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First report of Klebsiella pneumonia carbapenemase-producing Pseudomonas aeruginosa isolated from burn patients in Iran: Phenotypic and genotypic methods

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First report of *Klebsiella pneumoniae* carbapenemase-producing *Pseudomonas aeruginosa* isolated from burn patients in Iran: phenotypic and genotypic methods

Erster Bericht über *Klebsiella pneumoniae* Carbapenemase-bildende *Pseudomonas aeruginosa*-Stämme, isoliert von Verbrennungspatienten im Iran: phenotypische und genotypische Methoden

Abstract

Wound infection associated with carbapenem-resistant *Pseudomonas aeruginosa* in burn patients is a growing problem. One of the main mechanisms of resistance to carbapenem antibiotics is the ability of *P. aeruginosa* to produce carbapenemase enzymes. *Klebsiella pneumoniae* carbapenemase (KPC) is an important type of carbapenemase which can hydrolyze carbapenem antibiotics. The Modified Hodge Test (MHT) and boronic acid as a KPC inhibitor are two phenotypic methods used for detection of carbapenemase. The sensitivity and specificity of these two phenotypic tests for the identification of KPC can be measured by PCR.

In this study, 241 *P. aeruginosa* strains were isolated from wounds of hospitalized burn patients. Carbapenem-resistant *P. aeruginosa* isolates were determined by the disk diffusion method. KPC-producing carbapenem-resistant strains were examined using the Modified Hodge Test, followed by boronic acid. Further, strains with positive responses to MHT and boronic acid tests were analyzed with the PCR molecular method. One hundred eighty-six of 241 isolates were resistant to carbapenems and 75 were positive in the MHT. Three exhibited an at least 5-mm diameter difference when meropenem was combined with boronic acid vs meropenem alone in the boronic acid test. Two strains had a specific band with primer No.1 after gel electrophoresis.

This study showed that MHT, despite excellent sensitivity, has variable specificity independent of bacterial species. Further, the use of KPC inhibitors such as boronic acid did not yield favorable sensitivity and specificity among the specimens from Iranian patients. Thus, it seems that sequencing after PCR should be considered the gold standard for the detection of KPC-producing *P. aeruginosa*.

Keywords: *P. aeruginosa*, KPC, boronic acid, Modified Hodge Test, blaKPC

Zusammenfassung

Wundinfektionen bei Verbrennungspatienten mit Carbapenem-resistentem *Pseudomonas (P.) aeruginosa* sind ein wachsendes Problem. Einer der hauptsächlichsten Resistenzmechanismen gegen Carbapeneme ist die Fähigkeit von *P. aeruginosa*, Carbapenemase-Enzyme zu bilden. *Klebsiella pneumoniae* Carbapenemase (KPC) ist eine wichtige Carbapenemase, die Carbapeneme hydrolysieren kann. Der modifizierte Hodge-Test (MHT) und Boronsäure als KPC-Inhibitor sind zwei phenotypische Methoden zur Detektion der Carbapenemase. Die Sensitivität und Spezifität beider Tests zur KPC-Identifikation kann mittels PCR bestimmt werden.

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In der Studie wurden 241 *P. aeruginosa*-Stämme von Wunden hospitalisierter Verbrennungspatienten isoliert. Carbapenem-resistente *P. aeruginosa*-Isolate wurden mittels Plättchendiffusionstest bestimmt. KPC-bildende Carbapenem-resistente Stämme wurden mittels modifiziertem Hodgetest (MHT) und anschließend mit Boronsäure detektiert. Stämme mit positiver Reaktion wurden mittels PCR analysiert.

168 Isolate waren resistent gegen Carbapeneme und 75 waren im MHT positiv. Drei Isolate exhibierten mindestens 5-mm Durchmesser als Differenz, wenn Meropenem mit Boronsäure kombiniert wurde vs Meropenem allein im Boronsäuretest. Zwei Stämme hatten eine spezifische Bande mit Primer Nr. 1 in der Gelelektrophorese.

Die Studie zeigt, dass der MHT neben seiner hohen Sensitivität abhängig von der Bakterienart eine unterschiedliche Spezifität aufweist. Ferner erhöht der Einsatz eines KPC-Inhibitors wie Boronsäure nicht die Sensitivität und Spezifität bei den isolierten Stämmen. Daher scheint die Sequenzierung mittels PCR der Goldstandard für die Detektion KPC-bildender *P. aeruginosa*-Stämme zu sein.

Schlüsselwörter: *P. aeruginosa*, KPC, Boronsäure, modifizierter Hodge-Test, blaKPC

Background

Resistance to carbapenems such as broad spectrum betalactam antibiotics in *Pseudomonas aeruginosa* is an increasing challenge worldwide [1], [2], [3]. A growing incidence of carbapenem-resistant *P. aeruginosa* is associated with KPC production, especially in burn patients, and is an important concern in health-care systems [1]. The importance of this resistance is due to the potential for resistance to all betalactam antibiotics in KPC-producing microorganisms, which is one of the main choices for treatment of wound infection [4], [5], [6], [7]. KPC-producing bacteria are emerging in various countries and regions, such as Greece, Iran and Latin America [4], [8], [9]. Rapid and accurate detection of KPC-producing bacteria is necessary for preventing the spread of the *kpc* gene, given that it is located in transferable genetic elements (i.e. plasmids and transposons) [4], [5], [10]. According to CLSI (Clinical and Laboratory Standards Institute), MHT is one of the phenotypic methods of KPC confirmation ([11], Supplemental Table 2A-S2); however, some studies suggest that MHT may not have a high specificity for the identification of KPC and may only confirm carbapenemase enzymes but not carbapenemase types. Nevertheless, most researchers believe that MHT has a high sensitivity rate [12].

The use of boronic acid – a KPC inhibitor – is another phenotypic method for confirming KPC. Moreover, PCR for the *kpc* gene is utilized for molecular detection of this enzyme. The aim of this study was to evaluate these two phenotypic methods (MHT and boronic acid) for the detection of KPC by PCR as a molecular test.

Materials and methods

Bacterial strains

In this study, 241 *Pseudomonas* spp. were isolated from hospitalized burn patients in Motahari Hospital in Tehran, Iran. The species of bacteria were determined by specific biochemical tests such as oxidase, TSI, and gelatinase. PCR was used to confirm identification of *Pseudomonas aeruginosa* strains using specific primers for *oprI* for bacteria genus and *oprL* for species [13]. *Pseudomonas aeruginosa* ATCC 27853 and *Acinetobacter baumannii* ATCC 19606 were used as the positive and negative controls, respectively.

Antibiotic susceptibility testing was conducted against carbapenems (Imipenem, Meropenem and Ertapenem) using the disk diffusion method according to the CLSI recommendation. The modified Hodge test was performed for carbapenem-resistant strains.

Modified Hodge Test

According to CLSI, MHT is one of the confirmatory tests for phenotypic identification of the KPC enzyme. In this study, MHT was performed according to the CLSI recommendation by using *E. coli* ATCC 25922. *K. pneumoniae* ATCC BAA-1705 – MHT-positive was used as a positive control.

Use of KPC inhibitor

Using 400 µg 3-amino phenyl boronic acid (APBA) as a KPC inhibitor, per disk plus Meropenem vs Meropenem alone is another phenotypic method for confirming identification of KPC. Strains with an increase of at least 5 mm around Meropenem plus APBA vs Meropenem alone were

Table 1: Five different primer sequences used in this study

No.	Genes	Primers	Size	References
1	blaKPC-F	5' GTATCGCCGTCTAGTTCTGC 3'	636 bp	[19]
	blaKPC-R	5' GGTCGTGTTTCCCTTAGCCA 3'		
2	blaKPcA-F	5' GGCCGCCGTGCAATAC 3'	60 bp	[20]
	blaKPcA-R	5' GCCGCCCAACTCCTTCA 3'		
3	blaKPcB-F	5' CTGTCTTGTCTCTCATGGCC 3'	795 bp	[21]
	blaKPcB-R	5' CCTCGCTGTGCTTGTTCATCC 3'		
4	blaKPcC-F	'3 -- TGTCAGTGTATCGCCGTC '5	1009 bp	[22]
	blaKPcC-R	3-' CTCAGTGCTCTACAGAAAACC' 5-		
5	blaKPcD-F	'3- AACAAAGGAATATCGTTGATG' -5	915 bp	[23]
	blaKPcD-R	'3- AGATGATTTTCAGAGCCTTA' 5-		

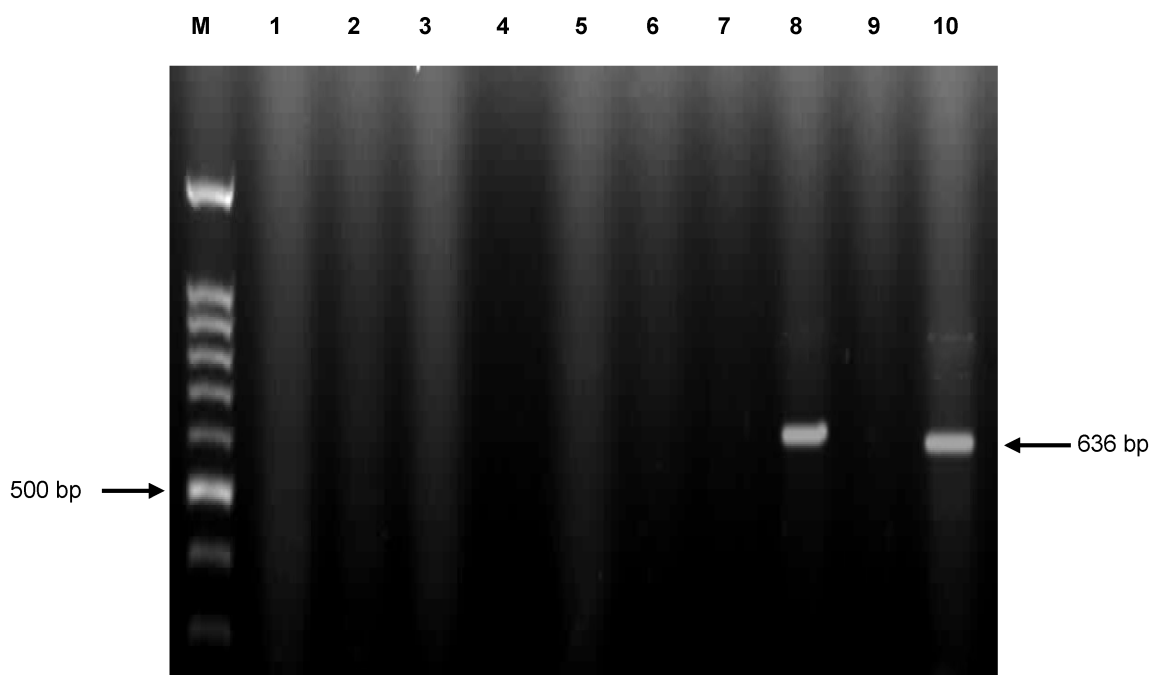


Figure 1: PCR amplification fragments for detection of the *kpc* gene among *Pseudomonas aeruginosa* isolates
M: 1kb DNA size marker; lane 1–3 and 5–7: punctuation here is confusing negative strains; lane 4: negative control; lane 8 and 10: positive strains.

considered KPC-producing strains. On the other hand, the synergistic effect of Meropenem plus Oxacillin (750 µg/disc) was used to eliminate false positive responses.

PCR of the *kpc* gene

PCR was used to confirm the *kpc* gene in MHT-positive *P. aeruginosa* with 5 different specific primers (Table 1, Figure 1). PCR program was performed as follows. Initial denaturation for each of the genes was performed at 95 °C for 5 min and thereafter 30 cycles with denaturation at 95 °C for 1 min. The annealing temperatures consisted of blaKPC 64 °C, blaKPcA 56 °C, blaKPcB 56 °C, blaKPcC 60 °C and blaKPcD 55 °C, the annealing time was 1 min. The extension time was 1 min at 72 °C. The final extension for all genes was done at 72 °C for 5 min.

Results

In this study, 241 strains were identified and confirmed as *P. aeruginosa*. One hundred eighty-six strains were resistant to all tested carbapenems (Imipenem, Meropenem and Ertapenem). Seventy-five strains had MHT-positive test results. A synergism effect between Meropenem and APBA was observed in 11 *P. aeruginosa* strains with positive MHT test results. On the other hand, only 3 strains had synergism with APBA alone and the remaining 8 strains had synergism with Oxacillin simultaneously. Further, PCR with different specific primers showed specific bands after gel electrophoresis in only two MHT-positive strains with primer No. 1. The proportion of positive results from molecular testing of carbapenem-resistant *P. aeruginosa* is 0.01 (1.07%). One of two strains had a synergistic effect with APBA.

Discussion

Resistance to carbapenems in *P. aeruginosa* is a growing problem worldwide [1], [2], [3]. Resistance to carbapenems associated with KPC production is an alarming problem for health care systems [1], because the *kpc* gene can transfer between *P. aeruginosa* strains or even from *P. aeruginosa* to enterobacteria [4], [5]. In addition, KPC-producing carbapenem-resistant *P. aeruginosa* strains have potential resistance to all betalactam antibiotics except Azthronam [5], [6]. This can cause complications in the treatment of infections related to KPC-producing bacteria.

Our findings showed that MHT had an excellent sensitivity (100%) but low specificity (2.6%) for the detection of KPC among Iranian bacterial specimens. We suggest that MHT can be used as a primary screening test, since only two out of 75 MHT-positive strains were confirmed by PCR. In Argentina, sensitivity and specificity of MHT for carbapenemase-producing *P. aeruginosa* were reported to be 78% and 57%, respectively [14], [15]. However, the use of inhibitor boronic acid led to a significant increase of 97% in sensitivity and specificity [14]. Despite high sensitivity, MHT can have variable specificity due to different incidences of carbapenemase in different geographical areas (57%–90%) [12], [14], [15]. For instance, in Colombia in 2007, three KPC-producing *P. aeruginosa* strains were identified [16], and in 2009, one KPC-producing *P. aeruginosa* strain was isolated [17]. A study conducted in the USA in 2009 reported one KPC-producing *P. putida* strain and one *E. cloacae* strain from one patient, where MHT was positive for both [18]. Additionally, in 2010, one KPC-producing *P. aeruginosa* strain was isolated in the USA [2]. Despite global emergence of KPC-producing strains of *P. aeruginosa*, it still uncommon and is thought to be sporadic (yet its rapid detection is necessary) [2], [16], [17], [18]. In our study, two KPC-producing *P. aeruginosa* strains were identified, which is the first report from Iran to the best of our knowledge. On the other hand, the results of this study confirmed that only one of three strains with positive result in combination disk method (meropenem plus APBA) confirmed by PCR as a KPC producer.

These findings indicate that the sensitivity and specificity of inhibitory test (use of boronic acid) could not be explained by our results. This is despite the fact that false positive results were eliminated using Oxacillin plus Meropenem in our methodology. Thus, our findings may suggest that MHT, despite its excellent sensitivity, has variable specificity dependent of species of isolated bacteria and also depending on different geographical areas. Also, the use of a KPC inhibitor such as boronic acid may not lead to a reasonably high sensitivity and specificity among Iranian bacterial specimens. Therefore, PCR should be considered as the gold standard for the detection of KPC-producing *P. aeruginosa* for the time being.

Notes

Competing interests

The authors declare that they have no competing interests.

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