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Evaluation of Phenotypic Methods for Detection of *Klebsiella Pneumoniae* Carbapenemase-Producing *K. Pneumoniae* in Tehran

Leila Azimi [1, 2], *Abdolaziz Rastegar Lari* [1, 2*], *Malihe Talebi* [2], *Amirmorteza Ebrahimzadeh Namvar* [1], *Somayeh Soleymanzadeh-Moghadam* [1]

[1] *Antimicrobial Resistance Research Center, Tehran University of Medical Sciences, Tehran, IR Iran*
[2] *Department of Microbiology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, IR Iran*

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ABSTRACT

Background: One of the main mechanisms of resistance to carbapenems is potential of *Klebsiella pneumoniae* to produce *K. pneumoniae* Carbapenemase (KPC). KPC is an important type of carbapenemase, which can hydrolyze carbapenems and other beta-lactam antibiotics. Modified Hodge Test (MHT) and use of boronic acid as a KPC inhibitor are two types of phenotypic methods, which are used for detection of carbanemase-producing bacteria. Specificity of these two phenotypic tests for identification of KPC was assessed in this study.

Methods: Forty-four *K. pneumoniae* strains were isolated from wound infections of burn patients. All isolates were identified with specific biochemical tests. Carbapenem-resistant *K. pneumoniae* isolates were identified by disc diffusion method and analyzed with cut off-points of CLSI 2011 guideline. For detection of KPC-producing strains, carbapenem-resistant isolates were examined with two different phenotypic (i.e. MHT and Boronic acid) methods. Subsequently, strains with positive phenotypic methods were examined by PCR as a molecular method.

Results: Twenty-eight (64%) out of 44 isolates were resistant to carbapenem according to CLSI breakpoints and 16 (36%) were susceptible. MHT was positive in all of carbapenem-resistant isolates but none of them have had the synergism effect between meropenem and boronic acid. Also, all isolates were negative for presence of KPC genes on gel electrophoresis. According to results MHT has not enough specificity for detection of KPC.

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* *Corresponding Authors:* Abdolaziz Rastegar Lari, PhD., Antimicrobial Resistance Research Center, Tehran University of Medical Sciences, Tehran, IR Iran, Department of Microbiology, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, IR Iran, *Tel:* +98 21 82943183, *Fax:* +98 21 82943183, *E-mail:* lari@tums.ac.ir

Introduction

Emergence of resistance to carbapenem antibiotics has been increased recently among clinical isolates of *Klebsiella pneumoniae*. Production of carbapenem-hydrolyzing enzyme is one of the major resistance mechanisms to this group of antibiotics (1). Emersion of resistance to these antibiotics as a last choice of antibiotic therapy has made the treatment of these infections more complicated. In the past, resistance mechanisms among *Enterobacteriaceae* was mainly related to decrease in the porins of the bacterial cell wall or the ability of bacterial strains to produce AmpC enzyme or broad-spectrum -lactamase, while resistance to carbapenem had been rarely reported (2). Carbapenems are one of the most important broad-spectrum -lactam antibiotics and have greater activity against many resistant bacteria such as those which have the ability to produce AmpC -lactamase. Recent studies in prevalence of KPC and metallo- -lactamase producing strains among *K. pneumoniae* in different countries have been carried out (2, 3). Transmission of these genes to other gram-negative bacteria should be considered because they are located on mobile genetic elements like transposons and plasmids (4, 5). Several phenotypic methods have been suggested to identify KPC in carbapenem-resistant strains (1, 2, 4, 5). Despite of their high sensitivity, different specificities have been reported for these methods in different studies (1, 2, 4, 5). The aim of this study was to evaluate the specificity of two phenotypic methods, Modified Hodge Test (MHT) and usage of Boronic Acid (BA) as a KPC inhibitor in comparison with PCR method in *K. pneumoniae* isolated from patients.

Materials and Methods

Bacterial isolates

Forty four *K. pneumoniae* strains were isolated from patients with burn wound infection that were hospitalized in the Shahid Motahari Hospital, Tehran from April to July 2013. All isolates were identified with routine biochemical tests such as TSI, SIM and urea.

Antibiotic susceptibility test

Resistance of *K. pneumoniae* isolates to carbapenem antibiotics was determined by disc diffusion method using imipenem, meropenem and ertapenem discs (Mast, Bootle, UK) and analyzed with cut-off points of CLSI 2011 guideline (6).

Modified Hodge Test (MHT) and Boronic Acid (BA)

For identification of KPC-producing bacteria, all carbapenem-resistant strains and some imipenem susceptible isolates were diagnosed and then examined with phenotypic MHT (6) and BA (7-8). MHT was performed according to CLSI 2011 (6). Briefly, a 0.5 McFarland standard suspension of *Escherichia coli* ATCC 25922 was prepared and diluted 1:10 in broth, then inoculate on Mueller-Hinton agar plates for the routine disc diffusion procedure. Appropriate number of ertapenem discs was placed on the plates, which followed by overnight growth of *Klebsiella* spp. on a blood agar and inoculation in a straight line from the edge of the disc to edge of plate. Finally all plates incubated at 37°C for 18-22 hrs. Strains with cloverleaf shape around

of ertapenem disc were considered positive for production of KPC.

A stock solution of BA was prepared as described previously (2, 6) by dissolving boronic acid (benzeneboronic acid; Sigma-Aldrich, Steinheim, Germany) in Dimethyl Sulfate (DMSO) and distilled water to a concentration of 20 mg/ml. From this solution, 20 µl (containing 400 µg boronic acid) was added into meropenem discs. Carbapenem-resistant strains which had at least 5 mm increase in inhibition zone diameter around meropenem plus boronic acid in comparison with meropenem alone were considered as KPC-producer isolates.

Molecular analysis of carbapenem-resistant strains

All positive MHT strains and some imipenem susceptible isolates as negative control were subsequently analyzed by PCR method using five different primers listed in Table 1. Each PCR reaction contained: 1X PCR buffer, 0.4 mM dNTP, 0.7 mM $MgCl_2$, 1.6 µM forward primer, 1.6 µM reverse primer, 1 unit Taq polymerase, 2 µl DNA template and distilled water up to 25 µl. For PCR method, the initial denaturation phase for each PCR assay with different primers was established on 95°C for 5 min also denaturation was 95°C for 1 min. The annealing time was 1 min for all primers and temperature was 64, 56, 56, 60 and 55°C for bla_{KPC}, $bla_{KPC\text{-}A}$, $bla_{KPC\text{-}B}$, $bla_{KPC\text{-}C}$ and $bla_{KPC\text{-}D}$, respectively. The extension time was 1 min in 72°C. The final extension for all genes was done at 72°C for 5 min.

Table 1. Primers used in this study

Genes	Primers	Size	References
bla_{KPC}-F	5'-GTATCGCCGTCTAGTTCTGC-3'-	635 bp	9
bla_{KPC}-R	5'-GGTCGTGTTTCCCTTTAGCCA-3'		
$bla_{KPC\text{-}A}$-F	5'-GGCCGCCGTGCAATAC-3'	60 bp	10
$bla_{KPC\text{-}A}$-R	5'-GCCGCCCAACTCCTTCA-3'		
$bla_{KPC\text{-}B}$-F	5'-CTGTCTTGTCTCTCATGGCC-3'	795 bp	11
$bla_{KPC\text{-}B}$-R	5'-CCTCGCTGTGCTTGTCATCC-3'		
$bla_{KPC\text{-}C}$-F	5'-TGTCACTGTATCGCCGTC-3'	1009 bp	12
$bla_{KPC\text{-}C}$-R	5'-CTCAGTGCTCTACAGAAAACC-3'		
$bla_{KPC\text{-}D}$-F	5'-AACAAGGAATATCGTTGATG-3'	915 bp	13
$bla_{KPC\text{-}D}$-R	5'-AGATGATTTTCAGAGCCTTA-3'		

Results

In this cross-sectional study, 28 (64%) out of 44 isolates were resistant to carbapenems according to CLSI break points and 16 (36%) were susceptible.

MHT was positive in all of carbapenem-resistant isolates *(Figure 1)* but none of them showed at least 5 mm diameter difference when meropenem was combined with BA in comparison with meropenem alone. All isolates were negative for presence of KPC genes on gel electrophoresis.

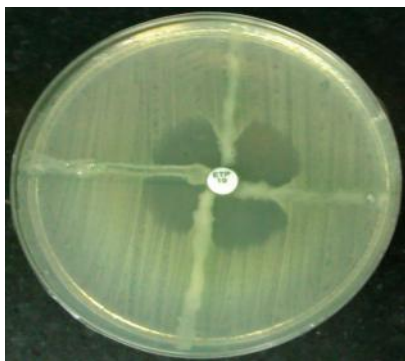

Figure 1. MHT positive strains

Discussion

Developments of resistance to carbapenem antibiotics association to the potential of KPC producing enzyme among *Enterobacteriaceae* have made a challenge and significant problem in treatment of the infections caused by these types of Gram-negative bacteria (2, 5). Rapid phenotypic detection of KPC-producing bacteria can prevent spread of this mechanism of resistance. Several phenotypic methods for detection of KPC have been suggested (14, 15). The specificities of these tests are different. In this study, the results on the usage of BA as a KPC inhibitor was concordant with PCR results, but results of MHT were not. Our results showed low specificity of MHT for detection of KPC. In 2011 in Taiwan, specificity of MHT for detection of KPC was 7%, which is similar to our results. The authors noted the low specificity of MHT for detection of KPC (16). In another study done in Argentina, 57% specificity was reported for MHT (17). On the other hand, according to CLSI 2011, MHT has shown more than 90% sensitivity and specificity for detection of KPC in the USA (18). Even in some studies, 100% sensitivity and specificity of MHT for detection of KPC was observed (19). Recently, Nordmann *et al* also noted that MHT has an excellent sensitivity for detection of KPC (20). In some countries the results of using BA as an inhibitor for detection of KPC were completely confirmed by molecular tests (2, 6, 20). It seems that the sensitivity and specificity of phenotypic methods, especially MHT for detection of KPC can be various and depends on prevalence of carbapenemase resistance and geographical location. Therefore, according to the results of this study, we suggest using MHT as a primary screening test for detection of KPC-producing *K. pneumoniae* in routine laboratories especially in patients whom meropenem and imipenem is used as the first line of antibiotic therapy.

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Conflict of interest

None declared conflicts of interest.

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