

# Detection of KPC Carbapenemase in *Pseudomonas aeruginosa* Isolated From Clinical Samples Using Modified Hodge Test and Boronic Acid Phenotypic Methods and Their Comparison With the Polymerase Chain Reaction

Saeed Falahat,<sup>1</sup> Mana Shojapour,<sup>2</sup> and Abdorrahim Sadeghi<sup>3,\*</sup>

<sup>1</sup>Molecular and Medicine Research Center, Arak University of Medical Sciences, Arak, IR Iran

<sup>2</sup>PhD Student, Department of Molecular Medicine, School of Advanced Technologies in Medicine, Tehran University of Medical Sciences, Tehran, IR Iran

<sup>3</sup>Molecular and Medicine Research Center, Department of Biochemistry and Genetics, Arak University of Medical Sciences, Arak, IR Iran

\*Corresponding author: Abdorrahim Sadeghi, Molecular and Medicine Research Center, Department of Biochemistry and Genetics, Arak University of Medical Sciences, Arak, IR Iran. Tel: +98-8634173502, E-mail: sadeghi@arakmu.ac.ir

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## Abstract

**Background:** Bacterial resistance to antibiotics has become a major source of concern for public health. *Pseudomonas aeruginosa* strains are important opportunistic pathogens. These bacteria have a high resistance to a wide range of existing antimicrobials and antibiotics.

**Objectives:** The present study was performed to evaluate the frequency of KPC in *P. aeruginosa* isolated from clinical samples of educational hospitals of Arak University of Medical Sciences, using the mentioned phenotypic and genotypic methods.

**Materials and Methods:** One hundred and eight non-duplicate clinical isolates of *P. aeruginosa* were collected from hospitals of Arak University of Medical Sciences, Arak, Iran. Antibacterial susceptibility was determined by the disk diffusion method. KPC production was confirmed by the Modified Hodge Test (MHT), which is a phenotypic test, and combined-disk test with boronic acid and the Polymerase Chain Reaction (PCR).

**Results:** In the present study, 13 isolates (12%) of *P. aeruginosa* were positive for KPC, using PCR. Comparison of the two phenotypic methods used in this study showed that boronic acid is more sensitive than MHT in identification of KPC-producing strains (84.6% vs. 77%).

**Conclusions:** Utilization of reliable methods for identifying carbapenemase-producing strains and determining their antibiotic resistance pattern could have a very important role in treatment of infections caused by these strains. A substantial amount of *P. aeruginosa* isolated from clinical samples of hospitals in Arak (Iran) produce KPC carbapenemase. Due to their low specificity, MHT and boronic acid phenotypic methods could not completely identify KPC-producing *P. aeruginosa*. However, the sensitivity of boronic acid phenotypic method in detection of KPC was higher than MHT.

**Keywords:** Carbapenemase, Phenotypic Method, Polymerase Chain Reaction, *Pseudomonas aeruginosa*

## 1. Background

*Pseudomonas aeruginosa* is a Gram-negative rod and belongs to the glucose non-fermentative bacteria family. It is an important opportunistic pathogen that causes infections in patients with weakened immune system and cystic fibrosis genetic defect, as well as patients at Intensive Care and Burn Units (1). These bacteria have a high resistance to a wide range of existing antimicrobials and antibiotics. Eradication of infections by *P. aeruginosa* is difficult due to their inherent resistance to a wide range of antibiotics, as well as their mobile genetic elements including resistance genes (2).

Emergence of carbapenem-resistant strains has expanded amongst Gram-negative bacteria in the recent years. The most important mechanisms of resistance to carbapenems are production of carbapenemase enzymes and secretory pumps, and lack of expression of external membrane proteins (porins) (3). Carbapenemases have the ability to hydrolyze penicillins, cephalosporins, monobactams and carbapenems (4). The presence of KPC in transferable plasmids and transposons can result in a rapid spread among bacteria including *Klebsiella pneumoniae*, *Enterobacter* species, *Acinetobacter baumannii* and *P. aeruginosa* (5). The world's first report of KPC-2 presence in *Pseudomonas* was reported by the International Center of Medi-

cal Research and Education in Columbia, during year 2006. Subsequently, a study from Puerto Rico reported on two types of KPC (KPC-2 and KPC-5) in *P. aeruginosa* (6).

Rapid detection of strains producing carbapenemases, such as KPC, is crucial for preventing incorrect treatment, controlling hospital infections, and preventing the spread of these strains. Modified Hodge Test (MHT) proposed by the Clinical and Laboratory Standards Institute (CLSI), and boronic acid inhibitor-based assay are among the many phenotypic methods used in microbiology laboratories for identification of Group A carbapenemases in the *Enterobacteriaceae* family (7).

## 2. Objectives

In Iran, no study has so far been carried out for evaluation of KPC frequency using phenotypic tests of MHT and boronic acid inhibitor-based assay and for their comparison with the genotypic method of Polymerase Chain Reaction (PCR) in *P. aeruginosa*. Therefore, the present study was performed to evaluate the frequency of KPC in *P. aeruginosa*, isolated from clinical samples of educational hospitals of Arak University of Medical Sciences, using the mentioned phenotypic and genotypic methods.

## 3. Materials and Methods

This project was approved by the Arak University of Medical Sciences Ethical Committee (No. 92-152-14).

### 3.1. Bacterial Isolates and Detection of Extended Spectrum $\beta$ -Lactamases

This study was a descriptive cross-sectional study carried out from August 2012 to July 2013, in which 108 *P. aeruginosa* isolates were collected from urine, sputum, and ulcer samples of hospitalized patients at the educational hospitals of Arak University of Medical Sciences, Arak, Iran. *Pseudomonas aeruginosa* was identified using standard biochemical methods, including Gram staining, catalase test, Oxidative-Fermentation (OF) test, triple sugar iron agar (Merck, Germany), growth at 42°C, and production of blue-green pigments. Antibiotic susceptibility pattern of isolates was also determined using the disk diffusion method (Kirby-Bauer), according to the standards of Clinical and Laboratory Standards Institute (CLSI) (8). All disks were made by the MAST Company (UK) and included the following, ceftazidime, ciprofloxacin, amikacin, gentamicin, imipenem and meropenem. The standard strain of *P. aeruginosa* ATCC 27853 was used as the antibiogram control. An intermediate growth inhibition zone was considered as resistant.

### 3.2. Modified Hodge Phenotypic Test

First, a suspension of the standard strain of *Escherichia coli* ATCC 25922 was prepared at a concentration of 0.5 McFarland and then diluted to 0.01 McFarland with normal saline. The suspension was then lawn cultured on Mueller Hinton medium (Merck, Germany) using a swab. A 10- $\mu$ g meropenem disc (Mast, UK) was then placed on the centre of the plate. *Pseudomonas aeruginosa* sample was cultured in a direct line from the disc edge towards the plate edge and the plates were incubated at 37°C for 16 to 24 hours. Cloverleaf-like structure indicated the production of carbapenemase (9).

Since KPC-producing *P. aeruginosa* inhibit *E. coli* on Muller Hinton medium and lead to unexplained results, *E. coli* standard strain and *K. pneumoniae* ATCC 700603 were cultured on separate Mueller Hinton medium and *Pseudomonas* was cultured on and the results of the two tests were finally compared (10).

All isolates were tested for KPC carbapenemases production on disks containing boronic acid. A disk containing imipenem (Mast, UK) and another containing imipenem with 400  $\mu$ g of boronic acid (Sigma-Aldrich, Germany) were placed on the agar. The diameter of the growth-inhibitory zone around the imipenem disk with boronic acid was compared with that around the corresponding imipenem disk without boronic acid. The test was considered positive for the detection of class A carbapenemase production when the diameter of the growth-inhibitory zone around the imipenem disk with boronic acid was  $\geq$  5 mm larger than that around the disk containing the imipenem substrate alone (11).

### 3.3. Polymerase Chain Reaction (PCR)

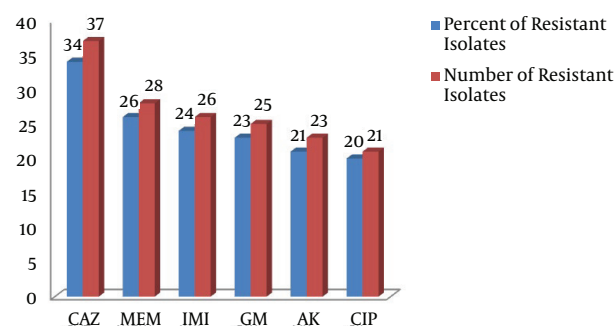
DNA was extracted from bacterial samples using the boiling method. The blaKPC gene was detected by PCR using the following specific primers; Forward: 5'CGTC-TAGTTCTGCTGCTTG3' and Reverse: 5'CTTGTCATCCTGT-TAGGCG3' (12). The DNA amplification, programmed for the blaKPC gene, consisted of an initial denaturation step (94°C, three minutes), followed by 40 cycles of denaturation (94°C, 30 seconds), annealing (58.2°C, 60 seconds) and extension (72°C, 30 seconds), and a single final extension (72°C, five minutes).

## 4. Results

Overall, 108 isolates of *P. aeruginosa* were collected from hospitalized patients' clinical samples at three educational hospitals of Arak University of Medical Sciences. The results of the antibiotic sensitivity pattern (number

and percentage of resistant isolates) of *P. aeruginosa* isolates, obtained through the disk diffusion method, are presented in Figure 1.

**Figure 1.** The Antibiotic Sensitivity Pattern (Number and Percentage of Resistant Isolates) of *P. aeruginosa* Isolates, Obtained by the Disk Diffusion Method



Gentamicin (GM), ceftazidim (CAZ), ciprofloxacin (CIP), amikacin (AK), meropenem (MEM), imipenem (IMI).

Assessment of carbapenemase production in *P. aeruginosa* through the MHT method, using the standard strain of *E. coli* ATCC 25922, resulted in only 27 examinable isolates; 81 isolates were unexplainable due to *Pseudomonas* inhibition of *E. coli* growth in the culture medium (Figure 2A). However, out of 108 isolates examined by the MHT test, using the standard strain of *K. pneumoniae* ATCC 700603, 38 isolates of *P. aeruginosa* produced carbapenemase and the results of this test were negative for 70 isolates (Figure 2B).

Using the combination-disk test with boronic acid, 26 isolates were identified as carbapenemase-producing strains, while 82 isolates were negative (Figure 2C). The characteristics of 13 isolates are presented in Table 1. Of the 108 isolates of *P. aeruginosa* studied by PCR, 13 isolates (12%) had the KPC carbapenemase encoding gene (*bla*KPC) (Figure 3).

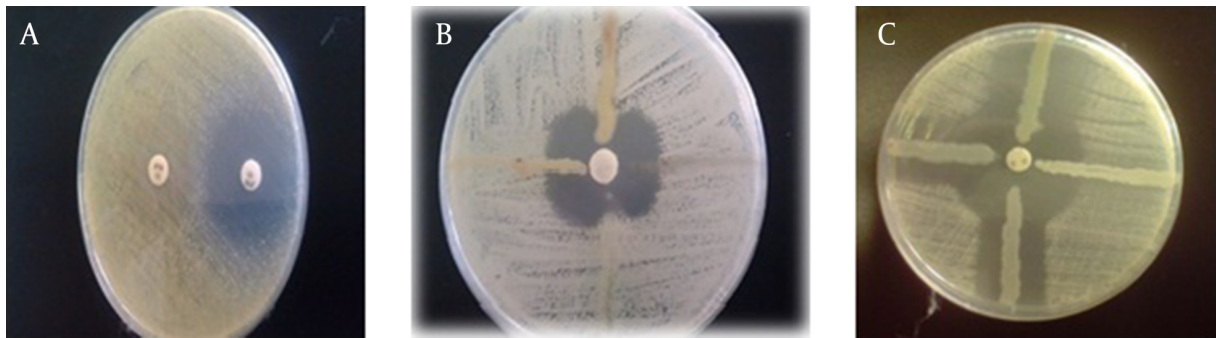
## 5. Discussion

Utilization of reliable methods for identifying carbapenemase-producing strains and determining their antibiotic resistance pattern could have a very important role in treatment of infections caused by these strains. This could be an important step in the control of hospital infections, in order to prevent patients' mortality and to reduce health care costs (3, 13). Since no study has so far been carried out for the evaluation of the frequency of *P. aeruginosa*-producing KPC carbapenemase in Iran, in the present study, the presence of KPC carbapenemase was examined in *P. aeruginosa* isolated from clinical samples of patients hospitalized at hospitals of Arak University of

Medical Sciences, using the genotypic method of PCR. The efficiency of MHT and boronic acid phenotypic methods for identification of KPC enzyme was compared with the PCR.

Due to their wide range of activities, carbapenems are commonly used to treat infections caused by multidrug-resistant bacteria, particularly Extended-Spectrum Beta-Lactamases (ESBLs)-producing bacteria and AmpC enzyme-producing strains (14). Spread of carbapenemase-producing bacteria throughout the world in the recent years has been considered as a major threat to public health. Among carbapenemases, KPC has a high frequency and has been commonly found in *K. pneumoniae* and *Enterobacter* (15). The enzyme is encoded by a gene located on mobile elements such as plasmids; this results in KPC gene transfer among bacteria. Furthermore, the KPC enzyme hydrolyses beta-lactam drugs, including monobactams, carbapenems, and third-generation cephalosporins (16). The presence of this enzyme in *P. aeruginosa* was first reported in Colombia, during year 2007 (17) and subsequently in other countries such as Puerto Rico (18), Trinidad and Tobago (19), the United States, and China (3, 6). Recent studies from Iran have also indicated the prevalence of KPC-producing bacteria (20). The highest prevalence of KPC gene in Iran, examined by PCR, was reported in *K. pneumoniae* by Hashemizadeh et al. as 11.9% (21). In the present study, 13 isolates (12%) of *P. aeruginosa* were positive for KPC, using PCR. This could indicate horizontal transfer of KPC genes among bacteria isolated from nosocomial infections in Iran. The prevalence of KPC in *P. aeruginosa* was 4.1% in Puerto Rico; this was lower than the present study (22). The pattern of antibiotic resistance in 13 KPC-producing isolates in the present study was such that they were all resistant to ceftazidime, gentamicin, amikacin, and ciprofloxacin. Thus, carbapenemase detection cannot be performed by antibiotic profiles in the laboratory. Phenotypic resistance caused by carbapenemase is affected by many factors such as strain, expression level, type, or enzyme variant, as well as the presence of other resistance mechanisms such as reduced permeability, secretory pumps, and other activities of beta-lactamases (4). In the present study, the disk diffusion method showed that of 13 KPC-producing isolates, six and five were resistant to imipenem and meropenem, respectively.

The Modified Hodge Test is a sensitive phenotypic method for detection of carbapenemases, however, it cannot identify the enzyme type. Instead of the standard strain of *E. coli* ATCC 25922, Pasteran et al. (10) used the standard strain of *K. pneumoniae* ATCC 700603 (lacking carbapenemases and sensitive to carbapenem) for the MHT. When the common indicator strain (*E. coli* ATCC 25922) was



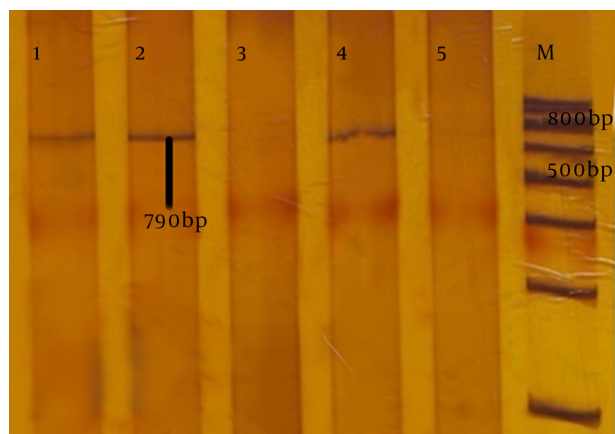
**Figure 2.** A, modified hodge test (MHT). Results for three *Pseudomonas* isolates, which were unexplainable when using *E. coli* ATCC 25922 standard strain. B, MHT test results using *K. pneumoniae* ATCC 700603 standard strain. C, The results of combination-disk test with boronic acid, which was positive for the examined strains.

**Table 1.** Characteristics of the Thirteen KPC-Positive Isolates Using the Polymerase Chain Reaction Method <sup>a</sup>

Number of Isolate	IMI	MEM	PCR for KPC	MHT	Boronic Acid
1	S	S	+	+	+
2	R	R	+	+	+
3	S	S	+	+	+
4	R	R	+	+	+
5	R	S	+	+	+
6	S	S	+	+	+
7	R	S	+	-	-
8	S	S	+	+	+
9	R	R	+	-	+
10	S	R	+	+	-
11	S	S	+	-	+
12	S	S	+	+	+
13	R	R	+	+	+

<sup>a</sup> Molecular studies detected 50% bla<sub>VIM-1</sub>, 56.6% bla<sub>VIM-2</sub>, and 6.6% bla<sub>IMP-1</sub> while none of the strains had bla<sub>IMP-2</sub> and bla<sub>SPM-1</sub> genes.

**Figure 3.** Polyacrylamide Gel Electrophoresis (PAGE) of Products of *Pseudomonas aeruginosa* KPC Gene, Amplified by the Polymerase Chain Reaction (PCR)



Lane M is the marker (DNA ladder, 100bp); lanes 1, 2 and 4, are amplified products of KPC gene in *P. aeruginosa*, isolated from hospital samples. Lane 5, is the amplified product of the negative control *K. pneumoniae* lacking the KPC gene. Lane 3, the amplified product of the KPC gene in *P. aeruginosa* lacking the KPC gene.

used, *P. aeruginosa* inhibited the growth of *E. coli* standard strains; this resulted in an unexplainable test and decreasing of the test sensitivity and specificity. This problem was resolved through exchanging the indicator strain with the standard strain of *K. pneumoniae* ATCC 700603. Of the 13 KPC-positive isolates obtained using the MHT method, 10 isolates were considered as KPC-producing strains. The results of the phenotypic method of boronic acid inhibitory effect on Group A carbapenemases identified 11 isolates, out of 13 KPC-positive isolates. The important point in interpretation of the results of phenotypic methods applied in the present study was their positivity in the strains being KPC negative in PCR. This represents the possible presence of other carbapenemase groups such as GES enzymes and various groups of metallo-beta-lactamase in the mentioned strains (given their resistance to imipenem and meropenem).

As a result, unlike the study carried out by Azimi et al. in Iran (20), who only used the MHT phenotypic method to assess the prevalence of KPC-producing strains, using



only the MHT method and its positivity in the examined isolates cannot be a reason for the presence KPC, and positive results can be caused by other carbapenemases, and in some cases, by CTX-M ESBLs along with a decrease in porins expression. Therefore, a definite confirmation of KPC-producing strains requires PCR followed by sequencing. The boronic acid method can detect KPC-producing strains, as well as GES amongst isolates. In isolates with AmpC overexpression and change in porins, boronic acid method may lead to false-positive results (9, 23). Comparison of the two phenotypic methods used in this study showed that boronic acid is more sensitive than MHT in identification of KPC-producing strains (84.6% vs. 77%).

Given the significant prevalence of KPC-producing *P. aeruginosa* and their drug multi-resistance, proper measures seem necessary for controlling infections of these strains, such as their rapid and reliable detection and administration of appropriate antibiotics for treatment. In future studies, the origin of infection and genetic relationships between strains obtained from different hospitals can be evaluated through epidemiologic molecular approaches. A substantial amount of *P. aeruginosa* isolated from clinical samples of hospitals of Arak (Iran) produced KPC carbapenemase. Due to their low specificity, MHT and boronic acid phenotypic methods cannot completely indicate KPC-producing *P. aeruginosa*. The sensitivity of boronic acid phenotypic method in detection of KPC was higher than MHT (84.6% vs. 77%).

## Footnotes

**Authors' Contribution:** Study concept and design: Saeed Falahat, Mana Shojapour and Abdorrahim Sadeghi; laboratory work, analysis and interpretation of data: Saeed Falahat; drafting of the manuscript: Mana Shojapour and Abdorrahim Sadeghi; critical revision of the manuscript: Saeed Falahat, Mana Shojapour and Abdorrahim Sadeghi.

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