Influence of Disk Preparation on Detection of Metallo- β -Lactamase-Producing Isolates by the Combined Disk Assay[†]

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The combined disk assay has been used for detection of metallo- β -lactamase-producing isolates. We have observed that the size of inhibition zones produced by many β -lactam/metallo- β -lactamase inhibitor (IMBL) combinations may differ depending on the way that the combined disks were prepared. Among the 10 β -lactam/IMBL combinations tested, only the imipenem/EDTA combination produced similar results.

Metallo- β -lactamase (MBL)-producing isolates have been increasingly reported in many geographic regions (12). Due to the ability of MBL-producing isolates to spread and to hydrolyze most β -lactam agents, accurate detection of this resistance phenotype by routine laboratories is essential to initiate adequate empirical therapy and to implement proper infection control practices.

There are no current Clinical Laboratory Standards Institute (CLSI) guidelines for screening bacterial isolates for acquired MBL production. Several phenotypic tests have been developed for MBL detection, such as the MBL Etest (AB Biodisk, Solna, Sweden) (9, 14, 18), double-disk synergy tests (1, 7, 9), combined disk (CD) assay (14, 15, 20), microdilution (11), and the Hodge test (7). All of these tests are based upon the ability of chelating agents, EDTA and thiol-based compounds, to inhibit the MBL activity.

The combined disk assay employs a β -lactam disk, usually a carbapenem or ceftazidime, to which an MBL inhibitor (IMBL) is added. The results are further compared with the inhibition zones produced by the corresponding β -lactam agent alone. Due to its objective interpretation, this test has been considered a good phenotypic resource (5). However, the incorporation of IMBL into the ceftazidime or imipenem disks has not been standardized yet. Different studies have either (i) added the IMBL solution directly on the β -lactam disk already placed on the agar plate (AD) (6, 20) or (ii) previously prepared the disks (PP) (5, 19), which are dried at ambient temperature and stored at 4°C or –20°C for future use (20). Therefore, it is not known if results obtained by the combined disk assay using distinct IMBL incorporations are different. Upon screening for MBL-producing isolates in our laboratory, we realized we could have obtained different results for some isolates, depending on the way that IMBLs were added to β -lactam disks. Thus, we have designed a study to verify inhibition zone results for (i) β -lactam/IMBL disks previously prepared, dried, and

stored (PP) and (ii) β -lactam/IMBL disks prepared by adding the IMBL solution after placing the β -lactam disk on a Mueller-Hinton (MH) agar plate (AD). We believed it was important to investigate whether AD and PP would display identical inhibition zone results, since screening of suspicious MBL-producing isolates by CD is directly influenced by the size of inhibition zones.

The isolates evaluated in this study are described in Table 1. Only genetically unrelated MBL producers were selected. A total of five IMP-1 producers, one of each species tested, were selected (*Pseudomonas aeruginosa*, *Acinetobacter* sp., *Serratia marcescens*, *Enterobacter cloacae*, and *Klebsiella pneumoniae*). Other isolates included two VIM-1-producing *E. cloacae* strains, one SPM-1-producing *P. aeruginosa* strain, one GIM-1-producing *P. aeruginosa* strain, and one SIM-1-producing *Acinetobacter baumannii* strain.

The IMBLs were purchased from Sigma (Steinheim, Germany), except for mercaptoethanol, which was obtained from Gibco (New York, NY). The IMBL solutions tested were 100 mM EDTA, 1.4 mM mercaptopropionic acid (MPA), 1.2 mM mercaptoacetic acid (MAC), 55 mM mercaptoethanol (MET), and 8 mM phenanthroline (PHEN). The size of the inhibition zones produced by β -lactam disks containing 10 μ l of each IMBL solution was assessed.

The phenotypic tests were performed by following the CLSI recommendations for the disk diffusion method. Briefly, a 0.5 McFarland bacterial suspension was inoculated on an MH agar plate (Oxoid, Basingstoke, England). For the AD combined disk assay, ceftazidime and imipenem disks were first placed on the inoculated MH plates, and 10 μ l of each inhibitor solution was directly added to the disks. For the PP combined disk assay, 10 μ l of the IMBL solutions was previously incorporated into ceftazidime and imipenem disks, which were immediately dried at room temperature and stored overnight at –20°C. The β -lactam/IMBL disks were then placed on MH agar plates inoculated previously with the test strain. After overnight incubation at 35°C, the size of the combined disk inhibition zone was measured and compared to that displayed by the β -lactam disk itself for both methodologies (AD and PP). Since there is no general consensus about which breakpoints should be used

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TABLE 1. Characteristics of the 10 MBL-producing bacterial isolates selected for this study

Organism	MBL	Reference
<i>P. aeruginosa</i>	IMP-1	16
<i>Acinetobacter</i> sp.	IMP-1	10
<i>P. aeruginosa</i>	GIM-1	4
<i>E. cloacae</i>	VIM-1	3
<i>S. marcescens</i>	IMP-1	13
<i>E. cloacae</i>	IMP-1	2
<i>K. pneumoniae</i>	IMP-1	3
<i>E. cloacae</i>	VIM-1	3
<i>P. aeruginosa</i>	SPM-1	17
<i>Acinetobacter</i> sp.	SIM-1	8

to classify a bacterial isolate as an MBL producer and we do not know which breakpoint would work better under the tested conditions, we only present results of mean inhibition zones of MBL-producing isolates. Thus, this study was not designed to determine which one (AD or PP) would be the best test to detect MBL-producing isolates.

For each of the 10 β -lactam/IMBL combinations, a total of 20 experiments were performed (10 PP and 10 AD), leading to 200 observations. The results of this study are shown in Table 2. AD and PP presented different results for MBL detection according to the β -lactam/IMBL combination employed. Among the MBL-producing isolates, larger inhibition zones were observed for the AD test, except for imipenem/EDTA (mean of AD and PP, 9.0 mm) and imipenem/MAC (mean of AD, 4.0 mm; mean of PP, 5.0 mm). The greatest discrepancy between AD and PP was noticed for the ceftazidime/MAC combination (15.9 mm versus 8.2 mm, respectively), followed by imipenem/MET (5.0 mm versus 0.0 mm, respectively) (Table 2).

We have noticed that the mean size of inhibition zones produced by combining EDTA and imipenem was the same for both AD and PP. This finding is significant, since differences in the preparation of the disks would not have influenced MBL detection. In this manner, studies that have employed this combination might have their final results compared.

On the other hand, we have demonstrated that the sizes of inhibition zones produced by many β -lactam/IMBL combinations differ after incubation, depending on the way the combined disks were prepared, with the AD test usually producing larger inhibition zones. These results might reflect, in part, a reduced ability of PP to keep the IMBL volumes dispensed into the β -lactams during the process of disk preparation and -20°C storage. For this reason, a given isolate may not be equally classified under the same category (MBL producer or non-MBL producer) by AD and PP CD assay when phenotypic MBL detection is being performed. Since the imipenem/EDTA combinations had produced comparable results independent of the EDTA incorporation into the β -lactam disks, the volatility of thiol-based compounds could be argued to be one of the possible reasons for having discrepant results between the AD and PP tests. However, the incorporation of EDTA into ceftazidime disks also produced distinct results, implying that factors other than volatility are involved.

This study did not intend to select the best CD methodology for detecting MBL-producing isolates but calls attention to the

TABLE 2. Mean increase in inhibition zone produced by β -lactam/IMBL disks prepared by different methodologies (AD and PP^a)

β -Lactam/IMBL combination ^b	Mean increase in inhibition zone (mm) over MBL-positive controls	
	PP	AD
Ceftazidime/EDTA	9.2	12.6
Ceftazidime/MPA	14.3	17.6
Ceftazidime/MAC	8.2	15.9
Ceftazidime/MET	4.3	7.5
Ceftazidime/PHEN	3.3	5.2
Imipenem/EDTA	9.0	9.0
Imipenem/MPA	5.0	7.0
Imipenem/MAC	5.0	4.0
Imipenem/MET	0.0	5.0
Imipenem/PHEN	0.0	1.0

^a AD refers to IMBL solution added directly to the disk placed on the agar plate. PP refers to β -lactam/IMBL disks previously prepared, dried, and stored.

^b A volume of 10 μl of each MBL inhibitor was added to the β -lactam disks.

fact that discordant results might occur. We believe both AD and PP may be used for MBL detection, but interpretation of results may vary according to the β -lactam/IMBL selected. It should be noted that the selection of an appropriate breakpoint for screening MBL-producing isolates is directly influenced by the β -lactam/IMBL combination and by the way IMBLs are incorporated into the β -lactam disks. Thus, standardization of either AD or PP testing of additional IMBL concentrations may be necessary for intra- and interlaboratory comparison. In addition, the employed methodology for preparation of the β -lactam/IMBL combination should be discriminated in future studies to help interpretation and comparison of their results.

In comparison to the other MBL tests, the disk has been reported to be a simple, inexpensive phenotypic resource for detection of MBL that could be easily incorporated into the routine of clinical laboratories (15). Since commercial preparations of combined disks are not available, further studies standardizing the performance and interpretation of the CD assay are of crucial importance to guarantee its intra- and interlaboratory reproducibility.

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