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Efficacy of Blue-CARBA Test for Detection of Carbapenemase in *Acinetobacter baumannii* Isolated from Burn Patients

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ABSTRACT

Background: Resistance to carbapenem as a most effective antibiotic for treatment of *Acinetobacter baumannii* infection can make a complication in treatment of patients. Several phenotypic methods have been introduced for detection of the carbapenemase producing strains. The aim of this study was determined the efficacy of the newest method that is called Blue-CARBA test for identification of the carbapenemase producing *Acinetobacter baumannii*.

Methods: In this cross sectional study 63 *Acinetobacter baumannii* have been collected from burn wounds infection. Carbapenem susceptibility testing has been conducted by disc diffusion agar method after identification The Modified Hodge test and Blue-CARBA test was performed for all carbapenem resistant strains. Vim, imp, oxa-23, oxa-48, NDM-1, SPM-1 and kpc genes have been detected as a most common carbapenemase in *Acinetobacter baumannii*.

Results: Sequence analysis showed that 54 isolates include oxa-23 gene. Eight and 5 strains carried vim and kpc genes respectively. MHT and Blue-CARBA test were positive in 27 and 28 of imipenem resistant strains, respectively.

Conclusion: According to the results of this study, Blue-CARBA test showed similar power of carbapenemase producing identification with Modified Hodge test and can be purposed use in place of MHT for detection of carbapenemase.

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Introduction

The beta-lactam antibiotics are an important family of antibiotics used for treatment of infection caused by various bacteria such as *A. baumannii*, because of their broad activity and safety (1, 2). Over the last decade, multi- drug resistance (MDR) and now extremely-drug resistance (XDR) of *A. baumannii* is increasing because of they have the greater ability to develop resistance against antibiotics (2-5).

Due to emergence of resistance to many of beta-lactam antibiotics in *A. baumannii*, the use of carbapenem is necessary for the treatment of *A. baumannii* infection, especially in burned patients (2, 6, 7). This group of antibiotics has broad spectrum activity against gram-negative bacteria is, even some of gram-positive ones (2, 8, 9). Carbapenems except ertapenem are active against *A. baumannii* and usual use for treatment of infection that cause by ESBL-producing bacteria because of the carbapenem resistant to more beta-lactamases (2, 6, 10).

In the recent decade, misuses of antibiotics are particularly being high in hospitalized patients, especially burned patients when physicians treat patient empirically (2, 11). On the other hand the use of carbapenem is increasing due to the high prevalence of MDR and now a days XDR *A. baumannii* (2). This overuse of carbapenems leads to increasing of carbapenem resistant in *A. baumannii* in the last decade (2, 11).

Generally, the resistance to carbapenems can be related to multiple mechanisms such as, production of carbapenemase enzymes, efflux pump and/or over production of AmpC combined with especial porins loss for carbapenem like oprD (1, 2, 5). The most mechanism of carbapenem resistance is associated with hydrolyzing of them belonging to the carbapenemase (2, 12). Carbapenemase producing bacteria are an increasing concern in global health care due to involve the emergence of resistance to beta-lactam antibiotics and the other class of antibiotics like aminoglycosides and fluoroquinolones (1, 2, 12). It can make more complications for treatment of infection associated with these MDR and even XDR strains (2). So, quick, simple and reliable method need for laboratory detection of carbapenemase (9, 13, 14). Recently, many methods use for detection of carbapenemase worldwide like Modified Hodge Test (MHT), use of carbapenem inhibitors, CARBA NP-Test (14) and the newest is Blue-CARBA test (9, 13, 14).

The aim of this study was determined the efficacy of the newest method that is called Blue-CARBA test for identification of the carbapenemase producing *A. baumannii*.

Materials and Methods

Bacterial strains

In this study 63 non replicated imipenem resistant *A. baumannii* have been collected from burn wounds infection in hospitalized burn patients. After the identification with biochemical and microbiological tests, confirmation carbapenem resistant testing has been conducted according to CLSI 2015 guidelines by disc diffusion method with Mast antibiotic disc (MAST Company, England). Standard antibiotic discs have been prepared from MAST Company (14). *A. baumannii* ATCC 19606 was used as a control for identification and *P. aeruginosa* ATCC 27853 used as control strain in the antibiotic susceptibility testing.

Molecular detection

Carbapenem-resistant strains in antibiotic susceptibility testing were considered for molecular assay to confirm carbapenemase-producing bacteria. Extraction of bacterial DNA was performed with a plasmid Mini kit (QIAGEN Plasmid Mini Kit (100), Cat No./ID: 12125, Germany) according to the manufacturer's instructions. Three different sets of multiplex PCR

for Vim, imp and oxa-23, oxa-48 and NDM-1, SPM-1 genes and conventional PCR for kpc gene were designed. The lists of primers used were shown in table 1. *A. baumannii* ATCC 19606 was used as a negative control in all PCR run and we used internal positive control for each gene. PCR was performed in a reaction mixture with the total volume of 25 Il, containing 5 Il template DNA (20 ng), 2.5 Il 10X Taq polymerase buffer [100 mM Tris/HCl (pH 8.3), 500 mM KCl, and 15 mM MgCl2], 0.25 Il (100 pmol/ Il) each of primers, 0.25 Il dNTPs (10 mM), 0.2 Il (5U/ Il) Taq DNA polymerase and 16.55 Il sterilized distilled water. PCR products were analyzed by electrophoresis on agarose 1.5% with Safe staining.

Modified Hodge Test

The modified cloverleaf test was performed according to the CLSI 2015 guidelines using *E. coli* ATCC 25922 and Ertapenem disk to identify carbapenemase producing strains (14).

Blue-CARBA test

Bromothymol blue was used as an indicator with the optimal pH range (6.0-7.6) for most beta-lactamases (pH=6.8), the key and important factor for a this method is the use of direct colony approach, and a commercially and widely available imipenem (Tienam® 500, Merck Sharp & Dohme, France) as substrate for carbapenemases. The test solution consisted of an aqueous solution of bromothymol blue at 0.04% (Merck Millipore, Germany) adjusted to pH=6.0, 0.1 mmol/l ZnSO4 and 3mg/ml of imipenem, with a final pH=7.0. A negative control solution (0.04% bromothymol blue solution, pH=7.0) was prepared to control 50 the influence of bacterial components or products in the pH of the solution.

A loop (approximately 5µ l) of a pure bacterial culture recovered from Mueller-Hinton agar (Merck Millipore, Germany) was directly suspended in 100 µ l of both test and negative control solutions in a 96-well microtiter plate and incubated at 37 ºC with agitation (150 rpm) for 2 hours. Carbapenemase activity was revealed when the test and negative control solutions were respectively: i) yellow vs. blue; ii) yellow vs. green; iii) green vs. blue. Non- carbapenemase producers remained blue or green on both solutions. The test was performed in triplicate for all isolates, yielding reproducible results (13, 15).

Results

According to results of identification tests and carbapenem susceptibility testing, 63 carbapenem resistant *A. baumannii* were confirmed. Direct sequencing of PCR amplified products was carried out using ABI 3730X capillary sequencer (Pishgam, Macrogen, Seoul, Korea). PCR and Sequence analysis showed that 54 (86%) isolates include oxa-23 gene. Eight (13%) strains carried vim and oxa-23 and 5 (8%) of them have kpc and oxa-23 genes, simultaneously. IMP, NDM-1 and SPM-1 have been not detected in any of isolates (Figure 1) and nine strains did not carry any carbapenemase genes.

MHT were positive in 27 (43%) of imipenem resistant strains. Twenty- three of 27 isolates harbored at least one carbapenemase gene (Table 2).

On the other hand, 33 strains which confirmed as carbapenemases-producing strains by PCR assay were showed negative MHT.

Blue-CARBA test was positive in 28 strains that 25 of them were carried different types of carbapenemase genes. On the other hand, 31 carbapenemases producer strains that confirmed by PCR were not shown positive reaction in Blue-CARBA (Table 2).

Table 1. Sequence of primers.

Primer	Sequence (5'→3')	PCR Product Size (bp)	Annealing Temperature (°C)	References
VIM F	TTGACACTCCATTTACDG	390	60	(1)
VIM R	GATYGAGAATTAAGCCACYCT			
Imp F	GATGGTGTTTGGTCGCATA	139		
Imp R	CGAATGCGCAGCACCAG			
OXA-23 F	GATGTGTCATAGTATTCGTCGT	1050	55	(2)
OXA-23 R	TCACAACAACTAAAAGCACTGT			
OXA-48 F	CCAAGCATTTTTACCCGCATCKACC	389		
OXA-48 R	GYTTGACCATACGCTGRCTGCG			
NDM-1 F	CCCGGCCACACCAGTGACA	129	60	(2)
NDM-1 R	GTAGTGCTCAGTGTCGGCAT			
SPM-1 F	GGGTGGCTAAGACTATGAAGCC	447		
SPM-1 R	GCCGCCGAGCTGAATCGG			
KPC F	GTATCGCCGTCTAGTTCTGC	636	56	(3)
KPC R	GGTCGTGTTTCCCTTTAGCC			

Table 2. Different patterns of PCR, MHT and BCT* results.

PCR+/BCT+	PCR+/BCT-	PCR+/MHT+	PCR+/MHT-
46%	57%	42.5%	61

Nine (14%) of carbapenem-resistant strains are PCR, MHT and Blue-CARBA test negative. MHT and Blue-CARBA test showed the Four (15%) and three (11%) false negative results, respectively.

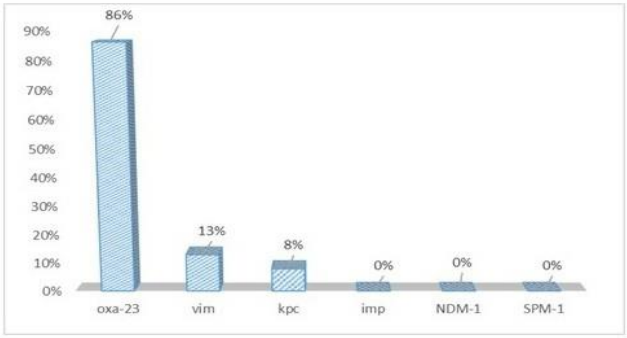

Fig 1. Percentage of strains with different type of carbapenemase genes.

Discussion

Over use of carbapenem is increasing a worldwide and make a complication in treatment of infection, which cause by carbapenem resistant microorganisms such as *A. baumannii* (1, 2). *A. baumannii* is one of the most important and common cause of health care association infection, especially in burn patients and resistance to imipenem in these bacteria mediated by different mechanisms (2, 5-7).

Potential of carbapenemases producing is one of the most important mechanisms in this way that can mediate resistant to all beta-lactam antibiotics (in some case except azthronam), and some other groups of antibiotics like aminoglycosides (2, 8, 9). The results of a study which was carried out in 2014 in China were indicated that 78.33% of carbapenem resistant strains were confirmed as MBL-producer (16). But in current study only 12.69% of tested isolates were confirmed as MBL-producer. This differentiation can be related to variation in the prevalence of different type of carbapenemase in different country and different regions worldwide.

The results of study that has been conducted by Owlia et al in Iran in 2012 showed 39% MBL producing *A. baumannii* (7) but the results of this study showed 12.69% MBL producing strains. This differentiation can be related to different methods that have been used in these two studies. Phenotypic MBL detection has been used in Owlia et al study but PCR assay as a molecular method used in this study with more accuracy. In the study in Argentina, the sensitivity and specificity of MHT for detection of carbapenemases-producer microorganisms were 78% and 57% respectively (17).

According to our results, 85.1% of MHT positive strains were carbapenemase producer and 14.9% of them were non carbapenemase producer. It can indicate the reasonable specificity of MHT for detection of carbapenemases. Fifty- nine percentage of carbapenem producing strains showed negative MHT. It can indicate that MHT has not 100% sensitivity for detection of carbapenemase. On the other hand, 89.2% of strains with positive results of Blue-CARBA test were indicated as carbapenemases producer and 10.8% of them did not carry any of carbapenemase genes.

These results were indicated that the specificity of Blue-CARBA test can be better than MHT but this differentiation is not significant ($P \geq 0.05$). Also, 55.3% of strains showed negative Blue-CARBA despite detection of carbapenemases genes by PCR. The sensitivity of Blue-CARBA test was approximately similar to MHT. According to these results, we purpose use of Blue-CARBA test in place of MHT for detection of carbapenemase, because of: i) more specificity of Blue-CARBA test in contrast of MHT, ii) the Blue-CARBA test can detect carbapenemase more quickly than MHT. Rapid detection of carbapenemase producing strains can play a key role for control speed of carbapenem resistant *A. baumannii*, due to the increasing of carbapenem resistant word wide and make more complication in antibiotic therapy (18-20).

Conclusion

Blue-CARBA test can detect carbapenemase rapidly and in two hours after culture bacteria despite MHT, which need at least 18 hours after culture bacteria. Rapid detection of carbapenemase producing strains can helpful for selection the best choice for antibiotic therapy and prevent the blind prophylaxis of antibiotic therapy. On the other hand, inhibition of the spread of carbapenemase producing strains can be possible by the rapid detection of carbapenemase producing bacteria by using rapid test like Blue-CARBA test.

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Ethics approval and consent to participate

The study was approved by the relevant ethics committee of Iran University of Medical Sciences. All experiments were conducted in accordance with the Helsinki Declaration of 1975, revised in 2000. Informed consent was obtained from all patients involved in the study. The study complies with institutional and national guidelines for research involving human subjects.

Conflict of interest

Ali Mohammad Mirdehghan declares that he has no conflict of interest. Leila Azimi declares that she has no conflict of interest. Amir Mahdi Paksaz declares that he has no conflict of interest.

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