

Comparison between phenotypic and PCR for detection of OXA-23 type and metallo-beta-lactamases producer *Acinetobacter* spp.

Vergleich zwischen Phänotypisierung und PCR für den Nachweis von OXA-23 Metallo-beta-Lactamase produzierenden *Acinetobacter* spp.

Abstract

Background: Resistance to carbapenems is developing around the world and can cause many problems for treatment of patients. Production of metallo-beta-lactamase (MBL) is one of the main mechanism for this type of resistance. So, detection of MBL-producer microorganisms can prevent the spread of this type of resistance.

Materials and methods: In this study 94 *Acinetobacter* spp. were investigated. Resistance to imipenem was conducted after purification and identification. Combination disc (CD) and Double Disc Synergy Test (DDST) were performed for phenotypic detection of MBL and the molecular PCR method was done for vim-1, vim-2, imp-1 and OXA-23 genes.

Results: According to TSI, SIM and oxidation-fermentation (OF) test and PCR assay 93 *Acinetobacter baumannii* and one strain *Acinetobacter lwoffii* were identified. 85% of them were resistant to imipenem. 34% of them have a positive combination disc test (CD) while Double Disc Synergy Test (DDST) was negative for all of them. The vim-1, vim-2 and imp-1 genes were not detected in PCR molecular method, however in 74% of strains with positive results in combination disc, were positive for the OXA-23 gene after PCR test. This study shows that the blaOXA-23 resistance determinant may become an emerging therapeutic problem.

Discussion: According to the results, it seems that combination disc does not have enough specificity for detection of MBL-producer *Acinetobacter* and using Double Disc Synergy Test (DDST) can be more convenient.

Keywords: metallo-beta-lactamase, *Acinetobacter*, combination disc Test, Double Disc Synergy Test, OXA23

Zusammenfassung

Hintergrund: Die Resistenz gegen Carbapeneme nimmt weltweit zu und verursacht viele Probleme bei der Behandlung von Patienten. Die Produktion von Metallo-beta-Lactamasen (MBL) ist einer der Hauptmechanismen dieser Resistenz. Daher kann die Erkennung vom MBL-bildenden Mikroorganismen die Ausbreitung dieses Resistenztyps verhindern.

Materialien und Methoden: In der Studie wurden 94 *Acinetobacter* spp. untersucht. Nach Reinigung und Identifizierung der Isolate wurde die Imipenem-Resistenz bestimmt. Der Combination Disc Test (CD) und der Double Disc Synergy Test (DDST) wurden zum phänotypischen Nachweis der MBL durchgeführt. Zum Nachweis der Gene vim-1, vim-2, imp-1 und OXA23 wurde die molekularbiologische PCR eingesetzt.

Ergebnisse: Es wurden mittels TSI-Medium, SIM, Oxidations-Fermentations-Test und PCR-Untersuchung 93 *Acinetobacter baumannii* und ein *Acinetobacter lwoffii* identifiziert. 85% waren resistent gegenüber Imipenem. 34% von diesen zeigten einen positiven Combination Disc Test

Leila Azimi¹

Abdolaziz Rastegar
Lari^{1,2}

Malihe Talebi^{1,2}

Amirmorteza

Ebrahimzadeh Namvar¹

Mosadegh Jabbari³

1 Antimicrobial Resistance
Research Center, Iran
University of Medical
Sciences, Tehran, Iran

2 Department of Microbiology,
Faculty of Medicine, Iran
University of Medical
Sciences, Tehran, Iran

3 Dept. of Nephrology, Rasoul
Akram Hospital, Iran
University of Medical
Sciences, Tehran, Iran

(CD), wohingegen der Double Disc Synergy Test (DDST) in allen Fällen negativ ausfiel. Die *vim-1*, *vim-2* und *imp-1* Gene wurden in der PCR-Methode nicht nachgewiesen, allerdings zeigten in der PCR 74% der Stämme, die im Combination disc Test positiv waren, das OXA-23 Gen. Die Untersuchung zeigt, dass die *bla*OXA-23 Resistenzdeterminante zu einem neuen therapeutischen Problem werden kann.

Diskussion: Aufgrund der Ergebnisse scheint der Combination Disc Test (CD) nicht genügend spezifisch für den Nachweis von MBL-bildenden *Acinetobacter* zu sein, wohingegen der Double Disc Synergy Test (DDST) geeigneter ist.

Schlüsselwörter: Beta-Laktamase, *Acinetobacter*, Combination Disc Test, Double Disc Synergy Test, OXA23

Introduction

The emergence of carbapenem-resistant *Acinetobacter* spp. in the last decade has become a serious problem in the health community [1], [2]. The ability of producing Carbapenemases enzymes such as metallo-beta-lactamase (MBL) are the most common resistant mechanism to carbapenem. The class B beta-lactamase (MBL) which is hydrolyzing carbapenem and other beta-lactams except Monobactam can cause resistance to this antibiotic family [3], [4], [5]. Transferring of mobile genetic elements like plasmids is the main reason for spreading of this resistance mechanism in gram negative bacteria [6], [7]. Several phenotypic methods for detection of MBL-producer isolates have been suggested by using chelating agents nevertheless the specificity and sensitivity of this method are diverse [8], [9]. The Combination Disc assay (CD) and Double Disc Synergism Test (DDST) by use of imipenem and EDTA as an MBL inhibitor are the most widespread phenotypic methods which are used in numerous studies [10], [11], [12]. Molecular method PCR with different primers have been used for confirmation of phenotypic assay since, *bla*_{VIM} and *bla*_{IMP} are the most common MBL, so detection of these gene use for evaluation of phenotypic methods [3]. Because resistance to carbapenems is an increasing problem among *Acinetobacter baumannii*.

The aim of this study is using the CD and DDST methods as two phenotypic methods and PCR for achieving *bla*_{VIM1}, *bla*_{VIM2} and *bla*_{IMP1} as a common MBL gene in imipenem-resistant *Acinetobacter baumannii* isolated from burned patients.

Methods

Bacterial specimen

In this study 94 *Acinetobacter* spp. was isolated from burned patients that were hospitalized 3–10 days in Motahari hospital as a referral burn unit in Tehran, Iran. Isolated bacteria were purified in EMB and then collected for species confirmation by specific biochemical tests such as oxidase, motility, OF, etc. PCR was used as a confirmatory test for identification *Acinetobacter bauman-*

nii strains by specific primers for OXA-51-like gene (Table 1) [13], [14], [15], [16]. *Acinetobacter baumannii* ATCC 19606 was used as positive control. PCR program followed by: Initial denaturation 94 °C for 5 min, denaturation 94 °C for 45 seconds, annealing 58 °C for 1 min, extension 72 °C for 1 min, this program repeated for 30 cycles and the final extension 72 °C for 5 min.

Antibiotic susceptibility test

The antibiotic susceptibility test was carried out according to CLSI 2011 recommendation and MAST company antibiotic discs. Strains by ≤13 mm zone size of inhibition were considered as imipenem-resistant. MIC was performed by macro dilution between the range of 0.5–128 µg/ml according to CLSI recommendation. MIC ≥16 µg/ml considered as an imipenem-resistant strain.

Phenotypic detection of metallo beta-lactamase

At first 0.5 M of EDTA reached by dissolving 186.1 grams in one liter distilled water and PH adjusted [8] by NaOH, then EDTA 750 µg/disc and 930 µg/disc were prepared. The inhibition zone of each disc was measured solitary. In the next step the DDST conducted by imipenem and EDTA distinctly for each disc with 750 and 930 µg/disc, which were placed on both sides of imipenem with a distance of 20 mm center to center, for eventual synergism effectiveness. Strains with increasing size in the imipenem inhibition zone towards EDTA are considered as MBL producers in DDST. In the CD assay usage of imipenem alone and imipenem plus two concentrations of EDTA, the strains with ≥7mm differences of zone inhibition between imipenem alone and imipenem plus EDTA in two concentrations are considered as MBL producer.

PCR molecular test for detection of MBL (*bla*_{VIM-1}, *bla*_{VIM-2} and *bla*_{IMP-1})

Strains with at least one positive phenotypic test that explained above were examined for *bla*_{VIM-1}, *bla*_{VIM-2} and *bla*_{IMP-1} genes by PCR [9]. In this study also detection of OXA-23 gene which is known as one of the common car-

Table 1: Primer sequences and amplicon sizes

Gene	Primer sequences	Amplicon size (pb)	Reference
<i>OXA-51-likeF</i>	TAATGCTTTGATCGGCCTTG	353 bp	[13]
<i>OXA-51-like-R</i>	TGGATTGCACTTCATCTTGG		
<i>blavim-1-F1</i>	ATGTAAAAAGTTATTAGTAGT	801 bp	[14]–[15]
<i>blavim-1-R1</i>	CTACTCGGCGACTGAGCGAT		
<i>blavim-2-F2</i>	CTACTCAACGACTGAGCGAT	801 bp	[15]
<i>blavim-2-R2</i>	ACC GCA GCA GAG TCT TTG CC		
<i>blaIMP1-F1</i>	ACC GCA GCA GAG TCT TTG CC	587 bp	[16]
<i>blaIMP1-R1</i>	ACA ACC AGT TTT GCC TTA CC		
<i>OXA-23-F</i>	GAT GTG TCA TAG TAT TCG TCGT	1058 bp	
<i>OXA-23-R</i>	TCA CAA CAA CTA AAA GCA CTG T		

Table 2: The PCR program for bla_{VIM-1}, bla_{VIM-2}, bla_{IMP-1} and OXA-23

PCR steps	Gene	Temperature	Time
Initial denaturation	blavim-1 blavim-2 blaIMP1 OXA-23	95°C	5 min
Denaturation	blavim-1 blavim-2 blaIMP1 OXA-23	95°C	60 sec 45 sec 60 sec 60 sec
Annealing	blavim-1 blavim-2 blaIMP1 OXA-23	25°C 58°C 55°C 57°C	60 sec 45 sec 60 sec 60 sec
Extension	blavim-1 blavim-2 blaIMP1 OXA-23	72°C	60 sec 45 sec 90 sec 60 sec
Final Extension	blavim-1 blavim-2 blaIMP1 OXA-23	72°C	5 min

bapenemase in *Acinetobacter* spp., was done by PCR for survey of false positive responds in CD assay. The Primers and PCR program were used in this study are shown in Table 1 and Table 2.

Polymerase chain reaction was performed for each sample with the following compounds. 1X concentration Specific PCR buffer, 0.4 mM of dNTPs mix, 0.7 mM of MgCl₂, 1.6 M of each primer, one unit of Taq polymerase enzyme, 2 µl of DNA and sterile distilled water to get 25 µl as a final volume. Finally, the PCR products were evaluated on a 1% agarose gel.

Results

Ninety-three strains were identified as *Acinetobacter baumannii* by specific biochemical test and confirmed by

PCR. According to CLSI 2011 guideline, 80 (85%) strains were resistant to imipenem. MIC verified these results (MIC 16 µg/ml: 20%, MIC 32 µg/ml: 26%, MIC 64 µg/ml: 46% and MIC 128 µg/ml: 8%). 31 (34%) of imipenem-resistant strains were positive in the CD test with 750 and 930 µg/disc concentration of EDTA. Simultaneously, 750 and 930 µg/disc EDTA alone made the inhibition zone up to 13 mm and 20 mm respectively (Figure 1). None of them had synergistic effects between these two charges of EDTA alone and imipenem alone. In the molecular test there was not detected any VIM1, VIM2 and IMP1 genes in expected size after gel electrophoresis. Conversely, OXA-23 gene was observed in 25 out of 31 strains in positive CD test (Figure 2).

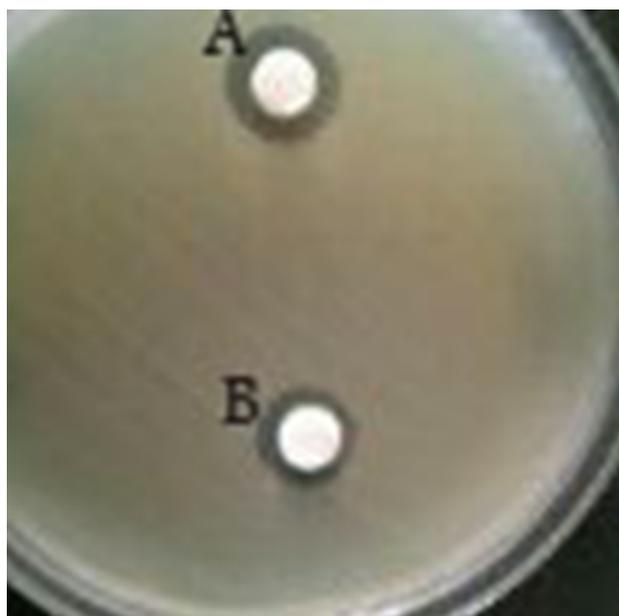


Figure 1: The inhibition zone of EDTA disc
A. 930 µg/disc, B. 750 µg/disc

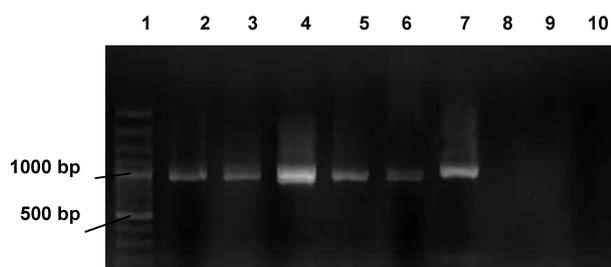


Figure 2: (From left to right) 1-Ladder 2-7 positive OXA-23 and 8-10 negative OXA-23

Discussion

The ability of producing metallo-beta-lactamase enzymes in gram negative bacteria is one of the resistance mechanisms, consequently the several methods have been suggested for phenotypic identification of metallo-beta-lactamase enzymes [2], [3], [7]. CD and Double Disc Synergism DDST methods with EDTA and imipenem are known as the most ordinary methods for phenotypic recognition of metallo-beta-lactamase [8], [9]. In the Eser and colleagues' study which was done on 124 strains of *Acinetobacter baumannii* in Turkey in 2009, 80% imipenem-resistant strains were detected as CD positive, however the results of PCR molecular tests for detection of bla_{VIM} and bla_{IMP1} were negative [11]. Results of recent studies are exactly similar to ours while the bla_{VIM1} , bla_{VIM2} and bla_{IMP1} genes in CD positive strains didn't distinguish, therefore our data shows that this technique is not specified for the detection of MBL. It is notable that the specificity of CD test was reported 98% in another study on *Enterobacteriaceae* family in Greece in 2008 [7], despite that the EDTA concentration was 2.5 times higher than ours. On the other hand in 2012 a Malaysian study was conducted on imipenem-resistant *Pseudomonas*

aeruginosa. The results showed that the specificity of the CD test in 43% of the strains was confirmed by molecular method while the specificity of DDST 97% and E-Test 62% reported respectively [8]. In 2008, a Brazilian study on gram-negative bacilli to identify the MBL enzyme using different inhibitors was performed. Test results showed that the CD test could not classify *Acinetobacter* species [10]. In the present study, no synergistic effect was observed in DDST techniques and molecular tests for the presence of genes $VIM1$, $VIM2$ and $IMP1$ was negative. According to the study, it was shown that EDTA alone was tested for *Acinetobacter* strains has an inhibition zone from 13 mm for 750 µg/disc and up to 20 mm for 930 µg/disc). Therefore, an increase of 7 mm zone of inhibition between imipenem alone and imipenem with EDTA can not explain the synergistic effect of these two materials. In 2011 in Iran the DDS test for the detection of MBL in *Pseudomonas* spp. showed that phenotypic tests for MBL detection was confirmed by molecular testing [3]. In 2002 in Italy 28 *Acinetobacter* imipenem-resistant strains were studied that 16 strains with DDST were positive for MBL production and the results confirmed by PCR. Maybe using CD test by other MBL inhibitors can partially compensate the problems caused by the use of Imipenem and EDTA simultaneously. MBL mediated imipenem resistance in *Acinetobacter baumannii* is a cause for concern in the treatment of infected burns patients. The rate of imipenem resistance due to MBLs was increased dramatically [8], [17]. The clinical significance of OXA-23 isolates, which were widespread [18], [19] in burn unit, is of great importance, since clinicians are advised against the use of extended-spectrum cephalosporins, aztreonam, cephamycins, imipenem, and aminoglycosides. In 2010 in China, an outbreak of OXA-23 producer *A. baumannii* was reported [18]. In 2013 in Nigeria 60% of imipenem-resistant isolated *A. baumannii* positive for OXA-23 [20].

This observation emphasizes the importance of having effective control measures in Iranian burn hospitals, such as early detection of colonized patients, isolation procedures, and a judicious use of antibiotics.

Conclusions

According to the results of this study and other studies, it seems that the DDS test for MBL detection is more specific than the CD test. On the other hand, using different charges of EDTA and zone inhibition in CD test is another reason that proves this phenotypic method is unreliable in the detection of MBL producing strains. It appears that the different species of bacteria and geographical area have important role in determining the specificity of the CD, also the variety of used concentrations and variables methods which have been applied in any of the studies mentioned above, confirm the instability of conditions and impossibility of standardization of the CD by EDTA tests. In addition, because the 80% of CD test responses in imipenem-resistant strains were fake, the

OXA-23 gene was detected instead of metallo-beta-lactamase.

Notes

Competing interests

The authors declare that they have no competing interests.

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Corresponding author:

Abdolaziz Rastegar Lari, PhD
Antimicrobial Resistance Research Center, Iran University
of Medical Sciences, P.O. Box 14515-717, Tehran, Iran,
Phone/Fax: +98-21-86703183
lari@tums.ac.ir

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