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Short Communication

Evaluation of tests to predict metallo-β-lactamase in cystic fibrosis (CF) and non-(CF) *Pseudomonas*

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Abstract

Double disks synergy test (DDST) and combined disks test (CD) were evaluated to predict the presence of metallo-β-lactamase in 70 *Pseudomonas aeruginosa* isolates recovered from cystic fibrosis and non-cystic fibrosis patients. $DDST_{CAZ\text{-}EDTA\ 1\ cm}$ and $CD_{IMP\text{-}EDTA}$ tests showed the best accuracy (94.3%). Furthermore, for other combinations, accuracy unsatisfactory was obtained.

Key words: *Pseudomonas aeruginosa*, metallo-β-lactamase, phenotypic tests.

Pseudomonas aeruginosa is one of the leading nosocomial pathogens worldwide. Infections caused by *P. aeruginosa* are often hard to treat mainly because of the intrinsic resistance and due to the high ability of this organism to acquire resistance to antimicrobial agents, including β-lactams (Giamarellou and Kanellakopoulou, 2008).

Production of β-lactamases is the major mechanism of acquired resistance to β-lactam agents. Carbapenem-hydrolyzing enzymes, such as metallo-β-lactamases, are among enzymes that occur in *P. aeruginosa*.

The production of these enzymes determines resistance to all β-lactams agents (including the carbapenems imipenem and meropenem) except aztreonam (Maltezou, 2009).

The prevalence of MBLs, notably among *P. aeruginosa*, has been increasing worldwide and it is a significant problem that limits therapeutic options for the treatment of patients (Maltezou, 2009). Recently, an increase of MBLs in *P. aeruginosa* isolated from cystic fibrosis patients was observed, limiting the therapeutic options for these patients (Pollini *et al.*, 2011; Perez *et al.*, 2012).

So far, no standardized phenotypic or molecular test for the detection of MBLs has been established by the Clinical and Laboratory Standards Institute (CLSI, 2012). Molecular detection of MBL by polymerase chain reaction using specific primers produce reliable and satisfactory results (Franklin *et al.*, 2006), however, its application in clinical laboratories is often limited due to high cost (Arakawa *et al.*, 2000). The need to develop simple, practical, and low cost tests for screening of MBL-producing bacterial isolates led to the study of various non-molecular techniques (Arakawa *et al.*, 2000; Lee *et al.*, 2003; Franklin *et al.*, 2006; Picão *et al.*, 2008). All these methods are based on inhibition of enzyme activity through the use of chelating agents (Picão *et al.*, 2008), such as EDTA and thiol esters (Arakawa *et al.*, 2000).

This study aimed to characterize isolates of *P. aeruginosa* MBL-producing by two phenotypic methods: double disks synergy test (DDST) and combined disks test (CD).

Isolates that presented resistance to at least ceftazidime (CAZ) or imipenem (IMP) according to CLSI guidelines (CLSI, 2012) were included in the study. A total

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of 70 isolates of *P. aeruginosa* were evaluated: forty-two isolates from patients admitted at Hospital Mãe de Deus (HMD), Porto Alegre, and 28 from cystic fibrosis patients admitted at Hospital de Clinicas de Porto Alegre (HCPA), both hospitals located in South of Brazil. To minimize possible clonal relatedness, only one isolate per patient was used in the study.

In DDST, 30 μg ceftazidime disk (CAZ) and 10 μg imipenem disk (IMP) were used as enzymatic substrates while blank disks containing EDTA (10 μL, 0.1 M) or 2-mercaptopropionic acid (2 μL) were used as enzymatic inhibitor agents. All combinations were tested in the distances of 1, 2 and 2.5 cm (center to center) between substrates and inhibitors agents. After incubation, isolates that presented inhibition of growth in the interface between antibiotics and disks containing inhibitors were considered positive for MBL (Picão *et al.*, 2008).

In CD, the follow combinations were determined: 30 μg CAZ disk plus 10 μL EDTA 0.1 M and 10 μg IMP disk plus 10 μL EDTA 0.1 M. The result was considered positive with the occurrence of an increase in the size of inhibition zone ≥ 5 mm in the disk plus chelator in comparison with disks containing only the substrate (Lee *et al.*, 2003).

For both tests, a 0.5 MacFarland bacterial suspension of each clinical isolate was made and inoculated on Mueller-Hinton agar (bioMerieux, Rio de Janeiro, Brazil), after 24 h of incubation at 35 °C plates were examined and results registered.

Isolates were tested for detection of the *bla*SPM--1-like, *bla*IMP-1-like and *bla*VIM-2 genes by PCR as previously described (Picão *et al.*, 2008).

Most (88.1%, 37/42) *P. aeruginosa* isolates recovered from patients at HMD were positive for the SPM-1 gene, whereas in the five remaining isolates PCR results were negative for the genes tested. On the other hand, only three isolates (10.7%, 3/28) recovered from cystic fibrosis patients were positive for MBL genes (two for IMP-1 gene and one for SPM-1 gene). The results for the different substrate/inhibitor combinations in the DDST and CD tests are shown in Table 1.

Thirty-seven isolates obtained from patients admitted at HMD, all SPM-1 positive, showed a positive reaction to at least one phenotypic test. It is interesting to note that twenty-eight positive isolates in the CD test (among SPM-1 positive isolates) for both substrates presented also positive result in $DDST_{CAZ\text{-}EDTA}$ at a distance of 1 cm between disks and in the $DDST_{IMP\text{-}EDTA}$ at a distance of 1 cm. Two isolates from non-CF patients, positive for SPM-1 gene, proved to be positive only by the $DDST_{CAZ\text{-}EDTA}$ at 1 cm combination, while another isolate harboring SPM-1 gene was negative for all combinations in the DDST tests, except for a CD test with CAZ (Table 1).

Two isolates harboring IMP-1 gene showed a distinct behavior in the different tests, however, negative results for $DDST_{CAZ\text{-}EDTA\ 2.5\ cm}$, $DDST_{IMP\text{-}EDTA\ 2.0\ cm}$, $DDST_{IMP\text{-}EDTA\ 2.5\ cm}$, $DDST_{CAZ\text{-}MPA\ 1\ cm}$, $DDST_{IMP\text{-}MPA\ 2.5\ cm}$ and $CD_{IMP\text{-}MPA}$ were observed in these two isolates. Combinations of EDTA with CAZ or with IMP at 2.5 cm were unable to detect MBL among the 35 MBL producing isolates (33 SPM-1 and 2 IMP-1). It is of note that the 2.5 cm distance between the antibiotic disk and the disk containing EDTA showed to be poorly accurate to predict MBL (accuracy ranging from 42.8% to 52.8%) (Table 2), mainly in isolates harboring SPM-1 gene. For the isolates IMP-1 positive the best results were obtained when performed with $CD_{IMP\text{-}EDTA}$ (Table 1). Similarly, for the isolates harboring SPM-1 gene, the best results were obtained with the CD test (regardless the antibiotic used as substrate) and with the $DDST_{CAZ\text{-}EDTA}$ combination and disks at 1 cm. A higher accuracy was observed with CD and $DDST_{\text{-}EDTA}$, especially with CAZ as substrate and applied at 1 cm of distance between the disks (Table 2). Another point to be considered is that among the twenty-eight imipenem non-susceptible isolates that were negative for those genes, in four of them at least one phenotype showed to be positive (Table 1).

Our results seem to be in opposition to the study of Arakawa *et al.* (2000) that demonstrated that the combination of CAZ and 2 MPA would be more sensitive in detecting isolates producing MBLs. On the other hand, a study conducted by Lee *et al.* (2003) the 2 MPA showed better sensitivity for isolates of *Acinetobacter* spp than *Pseudomonas* spp, however, the combination with EDTA and CAZ detected 100% of *P. aeruginosa* isolates, but failed in detecting the effect among isolates of *Acinetobacter* spp. Additionally, Chu *et al.* (2005) describes the poor effectiveness of the Etest and IMP-EDTA disk method for MBL detection in *P. aeruginosa* because the susceptibility of the microrganism to EDTA. Therefore, standardization is extremely important and it is desirable to select the appropriate test based upon studies that provide sensitivity and specificity for a specific pathogen (Strateva and Yordanov, 2009). Imipenem or ceftazidime-resistant *P. aeruginosa* isolates recovered from cystic fibrosis patients challenges the accurate detection of MBLs, because carbapenem-resistance among these isolates seems to be due to other mechanisms (impermeability or efflux) than carbapenemase production. For these isolates, discrepant results (phenotypic test positive for non-MBL producers) were originated from 4 distinct combinations: $DDST_{CAZ\text{-}MPA\ 2.5\ cm}$ (4 isolates); $DDST_{IMP\text{-}MPA\ 2.5\ cm}$ (4 isolates); $DDST_{CAZ\text{-}EDTA\ 1\ cm}$ (1 isolate) and $CD_{IMP\text{-}EDTA}$ (1 isolate). Thus, it is possible to speculate that there is influence of different variables involved (chelators, the test format, substrates, origin of the isolates, type of MBL involved, co-existence of other mechanisms of carbapenem resistance) in studies on the detection of MBLs.

Table 1 - Characterization of the *P. aeruginosa* isolates for the presence of MBL.

Source[a]	Nº of samples	Gene[b]	CAZ/IMP[c]	DDST[d] (EDTA and CAZ)			DDST (EDTA and IMP)			DDST (2 MPA and CAZ)			DDST (2 MPA and IMP)			CD[e] (plus EDTA)	
				1 (cm)	2 (cm)	2.5 (cm)	1 (cm)	2 (cm)	2.5 (cm)	1 (cm)	2 (cm)	2.5 (cm)	1 (cm)	2 (cm)	2.5 (cm)	CAZ	IMP
CF	1	*blaI*MP-1	S/R	neg[f]	neg	neg	pos[f]	neg	neg	neg	neg	neg	pos	neg	neg	neg	pos
CF	10	none	S/R	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
CF	1	none	R/I	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
CF	6	none	S/I	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
CF	1	*blaI*MP-1	I/R	pos	pos	neg	pos	neg	neg	neg	pos	pos	neg	pos	neg	neg	pos
CF	3	none	S/I	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
CF	2	none	S/I	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	pos	neg	neg
CF	1	*bla*SPM-1	R/R	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	pos
CF	1	none	S/R	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos
CF	2	none	I/S	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg	neg	pos	neg	neg
non-CF	3	*bla*SPM-1	R/R	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	pos
non-CF	2	*bla*SPM-1	R/R	pos	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg
non-CF	3	*bla*SPM-1	S/R	pos	neg	neg	pos	neg	neg	neg	neg	pos	neg	neg	neg	neg	pos
non-CF	1	*bla*SPM-1	R/S	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	pos	neg
non-CF	6	*bla*SPM-1	R/R	pos	pos	neg	pos	pos	neg	neg	neg	neg	neg	neg	neg	pos	pos
non-CF	2	*bla*SPM-1	R/R	pos	neg	neg	pos	neg	neg	neg	pos	pos	neg	neg	pos	pos	pos
non-CF	5	*bla*SPM-1	R/R	pos	neg	neg	pos	neg	neg	pos	pos	pos	pos	pos	neg	pos	pos
non-CF	5	*bla*SPM-1	R/R	pos	pos	pos	pos	neg	neg	pos	pos	neg	pos	pos	pos	pos	pos
non-CF	10	*bla*SPM-1	R/R	pos	pos	neg	pos	pos	neg	pos	neg	neg	pos	neg	neg	pos	pos
non-CF	5	none	R/R	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg	neg

[a]CF, cystic fibrosis; non-CF, non-cystic fibrosis.
[b]gene detected by a PCR procedure.
[c]S, susceptible; R, resistant, according to CLSI breakpoints (CLSI 2012).
[d]DDST, double-disk synergy test.
[e]CD, combined test.
[f]neg, negative; pos, positive.
The shaded results in the table represent the isolates producing MBL correctly characterized by the phenotypic test.
Results inside rectangle in the table represent the isolates non-producing MBL incorrectly characterized by the phenotypic test.

Table 2 - Sensitivity, specificity and accuracy of variations of approved screening tests for detecting the MBL gene.

	DDST (EDTA and CAZ)			DDST (EDTA and IMP)			DDST (2 MPA and CAZ)			DDST (2 MPA and IMP)			CD (plus EDTA)	
	1 (cm)	2 (cm)	2.5 (cm)	1 (cm)	2 (cm)	2.5 (cm)	1 (cm)	2 (cm)	2.5 (cm)	1 (cm)	2 (cm)	2.5 (cm)	CAZ	IMP
[a]Sensitivity (%)	92.5	55	12.5	82.5	40	0	50	32.5	27.5	52.5	72.5	17.5	82.5	92.5
[b]Specificity (%)	96.7	100	100	100	100	100	100	100	86.7	100	100	86.7	100	96.7
[c]Accuracy (%)	94.3	74.3	50	90	65.7	42.8	71.4	61.4	52.8	72.8	58.6	47.1	90	94.3

[a]Sensitivity; the percentage of MBL-positive strains correctly categorized.
[b]Specificity; the percentage of MBL-negative strains correctly categorized.
[c]Accuracy; the percentage of MBL-positive and -negative correctly categorized.

In our study, none combination evaluated was totally discriminatory for the presence of MBL (according to PCR procedure). It is of note that in some cases in which an MBL gene was identified and a phenotypic assay revealed a negative finding could be explained by lack or variability of gene expression at the protein (enzyme) level. So, the mere presence of an MBL gene does not imply a functional enzyme. A potential limitation of this study lies in the fact that the genetic background of the isolates is not clearly defined. However, selection of only one isolate per patient (with and without cystic fibrosis) denotes a probable clonal variability among these isolates.

Finally, our results showed that only two phenotypic tests - $DDST_{CAZ\text{-}EDTA\ 1\ cm}$ and $CD_{IMP\text{-}EDTA}$, from 14 different combinations, proved to be over 90% accurate to predict the presence of any MBL gene. Our results confirm that detection of MBLs in clinical laboratory by phenotypic methods is still a matter of debate and more studies are needed to clarify this issue.

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