



# Application of Multiplex PCR for the Identification of Oxacillinase Genes and Determination of Antibiotic Resistance Pattern in Environmental Isolates of *Acinetobacter baumannii* in ICU

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## Abstract

**Background:** *Acinetobacter baumannii* is a common cause of nosocomial infections. A prominent feature of these bacteria is resistance to carbapenems. This study aimed to identify *OXA* genes encoding oxacillinase in *Acinetobacter baumannii* isolates.

**Methods:** This cross-sectional descriptive study was performed on 25 environmental *A. baumannii* isolates collected from ICU over 8 months. Definitive identification of isolates was performed by biochemical tests and polymerase chain reaction (PCR) of *16s rRNA* gene. Antibiotic susceptibility testing was performed on Müller-Hinton agar medium by disk diffusion and E-test. Antibiogram and multiplex PCR data of beta-lactamase genes were collected and analyzed at a significance level of  $P < 0.05$  using SPSS 22.0.

**Results:** Except for one isolate, all isolates (96%) were sensitive to polymyxin B and 80% of isolates were sensitive to oxacillin. All isolates were sensitive to meropenem, ampicillin/sulbactam, gentamicin, amikacin, piperacillin, and carbenicillin. The results showed that 25 isolates (100%) had *OXA-51* gene, 21 isolates (84%) had *OXA-58* gene, one isolate (4%) had *OXA-24* gene, and none of the isolates contained *OXA-23* gene. Only isolate No.10 had three oxacillinase genes simultaneously and it was resistant to oxacillin, polymyxin B, and cephalothin.

**Conclusions:** The study showed that environmental isolates of ICU do not have pathogenic genes present in the clinical isolates, and how these genes are transferred to the peripheral isolates is an important point that should be studied. Identification of genes encoding carbapenem resistance may help to understand the mechanisms of resistance transfer in *A. baumannii*. The lack of the *OXA-23* gene plays an important role in the susceptibility of isolates to antibiotics and non-emergence of resistant strains.

**Keywords:** *Acinetobacter baumannii*, Carbapenems, Antibiotic resistance, Nosocomial infection



## Background

*Acinetobacter baumannii* is a gram-negative, oxidase-negative, obligate aerobic, non-fermentative, non-motile, and opportunistic coccobacillus that is abundant in soil and water. Bacteria are spread in the hospital environment on wet surfaces (such as mechanical ventilation) and dry surfaces such as human skin, live in the environment for a long time and are easily transmitted among patients. Due to the remarkable clinical properties of this bacterium and its ability to acquire drug resistance and high reproducibility, it is considered as a microorganism threatening patients hospitalized for treatment with antimicrobial drugs. The genus *Acinetobacter* is divided into two groups: glucose oxidizing species, the most common of which is *A. baumannii*, and non glucose-oxidizing species, the most prevalent of which is *Acinetobacter lwoffii* and *Acinetobacter haemolyticus*. The most common cause

of infections is *A. baumannii*, which accounts for most *Acinetobacter* infections in humans (1, 2).

*Acinetobacter baumannii* causes urinary tract infections, ulcers, meningitis, peritonitis, endocarditis, pneumonia, as well as sepsis in ICU, surgery, infection, burn sections. Those receiving broad-spectrum antibiotics, undergoing surgery, and using artificial respiration, as well as patients who have cystic fibrosis and patients with immunodeficiency are at risk for *A. baumannii* infections. *Acinetobacter* infections are difficult to treat because the organism is resistant to most antibiotics, such as carbapenems, due to the presence of *OXA* family genes, and specific treatment should be selected by *in vitro* susceptibility testing (1-5). *A. baumannii* is a pathogen with low sensitivity to antibiotics due to the presence and increased expression of efflux pumps, plasmid, and chromosomal beta-lactamase resistance genes, membrane

impermeability, drug binding site change, the presence of some drug-altering enzymes, or different cellular functions of mutants, biofilm production, the presence of simple and complex jumping genetic elements such as IS, transposon and integrons for horizontal transfer of genes and changes in the outer coat of bacteria (1-6). Class D beta-lactamases, also known as OXA-type enzymes or oxacillinases, have lower hydrolyzing activity on carbapenems than metallo-beta-lactamases (MBLs); however, if this group of OXA genes are located near insertion sequence or IS, they can increase their expressions under the influence of their strong promoters. The most important enzyme groups of this family are as follows (6-9):

The OXA-58 enzyme group was first found in France in 2003 and is now reported in many countries. This gene makes the bacterium resistant to penicillins and cephalothin and reduces sensitivity to carbapenems and moxalactam. This gene can be horizontally transferred by motile genetic elements such as plasmids, transposons, integrons, and phages and includes subgroups OXA-58, 96, 97, 164 (6-8).

The OXA-23 enzyme group is predominantly plasmid and was first identified in Scotland in 1993 as ARI-1 (*A. baumannii* resistant to imipenem-1) and then in Turkey, India, France, Germany, Spain, and the United Kingdom. It is originated from *A. radioresistance*, a symbiotic bacterium that lives in the skin. Bacteria containing these enzymes are resistant to cephalosporins, cefotaxime, oxacillin, cloxacillin, and other penicillins. This bacterium has 17 subgroups and IS elements that are involved in their activities. OXA-23 gene is the most common carbapenem resistance encoding gene which is translocated horizontally and plays a significant role in bacterial carbapenem resistance (6-8).

The OXA-51 enzyme group is mainly chromosome-encoded and was first identified in 2004 in Argentina. The two subgroups are OXA-51 and OXA-69. Members of this group behave as poor penicillinases and weak carbapenemases but are resistant to inhibition by clavulanic acid and tazobactam. This gene is now the identification marker of *A. baumannii* (6-8).

The OXA-24 enzyme group is transmitted via the plasmid and has a moderate hydrolytic activity against carbapenems. This enzyme is often active against *doripenem* (a type of carbapenem) and its configuration is similar to OXA-1. The gene was first identified in the chromosome of a carbapenem-resistant *A. baumannii* clinical strain isolated in Spain in 1997 and then reported in the Netherlands, the United States, England, and France. In strains with the OXA-24 gene, MIC for carbapenem increased up to 4-fold. OXA-24 is often found in combination with OXA-40 and is now found in other Gram-negative bacilli resistant to carbapenem and penicillins, including *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Stenotrophomonas maltophilia*, and *Burkholderia* (9-11). OXA-24 includes subgroups such as OXA-231, 143, 253, 72, 160, 40 (6-11).

Multiplex polymerase chain reaction (PCR) is a modified PCR method in which several mutations, genes, alleles,

and different infectious agents in different variants can be detected simultaneously using several different pairs of primers in one reaction (2,3,12). In the present study, the multiplex PCR method was used to identify 4 important subgroups of OXA-23, OXA-24, OXA-51, OXA-58 in 25 environmental isolates of *A. baumannii* in the laboratory plate taken from the ICU (equipment, devices, clothing, etc). The antibiotic resistance profile of the isolates was evaluated at a significance level of  $P < 0.05$  using SPSS 22.0.

## Materials and Methods

### Sample Collection and Culture

Twenty-five non-repetitive samples of bacteria in the plate collected from the ICU (equipment, tools, clothing, patient pillows) were transferred to the research laboratory of cellular and molecular biology over a period of 8 months in compliance with all ethical principles and international conventions. Blood agar and BHB (Brain Heart Infusion Broth) media were used to grow this bacterium. The bacteria were cultured in Müller-Hinton medium and then subjected to Gram staining, biochemical tests such as oxidase, catalase, oxidative-fermentative test, SIM (Sulfide, Indole, Motility medium), hemolysis, hydrolysis of succinylcholine, nitrate reduction, growth at 44°C, and PCR of *16s rRNA* gene (3,5,9). To extract plasmid DNA, Plasmid extraction mini kit (Yekta Tajhiz Azma Company, Cat No. FAPDE050) was used according to the instructions.

### Antibiogram

Antibiotic susceptibility testing was performed using Kirby-Bauer method and according to The Clinical & Laboratory Standards Institute (CLSI) 2020 standard guidelines. A 0.5 McFarland standard turbidity of bacteria was prepared and spread on Müller-Hinton agar medium (Merck, Germany) (13). The bacterial suspension was densely cultured by sterile cotton swab on Müller-Hinton agar medium. The antibiotic discs were then placed on the surface of the medium with sterile forceps and the culture medium was incubated for 37-24 hours at 37°C. Antibiotic susceptibility pattern was determined using disk diffusion and E-test for cefotaxime (30 µg), carbenicillin (100 µg), amikacin (30 µg), ampicillin/sulbactam (10/10 µg), cephalothin (30 µg), gentamicin (10 µg), meropenem (10 µg), piperacillin (100 µg), oxacillin (1 µg) and polymyxin B (300 µg) provided by Padten Teb Company, Iran. *Escherichia coli* ATCC 25922 and *A. baumannii* PTCC 1388 were used as standard strains in antibiogram test (2,3,5,9). Then, the diameter of the growth inhibition zone was accurately measured and the isolates were reported as resistant, semi-sensitive, and sensitive according to CLSI instructions. Statistical analysis was performed using chi-square and Fisher's exact test in SPSS 22.0 at a significant level of  $P < 0.05$  (2,14).

### Primers, PCR, and Electrophoresis

Total DNA extraction was performed by boiling method. In multiplex PCR method, the frequency of four oxacillinase

genes OXA-23, 24, 51, 58 was calculated simultaneously for each isolate using a thermocycler (Bio Rad, USA). Each reaction was performed in a volume of 25 µL containing 1X of PCR buffer, 1 U of Taq enzyme, 2 mM of MgCl<sub>2</sub>, 200 mM of dNTPs (CinnaGen, Iran), 0.2 µM of each primer, 1 µL of sample DNA and ddH<sub>2</sub>O for adjusting the reaction volume to 25 µL. Then multiplex PCR was done with the same temperature/time program. The primer sequences of the four oxacillinase genes and the 16s rRNA gene are presented in Table 1. *A. baumannii* ATCC 1388 was used as the standard strain in PCR reactions. PCR was carried out in a thermocycler as follow: Initial denaturation at 94°C for 4 minutes, 30 cycles of 94°C for 60 seconds, 53°C for 40 seconds, 72°C for 50 seconds, and final extension at 72°C for 5 minutes (12). PCR amplicons were electrophoresed (DeNa Gene Tajhiz, Iran) on 1.5% agarose gel, stained with DNA safe stain (2.5 µL/100 mL, Yekta Tajhiz Azma, Iran) and placed under a UV transilluminator.

The image obtained from electrophoresis was stored and compared with the 100 bp DNA ladder (Cat No. S-5090, Denazist, Iran).

**Results**

**Antibiogram Results**

Antibiotic susceptibility testing was performed by disc diffusion method (Figure 1). The antibiogram patterns of the isolates studied with 10 antibiotics are listed in Table 2. The results showed that except for one isolate, all isolates (96%) were sensitive to polymyxin B. Additionally, 80% of isolates were sensitive to oxacillin (PP=0.03) and all isolates were sensitive to meropenem, ampicillin/sulbactam, gentamicin, piperacillin, and carbenicillin.

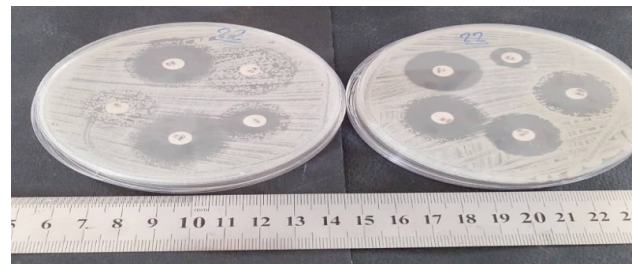
**Multiplex PCR Results**

Multiple PCR test was used to detect the presence of oxacillinase genes OXA-23, 24, 51, 58 according to the above-mentioned PCR program. After gel electrophoresis, normal PCR test was repeated three times to confirm the absence of genes in isolates without genes. The multiplex PCR products of ten isolates in 1.5% agarose gel

**Table 1.** The Primers Used in This Study (2, 12, 15)

| Gene Name | (5→3') Nucleotide seq.                                       | Amplicon Size (bp) |
|-----------|--|--------------------|
| OXA-51    | F-5'-TAATGCTTTGATCGGCCTTG-3'<br>R-5'-TGGATTGCACTTCATCTTGC-3' | 353                |
| OXA-58    | F-5'-AAGTATTGGGGCTTGTGCTG-3'<br>R-5'-CCCCTCTGCGCTCTACATAC-3' | 599                |
| OXA-23    | F-5'-GATCGGATTGGAGAACCAGA-3'<br>R-5'-ATTTCTGACCCGATTTCCAT-3' | 501                |
| OXA-24    | F-5'-GGTAGTTGGCCCCCTAAA-3'<br>R-5'-AGTTGAGCGAAAAGGGGATT-3'   | 246                |
| 16s rRNA* | F-5'-AGAGTTTGATCCTGGCTCAG-3'<br>R-5'-ACGGTACCTGTACGACTT-3'   | 1500               |

\* The 16s rRNA gene was not involved in the multiplex reaction but was used in separate PCR to molecularly identify the isolates.



**Figure 1.** Antibiotic Susceptibility Testing by Disc Diffusion Method.

electrophoresis are shown in Figure 2.

Multiplex PCR results showed that 25 isolates (100%) had OXA-51 gene, 21 isolates (84%) had OXA-58 gene, and one isolate (4%) had OXA-24 gene. No isolates contained the OXA-23 gene. Isolates containing two oxacillinase genes were not identified. Isolate No. 10 was the only isolate containing three genes of OXA-24, OXA-51, and OXA-58.

**Discussion**

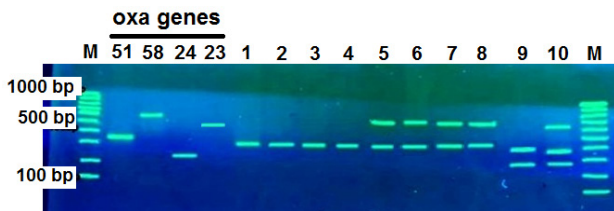
*Acinetobacter baumannii* is increasingly being identified as an important cause of nosocomial infections such as bacteremia and ventilator-associated pneumonia, surgical site infections, toxic sepsis, secondary meningitis, and urinary tract infections especially in ICU, infectious, and burn patients. This bacterium has a high potential for resistance to several classes of antimicrobial agents, which is mainly caused by the production of class D beta-lactamases with carbapenemase activity with the help of jumping genetic elements. The most common resistance-related genes in beta-lactam-resistant isolates include AmpC cephalosporinase genes, OXA-type carbapenemases, MBLs, adeA,S efflux pumps, targeted mutations that alter targets or cellular functions, decreased production of purines such as OprD and integrons which make the use of more antibiotics ineffective and also cause the emergence of strong strains of MDR (multidrug-resistant), XDR (extremely drug-resistant), and PDR (pandrug-resistant) (4,6,9,14-18).

**Table 2.** Antibiogram Profile of the Studied Isolates

| Antibiotic Name | No. (%) of Resistance Pattern of Isolates |               |           | P Value |
|-----------------|---|---------------|-----------|---------|
|                 | Sensitive                                 | Semisensitive | Resistant |         |
| OXA             | (80%)20                                   | -             | (20%)5    | 0.03*   |
| SAM             | (100%)25                                  | (0%) 0        | (0%)0     | 0.01*   |
| GM              | (100%) 25                                 | (0%)0         | (0%)0     | 0.01*   |
| AN              | (100%) 25                                 | (0%) 0        | (0%)0     | 0.01*   |
| MEN             | (100%) 25                                 | (0%) 0        | (0%) 0    | 0.01*   |
| CTX             | (96%)24                                   | (4%)1         | (0%)0     | 0.02*   |
| CF              | (96%) 24                                  | (0%) 0        | (4%)1     | 0.02*   |
| CB              | (100%) 25                                 | (0%)0         | (0%) 0    | 0.01*   |
| PIP             | (100%) 25                                 | (0%)0         | (0%)0     | 0.01*   |
| PB              | (96%) 24                                  | (0%) 0        | (4%) 1    | 0.01*   |

Abbreviations: CTX, cefotaxime; CB, carbenicillin; AN, amikacin; SAM, ampicillin/sulbactam; CF, cephalothin; GM, gentamicin; MEN, meropenem; PIP, piperacillin; OXA, oxacillin; PB, Polymyxin B. \* P < 0.05 and is significant.





**Figure 2:** Multiple PCR Reaction to Investigate the Presence of *OXA-58*, *OXA-23*, *OXA-24*, and *OXA-51* Genes on 1.5% Gel Agarose. Left to right M (100 bp marker) and positive control *OXA-51* (353 bp), *OXA-58* (599 bp), *OXA-24* (246 bp) and *OXA-23* (501 bp), isolates 1, 2, 3 and 4 have only *OXA-51* gene, isolates 5, 6, 7, and 8 have *OXA-51* and *OXA-58* genes, isolate 9 has *OXA-51* and *OXA-24* genes. Isolate 10 contained all three genes of *OXA-51*, *OXA-58*, and *OXA-24*, respectively.

The results of antibiotic susceptibility test showed that except for one isolate (4%), all isolates were sensitive to polymyxin B. Moreover, 80% of the isolates were sensitive to oxacillin ( $PP=0.03$ ). All isolates were sensitive to meropenem, ampicillin/sulbactam, gentamicin, piperacillin, and carbenicillin, and fortunately, carbapenem-resistant *A. baumannii* (CRAB) was not found in this study. These results are different from those of the studies conducted by Tarashi et al (5), Moghadasi et al, Rahmani, Vahhabi et al, and Sarhaddi et al and other studies conducted in different countries due to the clinical nature of the samples in most studies. In the present study, the isolates were derived from non-clinical samples such as equipment and devices used in ICU (2, 3, 5, 15, 19-30). In a study conducted by Goudarzi et al, the two groups of clinical and environmental samples were compared and it was revealed that most of the clinical samples were resistant to antibiotics (24). In 2016, Sarhaddi et al found that resistance to antibiotics was more than 95%. In addition, this study demonstrated high prevalence rates of *blaVIM*, *blaIMP*, *blaTEM*, *blaADC*, *OXA-Like23*, *OXA-Like24*, *OXA-Like51*, *OXA-Like58*, and *ISAbal* genes, which are resistant to beta-lactam antibiotics. These results suggest further studies on the rational administration of drugs in the treatment of infections caused by *A. baumannii* and the study of the mechanism of resistance transfer from pathogenic isolates to the environment, especially during antibiotic pressure (14,16,18,20,21,24,29).

Four subtypes of oxacillinase enzymes including *OXA-23*, *OXA-24*, *OXA-51*, and *OXA-58* have been identified in *Acinetobacter*. The *OXA-51* gene is inherently and chromosomally expressed in *A. baumannii*. The presence of this gene along with biochemical tests confirms *A. baumannii* (25,28,30). In our study, the *OXA-51* gene was present in all isolates under study, which was consistent with the study of other researchers. The relative frequency of *OXA-23* gene in studies conducted in Iran ranges from 4.3 to 100% (2,3,22-27) and in similar studies conducted abroad was reported to range from 31 to 100% (3,15,19,28-30). However, the results of a leading study showed that of the 25 isolates, all were negative for the *OXA-23* gene. Scientists attribute the emergence of CRAB strains to the presence of *OXA-23* gene (22, 27-30), and genetic elements play an important role in its expression (10,11,15,20).

According to the results of a study by Zhao et al, the presence of *OXA-23* gene is the cause of CRAB phenotype in this bacterium (28) and this may be the reason for the very good sensitivity of our isolates to the antibiotics used because the prevalence of this gene in the present study was 0%. Only one isolate (4%) had the *OXA-24* gene, which is consistent with the study conducted by Kooti et al (4.5%) (2). The frequency of this gene in Iran ranges from 0 to 74.1% (3,19,20,22-26) and in other countries, it ranges from 0 to 57.6% (19,21,26,29,30). According to a study conducted by Codjoe and Donkor in 2018, this gene is prevalent in *A. baumannii* isolates (14). One of the important reasons for this difference is the discrepancy in the type of sample (samples from burn wounds, blood infection, lung, urine, etc. with non-clinical samples used in our study). In the present study, bacterial samples were not obtained from patients but were isolated from the ICU (clothing, equipment, etc.). In our study, 21 isolates (84%) carried the *OXA-58* gene, which was consistent with a study by Rahmani et al (3). The frequency of this gene in various studies in Iran has been estimated to range from 0 to 82.1% (2,3,19,20,24,27) and in other countries it ranged from 0 to 60% (19,28-30). The only concern was isolate No. 10, which had three genes, *OXA-51*, 24 and 58 and was resistant to oxacillin, cephalothin, and polymyxin and relatively resistant to cefotaxime. However, resistance to drugs other than polymyxin is increasing sharply, but resistance to the strongest drug for the treatment of *A. baumannii* infections needs further epidemiological and molecular studies. *Mcr-1* gene plays an important role in resistance to polymyxin B and Colistin (5).

## Conclusion

Non-clinical ICU isolates lack important pathogenic genes present in clinical isolates and how these genes are transferred to environmental isolates is an important point that should be studied. Differences in the prevalence of resistance genes and subsequent change in antibiotic resistance patterns in many studies may result from geographical distance, sample size, design of the study, and type of biological sample. Therefore, knowledge of the resistance pattern of each region can help to reduce the incidence of antibiotic resistance, prevent treatment failure, and reduce treatment costs.

## Authors' Contribution

AF: Writing of original draft, investigation. MM: Validation, methodology, and consultation. SN: Data curation and analysis, as well as consultation. RY: Supervision, and editing.

## Conflict of Interests

None.

## Ethical Approval

Ethical principles were considered in relation to the proposed work and no ethical issues were found to be applied to this research.

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