried on gene cassettes inserted into class 1 integrons in clinical isolates of Pseudomonas spp. and Enterobacteriaceae. This finding suggests the horizontal dissemination of the class1 integron-associated MBL genes that may contribute to the emerging threat of carbapenem resistance among various Gram-negative isolates (Poirel et al., 2000).

Although bla<sub>IMP</sub> is considered to be the first and most widely prevailing gene all over the world since 1996, it was not detected in any of the local isolates. This could be attributed to presence of different alleles of IMP genes (Senda et al., 1996).
انتشار جينات الميتابيتالاكلتاميز في البكتيريا العصوية سارية الجراثيم والمقاومات للعديد من المضادات الحيوية

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في هذه الدراسة تم جمع 107 عزلة بكتيرية عصوية سارية جراثيم والمقاومة للسيكاربين من معمل الميكروبيولوجيا الطبية في مستشفى عمان بالقاهرة، مصر. تم استخدام أنماط مجهولة الحقيقة في اختبار الاحترام من حيث قدرتها على إنتاج الميتابيتالاكلتاميز باستخدام أكس أيبام واميلابينام مبتنية اسوات الإيثيلين دي أمين رادي حمض الألفا-ثناي مركبات حمض البروبيسام كمتحدة. تم اختبار 14 عزلة أتية التي تم تطهيرها بالاختبارين وليس مع الادنا لمزيد من الاختبارات. تم تقييم العزلات باستخدام شرائط API 20E (1)، سيدوموسا ثوان، سيدوموسا ميرابلس (2)، سيدوموسا نوج، سيدوموسا آريلاريا (3)، كوليا، كوليا ومجمعة CDC. وقد وجد أن العزلات البكتيرية المقاومة للسيكاربين تم تراوح بين 64 و32 ميكروغرام/مل على النواحي. استمرت نتائج استخدام.

المكونات:
- D. محمد عبد الله، قسم الميكروبيولوجي، كلية العلوم، جامعة عين شمس.
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