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Controlling False-Positive Results Obtained with the Hodge and Masuda Assays for Detection of Class A Carbapenemase in Species of *Enterobacteriaceae* by Incorporating Boronic Acid[▽]

Fernando Pasteran, Tania Mendez, Melina Rapoport, Leonor Guerriero, and Alejandra Corso*

Servicio Antimicrobianos, Departamento Bacteriología, Instituto Nacional de Enfermedades Infecciosas (INEI)-ANLIS Dr. Carlos G. Malbrán, Ministerio de Salud y Ambiente, Ciudad Autónoma de Buenos Aires, Argentina

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The modified Hodge method (MHT) has been recommended by the CLSI for confirmation of suspected class A carbapenemase production in species of *Enterobacteriaceae*. This test and the Masuda method (MAS) have advantages over traditional phenotypic methods in that they directly analyze carbapenemase activity. In order to identify the potential interferences of these tests, we designed a panel composed of diverse bacterial genera with distinct carbapenem susceptibility patterns (42 carbapenemase producers and 48 nonproducers). About 25% of results among carbapenemase nonproducers, mainly strains harboring CTX-M and AmpC hyperproducers, were observed to be false positive. Subsequently, we developed an optimized approach for more-accurate detection of suspicious isolates of carbapenemase by addition of boronic acid (BA) derivatives (reversible inhibitor of class A carbapenemases and AmpC cephalosporinases) and oxacillin (inhibitor of AmpCs enzymes). The use of the modified BA- and oxacillin-based MHT and MAS resulted in high sensitivity (>90%) and specificity (100%) for class A carbapenemase detection. By use of these methodologies, isolates producing KPCs and GES, Sme, IMI, and NMC-A carbapenemases were successfully distinguished from those producing other classes of ß-lactamases (extended-spectrum β-lactamases [ESBLs], AmpC β-lactamases, metallo-β-lactamases [MBLs], etc.). These methods will provide the fast and useful information needed for targeting of antimicrobial therapy and appropriate infection control.

Class A carbapenemases (*Klebsiella pneumoniae* carbapenemases [KPCs], Sme, NMC-A, IMI, and some allelic variants of GES/IBC) have become more prevalent within the *Enterobacteriaceae* family (23, 25). Early recognition of carbapenemase producers has become mandatory, as clinical failure associated with these enzymes has been described (8). Therefore, different strategies are needed for their detection in any attempt aimed for their control and eradication. An accurate identification of class A carbapenemases will therefore rely on the availability of specific, sensitive, and simple assays able to differentiate carbapenemase producers from nonproducers. The modified Hodge test (MHT) (5) and similar tests, such as the Masuda assay (MAS) (15), directly analyze the carbapenemase activity in unbroken cells and enzyme crude extracts, respectively. These tests preformed better than routine phenotypic methods for detection of carbapenemase-producing bacteria, especially when combined mechanisms were present. Additionally, a practical and fast approach that efficiently disrupts bacterial cells has become available to the routine clinical microbiological laboratory, making the MAS even easier to perform (12). Thus, given these features, these methods started to gain more adherents among microbiologists. Moreover, the CLSI has issued recommendations for phenotypic screening of carbapenemase producers among species of *Enterobacteriaceae* and included the MHT as a confirmatory assay (5). Thus, MICs of ertapenem (ETP), meropenem (MEM), and imipenem (IPM) of 2, 2 to 4, and 2 to 4 μg/ml, respectively (or an ETP- or MEM-induced zone of inhibition of ≤21 mm in diameter in the disk diffusion assay), may indicate isolates with carbapenemase production, and this phenotype should be confirmed by the MHT (5). However, we have reported false-positive rates of up to 25% for MHT results (carbapenemase-like patterns) among carbapenemase nonproducers (as defined by spectrophotometric assays and molecular methods) (21). These unexpected outcomes were observed among CTX-M-producing bacteria, with uniformly decreased susceptibility to ETP or AmpC hyperproducers. Recent reports have also questioned the specificity (SP) of the MHT, suggesting that AmpC hyperproducers and several members of the CTX-M ESBL family (mostly CTX-M-2 but also CTX-M-15 and CTX-M-59) were the main cause of the unexpected performance (1, 4, 18).

Cloxacillin, oxacillin (OXA), and boronic acid (BA) have long been known as reversible inhibitors of class C enzymes (2, 3, 26). More recently, it has been shown that an additional property of the BA derivatives, but not of OXA, is the ability to inhibit KPC enzymes (6, 21, 24) and the remaining members of the class A carbapenemase family (GES, Sme, IMI, and NMC-A) (21). Using a panel composed of diverse bacterial genera with distinct carbapenem susceptibility patterns, we designed strategies to improve the efficiency of the MHT and MAS for phenotypic confirmation of carbapenemase producers among species of *Enterobacteriaceae* by addition of BA and

* Corresponding author. Mailing address: Servicio Antimicrobianos, INEI-ANLIS Dr. Carlos G. Malbrán, Velez Sarsfield 563 Ave. (C1282AFF), Buenos Aires, Argentina. Phone and fax: 54-11-4303-2812. E-mail: acorso@anlis.gov.ar.

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TABLE 1. Resistance mechanisms, MICs, and results for microbiological methods observed for the panel of strains used in this study as positive and negative controls

β-Lactamase group and strain[a]	Bacterial species	β-Lactamase(s)/decreased porin(s)[b]	MIC (μg/ml)[c]			Microbiological assay result (carbapenemase activity)[d]						Reference or source
			IPM	MEM	ETP	MHT	BA-MHT	OXA-MHT	MAS	BA-MAS	OXA-MAS	
KPC producers (n = 20)												
9169	*Citrobacter freundii*	KPC-2, TEM-1	1	1	2	+	−	+	+	−	+	21
9171	*K. pneumoniae*	KPC-2, PER, TEM-1, SHV-1	64	32	128	+	−	+	+	−	+	21
D05/07	*K. pneumoniae*	KPC-2	2	2	16	+	−	+	+	−	+	CAP QC Assurance
9885	*K. pneumoniae*	KPC-2	16	16	16	+	−	+	+	−	+	21
9190 (TC9190)	*Salmonella* sp.[e]	KPC-2	1	1	1	+	−	+	+	−	+	21
9204 (TC9171)	*Salmonella* sp.[e]	KPC-2	1	0.5	1	+	−	+	+	−	+	21
9847	*Enterobacter cloacae*	KPC-2, hyperproduced AmpC	8	16	32	+	−	+	+	−	+	21
9884	*E. coli*	KPC-2	2	2	2	+	−	+	+	−	+	21
9988	*C. freundii*	KPC-2, inducible AmpC	1	2	1	+	−	+	+	−	+	21
10007	*E. coli*	KPC-2, TEM-1	2	1	2	+	−	+	+	−	+	21
10154	*Proteus mirabilis*	KPC-2, TEM-1	4	2	4	+	−	+	+	−	+	21
10155	*P. mirabilis*	KPC-2, TEM-1	4	2	4	+	−	+	+	−	+	21
11180	*E. cloacae*	KPC-2, CTX-M	2	2	16	+	−	+	+	−	+	21
11181	*Serratia marcescens*	KPC-2	8	8	16	+	−	+	+	−	+	21
11213	*K. pneumoniae*	KPC-2, CTX-M	8	64	128	+	−	+	+	−	+	21
11245	*K. pneumoniae*	KPC-2, PER-2	1	1	4	+	−	+	+	−	+	21
11268	*K. pneumoniae*	KPC-3	8	32	128	+	−	+	+	−	+	R. Melano
11269	*K. pneumoniae*	KPC-2	4	4	16	+	−	+	+	−	+	R. Melano
11270	*K. pneumoniae*	KPC-3	4	8	16	+	−	+	+	−	+	R. Melano
11271	*K. pneumoniae*	KPC-2	4	8	16	+	−	+	+	−	+	R. Melano
Sme producers (n = 10)												
3981	*S. marcescens*	Sme-1b	128	0.5	4	ND[f]	ND	ND	+	−	+	21
3989	*S. marcescens*	Sme-1b, CTX-M-2, OXA-2	4	2	0.5	ND	ND	ND	+	−	+	21
3991	*S. marcescens*	Sme-1b, OXA-2	256	2	2	+	−	+	+	−	+	21
5050	*S. marcescens*	Sme-1b, CTX-M-2, OXA-2, SHV-1	16	0.15	4	+	−	+	+	−	+	21
5061	*S. marcescens*	Sme-1b	1	0.5	0.5	+	−	+	+	−	+	21
5634	*S. marcescens*	Sme-1b	16	4	4	+	−	+	+	−	+	21
5635	*S. marcescens*	Sme	16	8	4	+	−	+	+	−	+	21
5636	*S. marcescens*	Sme	16	8	4	+	−	+	+	−	+	21
7596	*S. marcescens*	Sme	16	8	4	+	−	+	+	−	+	21
9247	*S. marcescens*	Sme	64	2	4	+	−	+	+	−	+	21
IMI or NMC-A producers (n = 2)												
3202	*E. cloacae*	IMI-1	4	8	1	+	−	+	+	−	+	Medieros A.
9967	*E. cloacae*	NMC-A	4	4	0.25	+	−	+	+	−	+	21
GES producers (n = 4)												
5401	*Enterobacter* sp.	GES-5	32	8	8	+	+	+	+	−	+	21
5825	*K. pneumoniae*	GES-3, CTX-M-2	32	64	128	+	+	+	+	−	+	21
5476	*K. pneumoniae*	GES-5	16	16	4	+	+	+	+	−	+	21
5490 (TC 5825)	*Salmonella* sp.[e]	GES-3	32	32	8	+	−	+	+	−	+	21
G1 (TC 5401)	*Salmonella* sp.[e]	GES-5	8	8	8	+	−	+	+	−	+	This work
G2 (TC 5476)	*Salmonella* sp.[e]	GES-5	4	8	4	+	−	+	+	−	+	This work
MBL producers (n = 4)												
7647	*K. pneumoniae*	VIM-2, CTX-M-2, TEM-1, SHV-1	0.5	1	4	+	+	+	+	+	+	11
7527	*K. pneumoniae*	VIM, TEM-1, SHV-1	32	8	32	+	+	+	+	+	+	11
9921	*E. cloacae*	IMP-8, PER-2	4	1	2	+	+	+	+	+	+	7
9959	*Proteus penneri*	VIM-2, PER-2, hyperproduced AmpC	256	256	256	+	+	+	+	+	+	7

Producers of AmpC (derepressed or plasmid mediated) (n = 21)												
C2	*K. pneumoniae*	FOX-5	0.12	0.06	0.06	−	−	−	−	−	−	L. Martinez-Martinez
9234	*E. coli*	AAC-1	0.12	0.03	0.03	−	−	−	−	−	−	L. Martinez-Martinez
9235	*E. coli*	CMY-2	0.06	0.06	0.03	−	−	−	+ (I)	−	−	L. Martinez-Martinez
9236	*K. pneumoniae*	DHA	0.03	0.03	0.5	−	−	−	−	−	−	L. Martinez-Martinez
7896	*Shigella flexneri*	CMY-2	0.06	0.03	0.06	−	−	−	−	−	−	21
9324	*P. mirabilis*	CMY	0.12	0.03	0.06	−	−	−	−	−	−	21
9140	*K. pneumoniae*	CMY	0.12	0.03	1	−	−	−	−	−	−	21
POS 9	*Morganella morganii*	Hyperproduced AmpC	2	0.12	0.12	−	−	−	−	−	−	21
POS15	*C. freundii*	Hyperproduced AmpC	0.12	0.06	0.12	−	−	−	+	−	−	21
3986	*C. freundii*	Hyperproduced AmpC	0.25	0.03	0.03	−	−	−	−	−	−	21
9251	*E. cloacae*	Hyperproduced AmpC	2	2	4	+	−	−	+	−	−	21
5485	*E. cloacae*	Hyperproduced AmpC/decreased porins	4	16	64	+	−	−	+	−	−	21
P99	*E. cloacae*	Hyperproduced AmpC	0.25	0.12	2	+	−	−	+	−	−	20
1769	*E. coli*	MIR-1	0.5	0.25	2	+ (I)	−	−	+	−	−	20
9808	*E. cloacae*	Hyperproduced AmpC	0.5	0.25	4	+ (I, E)	−	−	+	−	−	This work
9954	*E. cloacae*	Hyperproduced AmpC	0.25	0.25	4	−	−	−	+ (I)	−	−	This work
11294	*E. cloacae*	Hyperproduced AmpC/decreased porins	8	16	64	−	−	−	+	−	−	This work
11397	*E. cloacae*	Hyperproduced AmpC/decreased porins	16	8	64	−	−	−	−	−	−	This work
11151	*E. coli*	CMY/decreased porins	32	16	128	−	−	−	−	−	−	This work
11146 (TC 1151)	*Salmonella* sp.	CMY	0.5	0.25	4	−	−	−	−	−	−	This work
11154	*E. cloacae*	CMY, inducible AmpC	0.25	0.12	0.5	−	−	−	−	−	−	This work
ESBL producers (n = 10)												
1803	*K. pneumoniae*	PER-2, CTX-M-2, SHV-1, TEM-1	0.5	0.5	2	−	−	−	−	−	−	21
9375	*K. pneumoniae*	CTX-M-2, SHV-1, TEM-1	0.06	0.06	0.12	−	+[g]	−	−	−	−	21
9163	*K. pneumoniae*	CTX-M-2, SHV-1, TEM-1/ decreased porins	1	2	16	+	+	+	+	+	+	21
9391	*K. pneumoniae*	CTXM-2, SHV-1, TEM-1, porins	0.25	8	16	+	+	+	+	+	+	21
9310	*K. pneumoniae*	CTX-M-2, SHV-1, TEM-1/ decreased porins	0.12	8	16	+	+	+	+	+	+	21
29/C8	*E. coli*	CTX-M-13	0.06	0.03	0.015	−	−	−	−	−	−	R. Melano
31/C10	*K. pneumoniae*	CTX-M-15	0.12	0.06	0.03	−	−	−	−	−	−	R. Melano
H3	*K. pneumoniae*	CTX-M-15	0.12	0.03	0.03	−	−	−	−	−	−	R. Melano
700603	*K. pneumoniae*	SHV-18/decreased porin	0.06	0.12	0.12	−	−	−	−	−	−	ATCC
Cos15	*E. coli*	PER, TEM	0.03	0.03	0.12	−	−	−	−	−	−	21
OSBL producer strain 35218	*E. coli*	TEM-1	0.12	0.015	0.015	−	−	−	−	−	−	ATCC
Producers of low levels of K1, penicillinase, or cefuroximase (n = 3)												
7522	*Citrobacter koseri*	Penicillinase	0.015	0.015	0.008	−	−	−	−	−	−	21
HMC22	*P. penneri*	Cefuroximase	0.06	0.06	0.015	−	−	−	−	−	−	21
FLE17	*Klebsiella oxytoca*	Low-level K1	0.12	0.03	0.008	−	−	−	−	−	−	21
Producers of low basal levels of AmpC (inducible) (n = 5)												
GUT 8	*C. freundii*	Inducible AmpC	0.25	0.03	0.015	−	−	−	−	−	−	21
CEN31	*S. marcescens*	Inducible AmpC	0.12	0.03	0.06	−	−	−	−	−	−	21
HMI23	*E. cloacae*	Inducible AmpC	0.5	0.25	0.5	−	−	−	−	−	−	21

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TABLE 1—*Continued*

β-Lactamase group and strain[a]	Bacterial species	β-Lactamase(s)/decreased porin(s)[b]	MIC (μg/ml)[c]			Microbiological assay result (carbapenemase activity)[d]						Reference or source
			IPM	MEM	ETP	MHT	BA-MHT	OXA-MHT	MAS	BA-MAS	OXA-MAS	
POS B	*M. morganii*	Inducible AmpC	1	0.12	0.015	−	−	−	−	−		21
9113	*Providencia stuartii*	Inducible AmpC	0.06	0.06	0.03	−	−	−	−	−	−	21
Producers of ESBL(s) plus inducible AmpC (n = 4)												
9291	*S. marcescens*	CTX-M-2, TEM-1, inducible AmpC/decreased porins	2	4	64	+	+	+	+	+	+	21
9383	*S. marcescens*	CTX-M-2, inducible AmpC/decreased porins	2	1	32	+	+	+	+	+	+	21
109339	*E. cloacae*	PER-2, inducible AmpC	0.12	0.25	0.5	−	−	−	−	−	−	21
3972	*P. stuartii*	CTX-M-2, inducible AmpC	0.03	0.06	0.015	−	−	−	−	−	−	21
Strains with wild-type and/or marginal or low-level ß-lactamase activities (n = 4)												
1744	*Salmonella* sp.[h]	None	0.12	0.015	0.008	−	−	−	−	−	−	21
27508	*E. cloacae*	None (AmpC$^-$ mutant)	0.06	0.03	0.015	−	−	−	−	−	−	ATCC
HMI13	*P. mirabilis*	None	0.25	0.06	0.008	−	−	−	−	−	−	21
25922	*E. coli*	Basal AmpC	0.06	0.015	0.015	−	−	−	−	−	−	ATCC

[a] ESBL, extended-spectrum ß-lactamase; OSBL, "older-spectrum" ß-lactamase.

[b] Indicates a decreased amount of the evaluated OMP as depicted in the references.

[c] MICs determined by agar dilution in accordance with CLSI recommendations. IPM, imipenem; MEM, meropenem; ETP, ertapenem.

[d] For the modified Hodge test (MHT), "+" indicates that the tested strains produced carbapenemase activity, and "−" indicates that the tested strains did not produce carbapenemase activity. For the boronic acid-based MHT (BA-MHT) or the OXA-based MHT (OXA-MHT), "+" indicates that, compared with the level for the corresponding control, APB or OXA did not produce inhibition of the carbapenemase activity (i.e., carbapenemase activity persisted under the presence of the inhibitor), and "−" indicates that, compared with the level for the corresponding control, APB or OXA produced inhibition of the carbapenemase activity (i.e., carbapenemase activity disappeared under the presence of the inhibitor). For the Masuda test (MAS), "+" indicates that one or both of the volumes of crude extract tested produced carbapenemase activity, and "−" indicates that both volumes of crude extract tested did not produce carbapenemase activity. For the boronic acid-based MAS (BA-MAS) or the OXA-based MAS (OXA-MAS), "+" indicates that the strain displayed carbapenemase activity only with 15-μl disks and that this carbapenemase activity was not reverted with APB/OXA or that both volumes of extract tested (5 μl and 15 μl) displayed carbapenemase activity and that both carbapenemase activities were not inhibited by APB or OXA, and "−" indicates that, with one or both of the volumes of crude extract tested, carbapenemase activity was inhibited by APB/OXA, compared with the level for the corresponding blank disk.

[e] Transconjugant strain (brackets refer to the parenteral strain).

[f] ND, not determined, due to an indeterminate Hodge test result due to inhibition of growth of the indicator strain.

[g] The hemisulfate salt but not the monohydrate APB produced a partial hydrolysis of the imipenem load of the disk, reducing the expected inhibition zone by about 5 mm (not shown). The reduction of the IPM load potentiated a false-positive pattern for this carbapenemase nonproducer strain without interfering with the overall performance of the assay.

[h] Recipient strain for the conjugation assays.

OXA as inhibitors. The proposed procedures, designated the boronic acid-based modified Hodge test (BA-MHT) plus the oxacillin-based modified Hodge test (OXA-MHT) and the boronic acid-based Masuda assay (BA-MAS) plus the oxacillin-based Masuda assay (OXA-MAS) may be suitable as confirmatory tests, as they displayed high sensitivity (SN) and specificity (SP) for detection of class A carbapenemase producers, in addition to being relatively simple and inexpensive.

MATERIALS AND METHODS

Bacterial strains. Tests were performed with a panel consisting of diverse bacterial genera with distinct carbapenem susceptibility patterns. A total of 42 carbapenemase-producing isolates (including 38 producing class A carbapenemases) and 48 non-carbapenemase-producing isolates of *Enterobacteriaceae* were included in the panel (Table 1). The resistance mechanisms of the strains employed in this work, the carbapenem MICs for the strains, and the sources of the strains are given in detail in Table 1. All isolates were nonduplicates (molecular typing was performed to ensure that they were genetically unrelated), and except for the ATCC isolates and *Salmonella* species transconjugants, all isolates were isolated from clinical specimens. All isolates were identified by conventional techniques (19) and by use of an API 20E system (bioMérieux, Marcy l'Etoile, France). The genotypes of the isolates were characterized by molecular techniques (21). In addition, to exclude the possible presence of other, not-yet-described carbapenemases in the negative-control panel, the imipenemase activities of cell extracts from overnight broth cultures were determined by spectrophotometric assays (21).

Microbiological assays. (i) MHT, BA-MHT, and OXA-MHT. The MHT was performed as described previously (5). Briefly, a 1/10 dilution of an inoculum of the indicator organism *Escherichia coli* ATCC 25922, adjusted to a 0.5 McFarland turbidity standard, was used to inoculate the surfaces of plates (diameter, 145 mm; Greiner Laboratory, Germany) containing Mueller-Hinton agar (Difco, BBL) by swabbing. After the plates were allowed to stand for 10 min at room temperature, disks (Difco, BBL) containing IPM (10 μg), MEM (10 μg), or ETP (10 μg) (for the MHT) or one of those carbapenems supplemented with either 10 μl of a 3-aminophenyl-boronic acid (APB) solution (for the BA-MHT) or 10 μl of OXA solution (for the OXA-MHT) were placed onto the agar plates. We evaluated two APB formulations: a monohydrate, which was soluble in dimethyl sulfoxide (DMSO; Sigma Chemicals, St. Louis, MO), and hemisulfate salt, which was water soluble (Boron Molecular, Inc., Research Triangle Park, NC). Several APB concentrations of both formulations were tested: 0.3, 3, 30, 100, 200, and 300 mg/ml, rendering 3, 30, 300, 1,000, 2,000, and 3,000 μg/disk of APB, respectively. Also, several OXA concentrations (oxacillin sodium salt; Sigma Chemicals, St. Louis, MO) were tested: 10, 30, 50, and 100 mg/ml, rendering 100, 300, 500, and 1,000 μg/disk of OXA, respectively. Subsequently, by use of a 10-μl loop, three to five colonies of the test organisms, grown overnight on an agar plate, were inoculated onto the plate in a straight line from the edge of one disk to that of another containing the same carbapenem (Fig. 1). The presence of growth of the indicator strain toward the carbapenem disks was interpreted as a positive result for carbapenem hydrolysis screening (a carbapenemase-like pattern). A BA-inhibited or an OXA-inhibited carbapenemase pattern was distinguished due to the absence of growth of the indicator strain toward the carbapenem-plus-APB or -OXA disks, respectively, compared to the level for the corresponding disk without the inhibitor. More information for the interpretation of a BA- and/or OXA-inhibited carbapenemase pattern is described in Results.

Blank disks containing less than 1,000 μg of APB and 500 μg of OXA per disk did not inhibit or distort *E. coli* ATCC 25922 growth in preliminary assays. Higher concentrations of APB and OXA produced an inhibition of the growth of the indicator (with an inhibition zone of less than 10 mm). This effect did not interfere with the MHT, because the inhibition zones produced by the inhibitors were by far within the zone of inhibition expected for the carbapenems. As APB monohydrate was dissolved in DMSO (Sigma Chemicals, St. Louis, MO), we also tested the effect of DMSO by itself and found no effect on the growth of the indicator.

(ii) MAS, BA-MAS, and OXA-MAS. The MAS procedure described here was based on an adaptation of the assay originally developed by Masuda et al., with ulterior modifications (12, 15). Crude extracts were prepared by subjecting the cell suspension to mechanic rupture as described previously (12). Briefly, bacteria were grown overnight at 35°C in a tryptic soy agar plate. Two complete 10-μl loops of the test strain were harvested and then suspended in 300 μl of 50 mM Tris-HCl (pH 8) supplemented with 0.01 mM $ZnSO_4$ (ICN Biochemicals, Ltd., Thame, Berks, United Kingdom). Next, 300 mg of 0.1 mm zirconia-silica beads (Biospect Products, Bartlesville, OK) was added. Crude extracts were prepared by subjecting the cell suspension to mechanic rupture by a 3-min vortex at maximal speed in a Maxi-Mix II mixer-processor (Barnstead/Thermolyne, Inc., Waltham, MA). The cell extracts were clarified by centrifugation at 15,000 rpm for 15 min at 4°C and the supernatants stored at −20°C until processing. Before being used, crude extracts were thawed at room temperature. A set of two filter disks were loaded with 5 μl and l5 μl of crude extract of a test strain. The same volumes of extract were loaded on each of two blank disks previously supplemented with 10 μl of an APB or OXA solution. Several APB concentrations of both monohydrate and hemisulfate salt were tested: 3, 30, 100, and 300 mg/ml, rendering 30, 300, 1,000, and 3,000 μg/disk of APB, respectively. Also, several OXA concentrations were tested: 10, 30, and 50 mg/ml, rendering 100, 300, and 500 μg/disk of OXA, respectively. APB and OXA disks were prepared and stored at −20°C. Disks were allowed to dry at room temperature for 15 min. Then, an inoculum of the indicator organism *E. coli* ATCC 25922, adjusted to a 0.5 McFarland turbidity standard, was used to inoculate the surface of a plate containing Mueller-Hinton agar by swabbing. After the plate was allowed to stand for 10 min at room temperature, disks (Difco, BBL) with IPM (10 μg), MEM (10 μg), and ETP (10 μg) were placed onto the agar plate. Subsequently, each carbapenem disk was surrounded by extract-loaded disks that were applied within the expected susceptibility zone of the carbapenem tested (Fig. 2). The plates were incubated overnight at 35°C, and the growth patterns of the indicator *E. coli* ATCC 25922 strain were evaluated. The presence in the extract of any ß-lactamase that could hydrolyze carbapenems (a carbapenemase-like pattern) was revealed by the growth of the indicator *E. coli* cells around the blank disks loaded with the crude extract (with one or both of the volumes used). The interpretation of a BA-inhibited and/or OXA-inhibited carbapenemase-like pattern is described in detail in Results.

Characterization of the mechanisms of resistance. (i) PCR amplification and DNA sequencing. PCR analyses of all isolates were performed to confirm the presence of known carbapenemase genes. The isolation of total DNA was performed as described previously (21). Strains were analyzed for bla_{VIM}, bla_{IMP}, bla_{SPM}, bla_{KPC}, bla_{Sme}, bla_{IMI}, bla_{NMC-A}, bla_{CTX-M}, bla_{TEM}, bla_{PER}, bla_{SHV}, bla_{AmpC}, and bla_{OXA} genes (subgroups I, II, and III) as described previously (21).

(ii) OMP. Outer membrane protein (OMP) analysis was performed by SDS-PAGE as previously reported (14, 17).

(iii) AmpC production. First, suspected isolates were screened by isoelectric focusing (IEF) using a substrate-based development method as published previously (17). Those crude extracts with IEF bands that displayed *in situ* inhibition with OXA (1 mM) were subjected to spectrophotometric analysis. The ß-lactamase activities (in nanomoles of cephalotin [Laboratorios Richet, Argentina] hydrolyzed per minute per milligram of protein) were measured spectrophotometrically (SmartSpec 3000; Bio-Rad) on selected crude extracts previously incubated for 15 min in 5 μg/ml of clavulanate (Laboratorios Roemmers, Argentina) as described previously (10). A >95% reduction of cephalotin hydrolysis after OXA treatment (by incubation of crude extracts for 15 min in 50 μM OXA) was considered a positive result for class C ß-lactamases (10). *Acinetobacter baumannii* 5277 (bla_{OXA-58}) and *A. baumannii* FAV-1 (bla_{OXA-58} plus bla_{PER-2}) were used as control strains (22). Increments of AmpC ß-lactamase activity of >10-fold compared with the baseline activity derived from the ceftazidime susceptibility of the panel were considered indicative of AmpC hyperproduction.

SN and SP. Sensitivity (SN) and specificity (SP) calculations were done essentially as described in reference 9. The SN value is based on the ratio $a/(a + c)$, where a represents the number of strains that were correctly identified as producers of group class A carbapenemases by the tested assay and c represents the number of true class A carbapenemase producers incorrectly identified as nonproducing strains. The SP value is based on the ratio $d/(b + d)$, where d is the number of isolates correctly identified by the tested assay as nonproducers of group class A carbapenemases and b is the number of strains that were incorrectly identified as class A carbapenemase producers. The positive predictive value (PPV) and negative predictive value (NPV) are represented by $a/(a + b)$ and $d/(c + d)$, respectively.

RESULTS AND DISCUSSION

MHT, BA-MHT, and OXA-MHT. The MHT detects the activity of carbapenem-hydrolyzing enzyme in the unbroken cells of the analyzed bacteria. Besides the substrates recom-

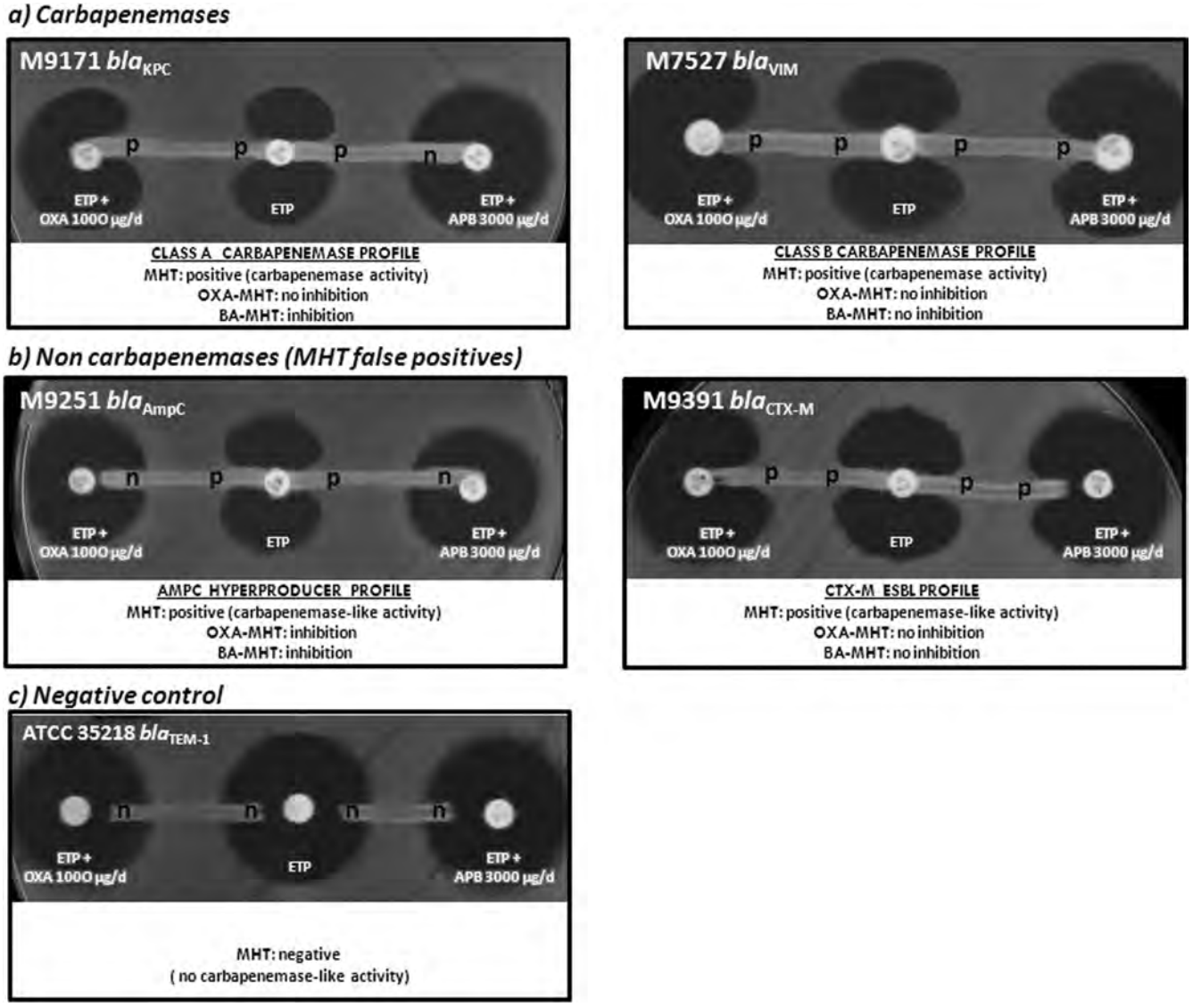

FIG. 1. Results obtained with the modified Hodge test (MHT), the boronic acid-based MHT (BA-MHT), and the OXA-based MHT (OXA-MHT) with ETP plus OXA (1,000 μg per disk; left disks), ETP (central disks) and ETP plus APB (3,000 μg per disk; right disks) for representative isolates (*K. pneumoniae* M9171 [producing KPC-2], *K. pneumoniae* M7527 [producing VIM], *Enterobacter cloacae* M9251 [producing high levels of AmpC]), *K. pneumoniae* M9391 [producing CTX-M-2], and *E. coli* ATCC 35218 [producing TEM-1]). The letter "p" indicates that ETP was hydrolyzed by the streaked cells; the letter "n" indicates that ETP was not hydrolyzed by the streaked cells. Final interpretations for the MHT, the BA-MHT, and the OXA-MHT are shown below each strain.

mended by the CLSI (ETP and MEM), we also tested IPM on the basis of the promising performance obtained previously (21). For standardization purposes, all strains (except two with indeterminate MHT results due to inhibition of the growth of the indicator strain) were subjected to APB and OXA inhibition regardless of whether they exhibited positive or a negative MHT results (Table 1). For clinical detection, it was adequate to test these inhibitors only for strains with positive MHT results. The optimal inhibitor concentrations for this assay (with the highest SN and SP) were 300 mg/ml of APB (or 3,000 μg APB/disk) and 100 mg/ml of OXA (or 1,000 μg OXA/disk). As mentioned previously, these APB and OXA concentrations affected the growth of the indicator strain only slightly, with only a few millimeters being overtaken by the inhibition zone of the carbapenems, thus not affecting the performance of the method. Both APB formulations performed equivalently.

The MHT displayed high efficiency for detection of class A carbapenemase-producing strains (Table 1), with an SN of 0.95, regardless of the substrate used (Table 2), corroborating our previous results (21). However, the major limitation of the MHT was the presence of discernible carbapenemase-like patterns among carbapenemase nonproducers, as was previously noticed (1, 4, 18, 21) (Table 1 and Fig. 1). These false-positive results corresponded mainly to strains producing CTX-M-2 ($n = 5$) and those with hyperproduction of AmpC (3, 4, and 5 strains with MEM, ETP, and IPM, respectively) (Table 1). Thus, SP showed slight differences according the substrate

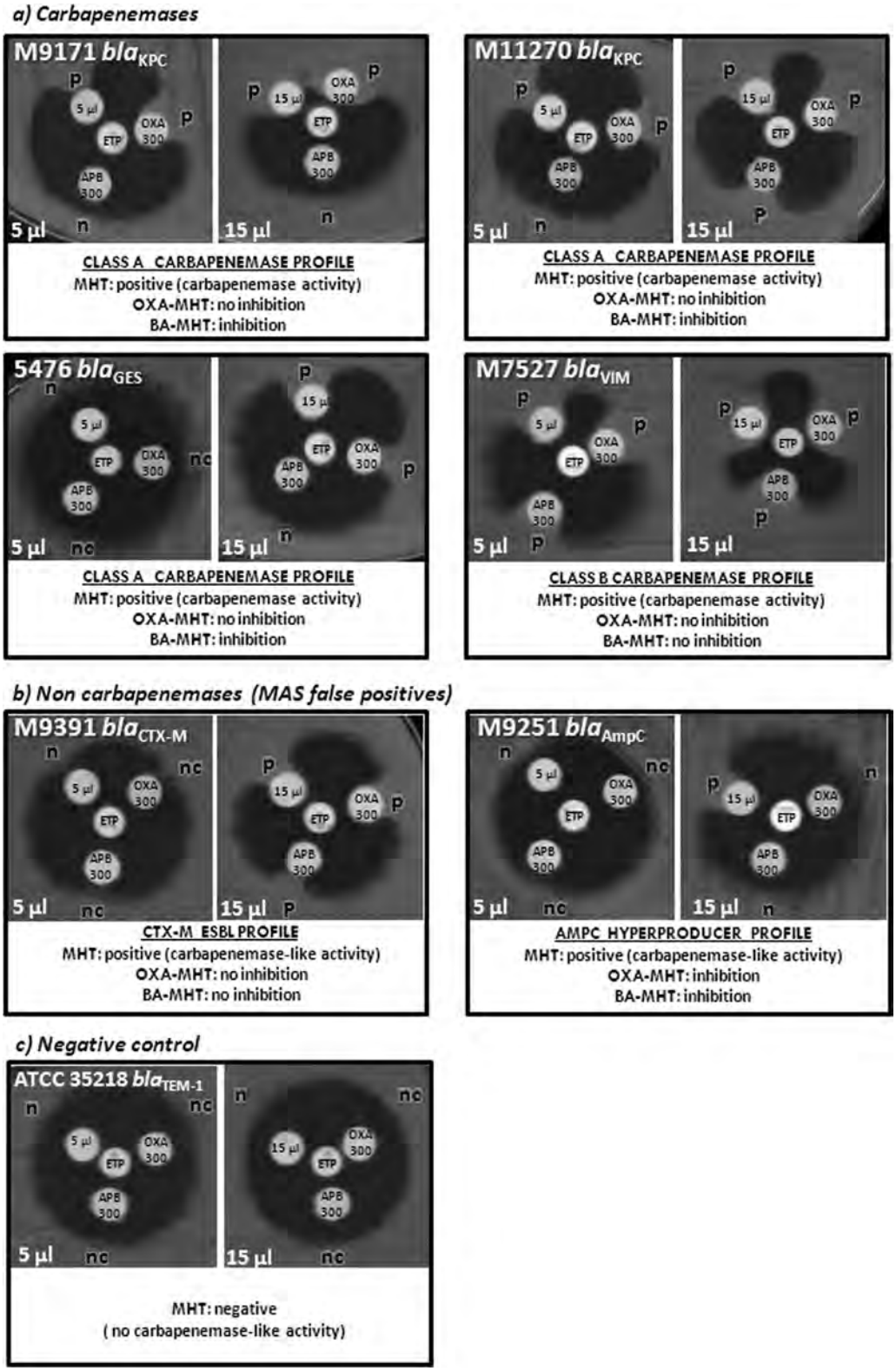

FIG. 2. Results obtained with the Masuda assay (MAS), the boronic acid-based MAS (BA-MAS), and the OXA-based MAS (OXA-MAS) with ETP (central disks). The left part of each photograph corresponds to the following disks, loaded with 5 μl of the crude extract: a blank disk (5 μl; left disks), an OXA disk (300 μg per disk; right disk), and an APB disk (300 μg per disk; lower disk). The right part of each photograph corresponds to the following disks, loaded with 15 μl of the crude extract: a blank disk (15 μl; left disks), an OXA disk (300 μg per disk; right disk), and an APB disk (300 μg per disk; lower disk). Representative isolates (*K. pneumoniae* M9171 [producing KPC-2], *K. pneumoniae* M11270 [producing KPC-2], *Salmonella* strain M5476 [producing GES-5], *K. pneumoniae* M7527 [producing VIM], *K. pneumoniae* M9391 [producing CTX-M-2], *Enterobacter cloacae* M9251 [producing high levels of AmpC], and *E. coli* ATCC 35218 [producing TEM-1]) are shown. The letter "p" indicates that ETP was hydrolyzed by the β-lactam content of the crude extract; the letter "n" indicates that ETP was not hydrolyzed by the β-lactam content of the crude extract. NC, no correspondence (the matching blank disk did not show carbapenemase activity). Final interpretations for the MAS, the BA-MAS, and the OXA-MAS are shown below each strain.

TABLE 2. Performance analysis of the confirmatory tests[a]

Assay (*n*)	SN	SP	NPV	PPV
Modified Hodge test (MHT) (90)	0.95	0.73 (IPM) 0.77 (MEM) 0.75 (ETP)	0.95	0.72 (IPM) 0.75 (MEM) 0.73 (ETP)
BA-MHT (88)[b]	0.92	0.90 (IPM) 0.94 (MEM) 0.92 (ETP)	0.94	0.88 (IPM) 0.92 (MEM) 0.90 (ETP)
BA-MHT plus OXA-MHT (88)[b]	0.92	1.0	0.94	1.0
Masuda test (MAS) (90)	1.0	0.67 (IPM) 0.69 (MEM, ETP)	1.0	0.69 (IPM) 0.72 (MEM, ETP)
BA-MAS (90)	1.0	0.85 (IPM) 0.88 (MEM, ETP)	1.0	0.83 (IPM) 0.86 (MEM, ETP)
BA-MAS[b] plus OXA-MAS (90)	1.0	1.0	1.0	1.0

[a] The performance levels and the most-favorable conditions for the phenotypic confirmatory tests described in this study for detection of class A carbapenemases among species of *Enterobacteriaceae* are summarized. When the substrate is not expressly stated (in parentheses), the 3 carbapenems (IPM, MEM, and ETP) showed the same performance.

[b] A total of 36 of 38 carbapenemase producer strains were analyzed (2 strains with indeterminate Hodge test results due to inhibition of growth of the indicator strain were excluded).

used, being as low as 0.73, 0.77, and 0.75 for IPM, ETP, and MEM, respectively (Table 2).

Thus, we designed the BA-MHT and the OXA-MHT to improve the performance of the MHT. Typical results observed for the proposed inhibitor-based MHT are shown in Fig. 1; a growth of the indicator *E. coli* strain toward the carbapenem disk, indicative of carbapenem hydrolysis (a carbapenemase-like pattern), was observed in four representative strains. Class A carbapenemases were identified by comparing the growth of the indicator *E. coli* cells toward the disk containing the carbapenem alone with that of the cells toward the disks supplemented with the inhibitors. The figure shows the results obtained with the ETP disk, as representative of the carbapenems, since the results obtained with the BA-MHT and the OXA-MHT were identical regardless of the carbapenem tested.

The typical situation found for a class A carbapenemase is shown for a representative KPC-producing strain (M9171), which was revealed by APB inhibition, as inferred from the absence of growth of the indicator *E. coli* strain toward the ETP-plus-APB disk only (Fig. 1). OXA failed to inhibit the carbapenemase pattern, as inferred from the growth of the indicator *E. coli* strain toward the ETP-plus-OXA disk. This pattern (inhibition by APB and refractory action with OXA) corresponded unequivocally to class A carbapenemases.

The typical pattern for class A carbapenemase nonproducers is shown for VIM (M7527) and CTX-M (M9391) representative strains where the indicator strain grew toward the ETP, ETP-plus-APB, and ETP-plus-OXA disks as well (Fig. 1). This revealed the presence of a non-class A carbapenemase enzyme due to the lack of APB inhibition. As no APB effect was observed, OXA results became irrelevant. Strains showing these carbapenemase patterns (no inhibition by OXA and APB) should be screened for class B carbapenemases by using appropriate methodologies, such as those previously published (13, 16).

Finally, several AmpC hyperproducer strains included in the panel displayed carbapenemase-like patterns (Table 1), as shown for M9251, a representative strain, where the growth of the indicator strain toward the ETP but not the ETP-plus-APB disk may indicate the presence of class A carbapenemase. However, the strain also exhibited OXA inhibition, as revealed by the lack of growth of the indicator strain toward the ETP-plus-OXA disk (Fig. 1). This unique pattern (inhibition by both APB and OXA) corresponded unequivocally to AmpC strains.

When the whole panel was analyzed, all of the KPC-, Sme-, IMI-, and NMC-A-producing strains, but only half of the GES-producing strains, were found by the MHT to be inhibited only by APB (Table 1). Thus, the SN of the BA-MHT was 0.92 (Table 2). The low performance level for GES was observed only among strains of clinical origin, not among the respective transconjugant strains, indicating that these enzymes were not refractory to APB inhibition. The mucoid phenotype observed for the parental strains (no other carbapenemase-producing strain of the panel had this phenotype) that might interfere with the enzyme and/or APB diffusion from the unbroken cells is probably responsible for these results for GES-producing strains. Among the 14 strains in the control group with positive MHT results, 9 of them (CTX-M and metallo-β-lactamase [MBL] producers) remained with positive carbapenemase patterns after the addition of APB, being properly classified as class A carbapenemase nonproducers (Table 1). The remaining 5 strains (AmpC hyperproducers) were finally resolved as class A carbapenemase nonproducers with the incorporation of OXA as an inhibitor (Table 1). Thus, with the simultaneous use of both inhibitors, a major increase in the SP of the MHT was observed (from 0.70 to 1.0) (Table 2).

MAS, BA-MAS, and OXA-MAS. The MAS procedure tested the presence of carbapenem-hydrolyzing activities using cell-free crude extracts of the analyzed bacteria. We evaluated the behavior of the identified enzymes toward the inhibitors by addition of the crude extract onto APB or OXA disks. We

found some limitations for the development of this technique. (i) It was not possible to use disks with ≥1,000 μg or 500 μg of APB or OXA, respectively, because the zone of inhibition produced around these disks became relevant for interpretation of MAS results. Therefore, we had to use disks with 300 μg of either APB or OXA for the standardization of the BA-MAS or the OXA-MAS, respectively, a significantly lower concentration than that required by the MHT to achieve its best performance. (ii) A high level of variability in crude-extract protein content was observed (data not shown). Thus, due to the competitive, mass-dependent nature of APB/OXA inhibition and the restriction of increase in disk load, we were forced to analyze 2 volumes of the crude extract (5 and 15 μl) simultaneously to try to achieve adequate inhibition. The different APB formulations performed identically.

Characteristic results observed for the proposed assay are shown in Fig. 2; the observation of the typical carbapenemase pattern is indicated by the growth of the indicator *E. coli* strain around a blank disk charged with either 5 or 15 μl of extract, as shown for representative isolates (highly loaded crude-extract disks tend to produce carbapenemase-like patterns more frequently than disks with 5 μl). Class A carbapenemases were identified by comparing the growths of the indicator *E. coli* cells around blank and OXA disks but not around disks with APB (comparison should be done between disks loaded with the same volume of crude extract, and when inhibition of the carbapenemase-like pattern was observed with at least one of the volumes of crude extract used, the test result was considered positive for that inhibitor). Figure 2 shows the results obtained with the ETP disk as representative of the carbapenem, since the results obtained with the BA-MAS and the OXA-MAS were identical regardless of the carbapenem tested.

The most typical situation found for a class A carbapenemase is shown for M9171, a representative KPC-producing strain: hydrolysis of ETP was revealed by growth of the indicator *E. coli* strain around disks loaded with either 5 or 15 μl of extract. The presence of a class A carbapenemase was exposed by the presence of the characteristic profile that included both the presence of APB inhibition, as inferred from the absence of growth of the indicator *E. coli* strain around APB disks loaded with either 5 or 15 μl of extract, and the absence of OXA inhibition, as indicated by the growth of the indicator *E. coli* strain around the OXA disks. This major pattern was observed for all the Sme strains, most of the KPC strains (13/20 strains) and GES strains (5/6 strains), and one IMI-1 strain.

Another situation for class A carbapenemase was observed among 7 of the 20 KPC strains, as shown for M11270, another representative KPC strain: hydrolysis of ETP was revealed again around blank disks with either 5 or 15 μl of extract, but in this case, the presence of the class A carbapenemase characteristic pattern was deduced to occur only around disks loaded with 5 μl of extract, as indicated by the absence of growth of the indicator strain around the corresponding APB disk but not around that with OXA. Appreciable growth around the 15-μl-supplemented APB disk, similar to the level for the corresponding control (15 μl of bacterial extract alone), was observed, probably indicating an excess of ß-lactamase over the APB load. Finally, another situation was found for one GES strain and one NMC-A strain, as shown for the M5476 representative strain (Fig. 1b); null hydrolytic activity toward ETP was obtained using 5 μl of extract, as an absence of growth of the indicator *E. coli* strain was observed around the disk. But ETP hydrolysis was visible when disks loaded with 15 μl of extract were used, suggesting a weak or poorly extracted ß-lactamase. Class A carbapenemase was therefore revealed only among disks loaded with 15 μl of crude extract, as indicated by the typical profile showing the absence of a zone of *E. coli* around the APB disk (APB inhibition), together with the growth of the indicator strains toward the OXA disk. Because of these two situations, a potential excess of β-lactamase over the APB load or the presence of a weak or poorly extracted ß-lactamase, we strongly recommended the simultaneous use of 2 volumes (5 and 15 μl) of extract in the tests.

Among the control group, the typical pattern for MBL producers is shown for M7527, a representative strain: growth of the indicator *E. coli* strain was observed around both blank disks (5 and 15 μl), and similar growth of the reference strain was maintained around both APB and OXA disks. These results reveal the lack of APB and OXA inhibition due to the presence of a non-class A carbapenemase enzyme. Another representative pattern for class A carbapenemase nonproducers was observed for several CTX-M producers (Table 1), as shown in Fig. 2 for M9391, a representative strain; this pattern showed null hydrolytic activity toward imipenem with the use of 5 μl of extract, but significant imipenem hydrolysis was observed with 15 μl. However, growth of the indicator strain was also observed around the corresponding APB and OXA disks (15 μl), revealing the lack of APB and OXA inhibition due to the presence of a non-class A carbapenemase enzyme. As this lack of inhibition obtained with APB and OXA of CTX-M producers is shared with MBLs, strains with this typical profile should be screened for class B carbapenemases (13, 16). Several AmpC hyperproducer strains (Table 1) showed a carbapenemase-like pattern, but only with 15-μl extract disks, as shown for the M9251 representative strain (Fig. 2). In parallel, significantly reduced growth of the indicator strain occurred around the corresponding 15-μl APB disk, initially suggesting the presence of a class A carbapenemase. However, the lack of growth of the indicator strain toward the OXA disk revealed the presence of a dually inhibited (OXA and APB) enzyme, a feature that rules out class A carbapenemases.

After assessing the panel, we obtained an SN of 1.0 for the MAS, together with a noticeable improvement of the SP for the classic assay, from 0.67 and 0.69 for IPM and MEM/ETP, respectively, to 1.0 for the three substrates, when the combined BA/OXA-MAS was used (Table 2).

Although in the present study, the MAS combination performed with slightly greater SN values than the MHT one, for a voluminous work burden, we find the MHT more practical because fewer disks are required and there is no need to prepare crude extracts. However, MAS becomes mandatory for strains with indeterminate MHT results and for strains with a marked mucoid phenotype that might have difficulty achieving APB inhibition with the MHT.

Finally, it should be noted that the MHT and the MAS did not include strict standardization for the inoculum size and the enzyme concentration used, respectively. Although this appears to be a major design flaw in these tests, as it may be one

of the factors causing false-positive results with the original techniques, the result was not critical when the BA/OXA-MHT/MAS was used under the methodological standardization described in this work.

Concluding remarks. Given the clinical and public health impact involved in the discovery of a strain carrying carbapenemase, screening and confirmation in clinical microbiology laboratories of carbapenemase producers should include efficient and inexpensive methods that cover the different needs and complexities of the local and regional epidemiology. The MHT and the MAS, as they are able to expose the carbapenemase activity of suspected isolates easily, have gained adherents throughout the world. Moreover, the MHT has been included in the last CLSI edition for phenotypic confirmation of suspected carbapenemase-producing strains (5). However, these methods, as known to date, should be used with caution because of the presence of false-positive results, especially in those scenarios with high proportions of strains expressing non-carbapenemase-mediated carbapenem nonsusceptibility (such as that generated by endemic CTX-M or AmpC hyperproduction) (1, 4, 18, 20). Thus, for improvement of their performances for carbapenemase confirmation, we standardized several strategies, with the addition of BA and OXA as inhibitors, to circumvent these main interferences. On the basis of the results obtained in this work, the use of the revised inhibitor-based methods (BA/OXA-MHT and BA/OXA-MAS) resulted in significant reductions in false-positive results, in addition to distinguishing strains producing class A carbapenemases from strains producing other classes of carbapenemases. Thus, with these modifications, these methods can be used by routine clinical laboratories as confirmatory tests with performance levels comparable to those obtained with the reference (molecular) methods. The proposed methodology will provide fast and useful information needed for targeting antimicrobial therapy and appropriate infection control, especially for regions where carbapenem resistance mediated by carbapenemase nonproducers is high or is increasing.

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