Designing a Multiplex PCR for Rapid and Accurate Detection of Metallobetalactamases Resistant Genes from Acinetobacter baumannii Isolates in Tehran City, Iran

Zahra Mottaghiyan1, Davoud Esmaeili2, Mohammad Hossein Ahmadi1, Mohammad Niakan1*

1. Department of Microbiology, Faculty of Medicine, Shahed University, Tehran, Iran
2. Bagiyatallah University of Medical Sciences, Tehran, Iran

KEYWORDS
Acinetobacter baumannii; MBL genes; Multiplex PCR;

ABSTRACT

Background & Objective: Acinetobacter baumannii strains harboring Metallobetalactamases (MBL) pose a significant threat in the context of nosocomial infections. The present investigation was undertaken with the objective of devising a Multiplex PCR methodology for the concurrent detection of MBL genes within A. baumannii strains prevalent in Tehran City, Iran.

Methods: Between October 2020 and February 2021, 100 strains of A. baumannii were procured from burn specimens of hospitalized patients at Motahhari Hospital in Tehran. The identification of A. baumannii strains involved conventional biochemical techniques, coupled with confirmation of the presence of the bla OXA-51 gene. Antibiotic susceptibility was assessed using the Kirby–Bauer disc diffusion test. MBL-producing strains were characterized through a phenotypic approach employing the combined disk test, alongside Multiplex PCR for the simultaneous identification of bla VIM, bla IMP, bla GIM, and bla NDM genes. Statistical analyses were conducted using the chi-square test, with SPSS version 20.0 employed for data processing.

Results: Among 100 strains examined, 96.1% exhibited positivity for MBL, as determined by the combined disk test. The study revealed a predominance of extensively drug-resistant (XDR) strains, with colistin demonstrating the highest level of sensitivity. The genotypic assay unveiled that Multiplex PCR identified bla VIM, bla NDM, and bla IMP in 20 strains, bla VIM and bla NDM in 30 strains, and exclusively the bla NDM gene in 45 strains. Notably, the Multiplex PCR technique exhibited the capacity to concurrently detect MBL genes (bla VIM, bla IMP, bla GIM, and bla NDM) in 2 strains.

Conclusion: The current investigation underscores prevalence of the bla NDM gene within clinical strains of A. baumannii. Furthermore, Multiplex PCR emerges as a robust and highly sensitive technique for rapid discernment of the MBL genes within in A. baumannii strains.

Introduction

Acinetobacter baumannii is a life-threatening and significant opportunistic pathogen commonly found in hospitals (1). Its inherent capabilities including survival in challenging environmental conditions and acquisition of antibiotic resistance mechanisms (2, 3), have resulted in the emergence of multidrug resistance (MDR), extensive drug resistance (XDR), and even PDR phenotypes, posing considerable challenges to healthcare systems and the health community. Notably, the ability to develop resistance to carbapenems, which are broad-spectrum β-lactam antibiotics, is a critical characteristic of A. baumannii (4, 5).

The carbapenems class are the most effective antibiotics for treatment of A. baumannii infections. The primary mechanism for carbapenem resistance involves the production of β-lactamase enzymes (6, 7). In A. baumannii, four molecular classes of β-lactamases (A, B, C, and D) have been identified (8). Among these, Class B β-lactamases, known as Metallo-β-lactamases (MBL), need zinc ions for their catalytic activity (9). MBLs exhibit the ability to hydrolyze all beta-lactam classes except monobactams (10). Examples of MBLs in A. baumannii include bla IMP, bla VIM, bla GIM, bla DIM, bla SPM, bla SIM, and bla NDM that bla IMP and bla VIM allelic variants as predominant MBLs globally (10, 11).
Infectious diseases caused by MBL-producing organisms are associated with elevated mortality and morbidity rates (12). In recent years, due to the Sars-Cov-2 pandemic, there has been a surge in the prevalence of MDR, XDR, and even PDR A. baumannii infections, posing significant challenges for hospitalized patients. Therefore, utilization of rapid diagnostic methods, such as Multiplex PCR, enables simultaneous detection of the drug resistance genes, aiding physicians in selecting appropriate antibiotics. The current study represents the first attempt to design a multiplex PCR method for the concurrent detection of MBL genes (bla VIM, bla IMP, bla GIM, bla NDM) from A. baumannii strains isolated in Tehran, Iran.

Material and Methods

Table 1. Prevalence of A. baumannii isolated from the Motahhari Hospital patients to type of infections.

<table>
<thead>
<tr>
<th>Sample type</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum</td>
<td>25</td>
</tr>
<tr>
<td>Urine</td>
<td>26</td>
</tr>
<tr>
<td>Catheter</td>
<td>5</td>
</tr>
<tr>
<td>Trachea</td>
<td>12</td>
</tr>
<tr>
<td>Wound</td>
<td>10</td>
</tr>
<tr>
<td>Blood</td>
<td>5</td>
</tr>
<tr>
<td>Cerebrospinal fluid (CSF)</td>
<td>7</td>
</tr>
<tr>
<td>Respiratory tract</td>
<td>1</td>
</tr>
</tbody>
</table>

Identification of Acinetobacter baumannii Strains

For the initial identification of A. baumannii strains, conventional biochemical techniques including growth on MacConkey agar, motility, sugar fermentation, triple sugar iron (TSI), colony morphology, positive catalase, citrate, negative oxidase, growth at 44°C, and sulfide indole motility (SIM) were employed. The \( \text{bla}_{\text{OXA-51}} \) gene was used to validate the strain identification. The PCR method was used to detect the \( \text{bla}_{\text{OXA-51}} \) gene in A. baumannii isolates using specific primers (Table 2). PCR conditions were consistent with those of the Multiplex PCR. Each verified strain was inoculated into a vial containing Brain Heart Infusion broth with 20% glycerol and stored at -80°C.

Antimicrobial Susceptibility Testing

Antimicrobial susceptibility was assessed by conducting a Kirby-Bauer disc diffusion test with a 0.5 McFarland bacterial suspension on Mueller Hinton agar. In accordance with CLSI recommendations (13), nine antibiotic discs were utilized to evaluate isolates for MDR and XDR using ampicillin (10 μg), cefotaxime (30 μg), ceftriaxone (30 μg), imipenem (10 μg), ciprofloxacin (5 μg), amikacin (30 μg), gentamicin (10 μg), cefepime (30 μg), and colistin (10 μg). Escherichia coli ATCC 25922 and A. baumannii ATCC 19606 were employed as negative and positive controls, respectively.

Phenotypic Detection of the MBL-producing Strains by Combined Disc Test

Initially, a bacterial suspension was prepared based on the 0.5 McFarland standard and streaked on Muller Hinton agar medium. The beta-lactam disc was immersed in 0.5 M EDTA, placed on the Muller Hinton agar medium, and positioned alongside the beta-lactam disc alone. Following an 18-hour incubation at 37°C, the diameter of the inhibition zone around the discs was measured. If the diameter around the imipenem/EDTA disc compared to imipenem alone increased by 7 mm or more, it indicated the presence of the metallo-beta-lactamase enzyme.

Multiplex PCR for Detection of bla VIM, bla IMP, bla GIM, and bla NDM Genes

The MBL gene sequences were initially verified on the NCBI website. Primers were designed using the Genscript program. Results of the blasting of the Forward and Reverse primers (https://blast.ncbi.nlm.nih.gov/Blast.cgi) indicated the suitability of the proposed primers. Primers were subsequently tested in an online in silico PCR amplification program after evaluation by the Oligo Analyzer software for specifically generated primers (http://insilico.ehu.es). As a result, specific primers for VIM, IMP, GIM, and NDM (Table 2), were employed to amplify the genes using multiplex PCR. Various genome dilutions were used to assess PCR sensitivity. Sequential dilutions of nucleic acids were created, and
subsequent genomic dilutions underwent PCR. The
dilution at which PCR results were obtained at the
lowest concentration was considered. PCR reactions
were carried out on the nucleic acids of *Bacillus
subtilis* and *Staphylococcus aureus* to establish primer
specificity. Genomic DNA was extracted using the
Bioneer kit (Bioneer, South Korea), and the
concentration of collected DNA was measured using
Nanodrop spectrophotometry. The MBL genes *
bla* _VIM_, *bla* _IMP_, *bla* _GIM_, and *bla* _NDM_ were identified using the
multiplex PCR approach. The reaction mixture
comprised template DNA, Master mix (cat. No.
180301-50), and Forward/Reverse primers. The total
reaction volume was 25 μL. Amplification conditions
for *bla* _VIM_, *bla* _IMP_, *bla* _GIM_, and *bla* _NDM_ included an
initial denaturation for 5 minutes at 94°C, followed by
35 cycles at 94°C for 40 seconds, annealing at 60°C for
25 seconds, extension at 72°C for 1 minute, and a final
extension at 72°C for 5 minutes. Agarose gel
electrophoresis at 1% (w/v) was conducted at 80 V for
60 minutes in 1X TBE, followed by multiplex PCR
analysis to confirm the presence of MBL genes.

### 2.6. Statistical Analysis

Data were analyzed using a Chi-square test using
SPSS software version 20.0 (SPSS Inc., Chicago, IL.,
USA). A *P*-value < 0.05 was considered statistically
significant.

### Table 2. Primers are used in amplification of the selected genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Nucleotide sequence</th>
<th>Amplicon size (bp)</th>
<th>Tm(°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMP</td>
<td>F</td>
<td>TCCAGAAACCTTGACCGAAGC</td>
<td>466</td>
<td>60</td>
</tr>
<tr>
<td>IMP</td>
<td>R</td>
<td>CAGCTCCAAACAAAGCTG</td>
<td>466</td>
<td>60</td>
</tr>
<tr>
<td>GIM</td>
<td>F</td>
<td>CAGCAAGCTGTAGCGCTC</td>
<td>928</td>
<td>60</td>
</tr>
<tr>
<td>GIM</td>
<td>R</td>
<td>GACTATCGTCGCCGACTTA</td>
<td>928</td>
<td>60</td>
</tr>
<tr>
<td>VIM</td>
<td>F</td>
<td>CCACTGCGATCCCCGGAAA</td>
<td>232</td>
<td>63</td>
</tr>
<tr>
<td>VIM</td>
<td>R</td>
<td>ACAGGCCAGCCATTAGCTTC</td>
<td>232</td>
<td>63</td>
</tr>
<tr>
<td>NDM</td>
<td>F</td>
<td>CGGCACCACATCGCTTTTG</td>
<td>160</td>
<td>63</td>
</tr>
<tr>
<td>NDM</td>
<td>R</td>
<td>GCCGGGAATGGCTCATACGA</td>
<td>160</td>
<td>63</td>
</tr>
<tr>
<td><em>bla</em> <em>OXA-51</em></td>
<td>F</td>
<td>AGGACATGACCCATGGCGAT</td>
<td>166</td>
<td>60</td>
</tr>
<tr>
<td><em>bla</em> <em>OXA-51</em></td>
<td>R</td>
<td>AAAGGACCACCGACCACCGA</td>
<td>166</td>
<td>60</td>
</tr>
</tbody>
</table>

### Results

#### Characteristics of the Clinical Strains

In this study, 100 *A. baumannii* strains were
gathered from different clinical sources in burn patients
of Motahhari Hospital as follows: sputum 25%, urine
26%, trachea 12%, wound 10%, cerebrospinal fluid
(CSF) 7%, catheter 5%, blood 5%, and respiratory tract
1% (*Table 1*).

#### Antimicrobial Sensitivity Testing

The Kirby-Bauer disc diffusion technique was used
to test all 100 *A. baumannii* strains on Mueller-Hinton
agar against a panel of 9 antibiotic discs. The findings
were then analyzed by CLSI recommendations (13).
Numerous isolates were XDR (*Figure 1*).
The proportion of strains that were resistant to ampicillin
(100%), ceftazidime (100%), and imipenem (100%)
was notably high. Additionally, the percentage of
antibiotic resistance among isolates to other antibiotics
was as follows: cefotaxime (97%), ciprofloxacin
(95%), ceftiraxone (94%), amikacin (89%), and
genamycin (85%). According to the results, *A.
baumannii* strains exhibited higher sensitivity (100%)
to colistin.

#### Phenotypic Detection of MBL-Producing Strains (Combined Disc Test)

The Combined Disk Test method was employed to
determine MBL-positive isolates under phenotypic
conditions. If the difference between the inhibition
zones of the imipenem/EDTA disc and imipenem disc
was ≥7mm, the combined disk test was interpreted as
positive for MBL. Detection of Metallo β-Lactamase
in phenotype conditions elucidated that 96.1% of
strains could produce MBL.

#### Detection of *bla* _VIM_, *bla* _IMP_, *bla* _GIM_, and *bla* _NDM_ Genes

Detection of the MBL (*IMP*, *VIM*, *GIM*, *NDM*)
genewes with specially designed primers by multiplex
PCR is presented in *Table 3*. To designate sensitivity
of the MBL primers, serial dilutions ranging from 10^-14_
to 10^-16_ were used. The lowest dilution at which the
PCR result was positive was considered as PCR
sensitivity. Moreover, a significant correlation
(*P*<0.05) between Ciprofloxacin (5 μg) and NDM was
observed (*Table 4*).
Table 3. Frequency of the MBL genes in 100 XDR Acinetobacter baumannii strains.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bla IMP, bla VIM, bla GIM, bla NDM</td>
<td>2</td>
</tr>
<tr>
<td>bla IMP, bla VIM, bla NDM</td>
<td>20</td>
</tr>
<tr>
<td>bla VIM, bla NDM</td>
<td>30</td>
</tr>
<tr>
<td>bla NDM</td>
<td>45</td>
</tr>
</tbody>
</table>

Table 4. Relation between antimicrobial agents and gene frequency in A. baumannii strains (R= Resistance, I= Intermediate, S= Sensitive, P= Positive, N= Negative)

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>VIM</th>
<th>IMP</th>
<th>GIM</th>
<th>NDM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>N</td>
<td>P</td>
<td>N</td>
</tr>
<tr>
<td>Cefotaxime (30μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>I</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>R</td>
<td>52</td>
<td>45</td>
<td>22</td>
<td>75</td>
</tr>
<tr>
<td>p-v</td>
<td>0.65</td>
<td>0.66</td>
<td>0.86</td>
<td>0.86</td>
</tr>
<tr>
<td>Ciprofloxacin (5μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>4</td>
<td>1</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R</td>
<td>50</td>
<td>45</td>
<td>23</td>
<td>72</td>
</tr>
<tr>
<td>p-v</td>
<td>0.23</td>
<td>0.21</td>
<td>0.81</td>
<td>0.00</td>
</tr>
<tr>
<td>Gentamicin (10μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>4</td>
<td>6</td>
<td>3</td>
<td>7</td>
</tr>
<tr>
<td>I</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>R</td>
<td>47</td>
<td>38</td>
<td>19</td>
<td>66</td>
</tr>
<tr>
<td>p-v</td>
<td>0.63</td>
<td>0.85</td>
<td>0.91</td>
<td>0.91</td>
</tr>
<tr>
<td>Amikacin (30μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>3</td>
<td>6</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>I</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>R</td>
<td>49</td>
<td>40</td>
<td>19</td>
<td>70</td>
</tr>
<tr>
<td>p-v</td>
<td>0.19</td>
<td>0.21</td>
<td>0.93</td>
<td>0.93</td>
</tr>
<tr>
<td>Cefepim (30μg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>I</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>R</td>
<td>51</td>
<td>43</td>
<td>20</td>
<td>74</td>
</tr>
<tr>
<td>p-v</td>
<td>0.83</td>
<td>0.1</td>
<td>0.8</td>
<td>0.8</td>
</tr>
</tbody>
</table>

Fig. 1. Antibiotic susceptibility testing of 100 A. baumannii isolates against a panel of 9 Antibiotics.
Discussion

Acinetobacter baumannii, identified as a critical nosocomial pathogen, poses a significant threat to human health. MBLs represent the most effective β-lactamases capable of hydrolyzing all β-lactams, with the exception of monobactams, and remain unhampered by any known inhibitor (14, 15). Given the exception of monobactams, and remain -lactams, with lactamases capable of hydrolyzing all β-

human health. MBLs represent the most effective β-
nosocomial pathogen, poses a significant threat to

OXA-48, bla multiplex PCR approach targeting
MBL gene detection. Ranjbar et al., with unerring specificity for simultaneous
A. baumannii strains (16).

Numerous investigations have documented prevalence of the MDR and XDR strains of A. baumannii, with the majority exhibiting sensitivity solely to colistin. Our findings align with those of Soltani et al., (17), who reported a 92.4% incidence of XDR strains in their study. Asadian et al., (18) observed 100% XDR prevalence among their 79 strains. In 2019, Girija et al., (19) noted rates of 71.23% MDR and 39.72% XDR. Another study reported MDR and XDR frequencies of 83.9% and 16.1%, respectively, with colistin-resistant A. baumannii identified in 7.6% of cases. In spite of these findings, Monfareed concluded that colistin remains the most efficacious treatment option presently available (20).

Our investigation demonstrated that 96.1% of isolates exhibited phenotypic MBL production, as determined by the Combined Disc Test. A comparable study by Saleh et al., investigated MBL phenotypic detection via the Combined Disc Test in 52 imipenem-resistant A. baumannii strains, revealing that 90.4% produced MBL (21). In a 2022 Indian study, 70.5% of carbapenem-resistant A. baumannii strains were identified as MBL producers (CDT positive). Rouf et al., underscored the cost-effectiveness and routine applicability of combined disk tests and other phenotypic assays for carbapenemase producers. However, for a comprehensive assessment, they advocated exploration of the resistance genes through genotypic methods (22).

In our study, specific primers were employed for simultaneous and rapid detection of the MBL genes, encompassing bla VIM, bla IMP, bla GES, and bla NDM. The outcomes evinced concordance between phenotypic and genotypic tests for clinical A. baumannii strains, affirming the 100% specificity of the designed primers. While numerous studies have detected MBL genes in A. baumannii isolates using PCR, only a minority have undertaken simultaneous identification of these genes. Given the high prevalence of antibiotic resistance in A. baumannii strains in Iran, we opted for designed primers with unerring specificity for simultaneous MBL gene detection. Ranjbar et al., employed a multiplex PCR approach targeting bla OXA-48, bla NDM, and bla OXA-23 genes, and reported reasonable specificity for concurrent MBL gene detection in A. baumannii strains (23). Massik et al., identified MBL genes, including bla IMP, bla OXA-23, bla VIM, and bla OXA-51, via multiplex PCR. They ascertained presence of bla OXA 51 gene in all strains, while bla OXA-23 gene was detected in 53 isolates (91%). However, MBL genes were not identified using multiplex PCR. In light of these discordant genotypic and phenotypic results, the authors posit two potential explanations: firstly, EDTA may serve as a source of false positives, as prior studies have suggested its permeabilizing effect may enhance membrane sensitivity in Gram Negative Bacteria (GNB); secondly, a more comprehensive exploration of additional MBL genes would have facilitated a more accurate conclusion (24). In a study by Raieszadeh et al., primers targeting ESBL genes (KPC, AMPC, TEM) were able to concurrently detect positive control samples through multiplex PCR. Raieszadeh concluded that rapid identification methods are imperative for a precise and swift detection of antibiotic resistance genes. Diverse factors, including improper usage of antibiotics, prolonged hospitalization, sample type, divergent diagnostic methodologies for gene identification, geographical variables, as well as patient gender and age, account for variability in the research outcomes (25, 26).

Conclusion

This study showed that the Multiplex PCR method, boasting 100% specificity, offers a reliable means of detecting MBL genes in A. baumannii isolates. The findings of this study, in conjunction with other pertinent research, underscore an escalating trend in antibiotic resistance among clinical strains of A. baumannii. The temporal alignment of patient sampling with the Sars-Cov-2 pandemic led to heightened usage of broad-spectrum antibiotics, consequently exacerbating resistance to conventional antibiotics in hospital strains of A. baumannii.

Acknowledgments

None.

Funding

No funds, grants, or other support was received.

Ethics approval:

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of University B (July 27, 2020/ IR.SHAHED.REC.1399.064).

Informed consent was obtained from all individual participants included in the study.

Conflict of Interest

The authors have no competing interests to declare that are relevant to the content of this article.
References


19. As SG. CLSI based antibiogram profile and the detection of MDR and XDR strains of Acinetobacter baumannii isolated from urine samples. Med J the Islamic Republic Iran. 2019;33:3.

20. Monfared AM, Rezaei A, Poursina F, Faghri J. Detection of genes involved in biofilm formation


