

Sensitive Screening Tests for Suspected Class A Carbapenemase Production in Species of *Enterobacteriaceae*[∇]

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The detection of class A serine-carbapenemases among species of *Enterobacteriaceae* remains a challenging issue. Methods of identification for routine use in clinical microbiology laboratories have not been standardized to date. We developed a novel screening methodology suitable for countries with high basal levels of carbapenem resistance due to non-carbapenemase-mediated mechanisms and standardized several simple confirmatory methods that allow the recognition of bacteria producing class A carbapenemases, including KPC, Sme, IMI, NMC-A, and GES, by using boronic acid (BA) derivatives. A total of 28 genetically unrelated *Enterobacteriaceae* strains producing several class A carbapenemases were tested. Thirty-eight genetically unrelated negative controls were included. The isolates were tested against imipenem (IPM), meropenem (MEM), and ertapenem (ETP) by MIC and disk diffusion assays in order to select appropriate tools to screen for suspected carbapenemase production. It was possible to differentiate class A carbapenemase-producing bacteria from non-carbapenemase-producing bacteria by using solely the routine IPM susceptibility tests. The modified Hodge test was evaluated and found to be highly sensitive, although false-positive results were documented. Novel BA-based methods (a double-disk synergy test and combined-disk and MIC tests) using IPM, MEM, and ETP, in combination with 3-aminophenylboronic acid as an inhibitor, were designed as confirmatory tools. On the basis of the performance of these methods, a sensitive flow chart for suspicion and confirmation of class A carbapenemase production in species of *Enterobacteriaceae* was designed. By using this methodology, isolates producing KPC, GES, Sme, IMI, and NMC-A carbapenemases were successfully distinguished from those producing other classes of β -lactamases (extended-spectrum β -lactamases, AmpCs, and metallo- β -lactamases, etc). These methods will rapidly provide useful information needed for targeting antimicrobial therapy and appropriate infection control.

Class A carbapenemases (KPCs, Sme, NMC-A, IMI, and some allelic variants of GES/IBC) have become more prevalent within the *Enterobacteriaceae* family (30, 33). Early recognition of producers of carbapenemases has become mandatory, as clinical failure associated with these enzymes has been described previously (12) and as recognition is crucial for controlling the spread of carbapenemase-producing bacteria. A uniform and standardized phenotypic tool for the detection of class A carbapenemases is still lacking. Recently, the CLSI issued recommendations for the phenotypic screening of carbapenemase producers among species of *Enterobacteriaceae*: MICs of ertapenem (ETP), meropenem (MEM), and imipenem (IPM) of 2, 2 to 4, and 2 to 4 μ g/ml, respectively (or a zone of inhibition by ETP or MEM of ≤ 21 mm in diameter in the disk diffusion [DD] assay), may indicate isolates with carbapenemase production, and this phenotype should be confirmed by the Hodge method (5). In Argentina, large proportions of nosocomial *Enterobacteriaceae* strains meet the CLSI criteria for suspected carbapenemase production according to ETP and MEM DD assay results (about 14 and 5% of 6,700 tested strains, respectively, as reported by the WHONET-Argentina Network in 2007 (<http://www.paho.org>). Up to one-

third of these isolates had a positive Hodge test result suggestive of carbapenemase production; still, almost all of them did not produce carbapenemases as determined by molecular methods (27). Therefore, in this work, we designed a panel composed of diverse bacterial genera with distinct carbapenem susceptibility patterns to identify potential problems in the current recommendations. In addition, we developed an optimized approach for the more accurate detection of isolates with possible carbapenemase production, suitable for countries in which strains have high baseline levels of resistance to carbapenems due to non-carbapenemase-mediated mechanisms, such as those resulting from the combination of extended-spectrum β -lactamases or AmpCs and porin mutations (2, 10, 15, 34).

Boronic acids (BA) were reported in the early 1980s to be reversible inhibitors of class C enzymes (1). Unexpectedly, we discovered unusual inhibition of organisms possessing KPC by 3-aminophenylboronic acid (APB), which may be attributed solely to the presence of this carbapenemase (25). Afterwards, Doi et al. and, in a subsequent study with a larger series of strains, Tsakris et al. demonstrated the overwhelming effectiveness of APB for the detection of *Klebsiella pneumoniae* possessing KPC (7, 32). Given these promising results, we decided to explore the potential of APB methods as confirmatory tests for class A carbapenemase detection. To date, information on the efficiency of APB methods for KPC-possessing bacterial species different from *K. pneumoniae* is missing. Moreover, boronic methods for carbapenemase detection de-

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scribed in the literature so far have been limited to KPC enzymes and have not been evaluated for the remaining class A members. Thus, we examined the capabilities of BA-based methods to detect the entire class A carbapenemase family by using these methods to test a panel of well-characterized strains, including several bacterial hosts with KPC and also GES-, Sme-, IMI-, and NMC-A-producing bacteria.

Thus, in this work we propose novel and accurate phenotypic methods, appropriate for clinical laboratories, to both screen for and confirm the presence of class A carbapenemases in species of *Enterobacteriaceae*.

MATERIALS AND METHODS

Bacterial strains. Tests were performed with a panel consisting of diverse bacterial genera with distinct carbapenem susceptibility patterns. A total of 30 carbapenemase-producing isolates (including 28 producing class A carbapenemases) and 36 non-carbapenemase-producing isolates of *Enterobacteriaceae* were included in the panel (Table 1). The resistance mechanisms of the strains employed in this work, carbapenem MICs for the strains, and the sources of the strains are described in detail in Table 1. All isolates were nonduplicates (molecular typing to ensure that they were genetically unrelated was performed previously), and except for ATCC isolates and *Salmonella* species transconjugants, all were isolated from clinical specimens. All isolates were previously identified by conventional techniques (19) and by use of an API 20E system (bioMérieux, Marcy l'Etoile, France). Although the genotypes of most of the isolates were characterized previously by molecular techniques, PCR analyses of all isolates were performed again to confirm the carbapenemase status (see below). 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 (see below).

Susceptibility tests and phenotypic carbapenemase assays. The DD assay was performed as described previously (4). Plates of Mueller-Hinton agar (Difco; BBL) were inoculated with samples of the tested strains, adjusted to a turbidity equivalent to a 0.5 McFarland standard. Disks (BBL) were applied to the surface of the inoculated agar, and plates were incubated overnight at 35°C. Each strain was tested in duplicate assays, and the average diameter of the zone of inhibition was registered.

Two further APB-based methods for phenotypic detection were designed. Each one was performed in duplicate assays. (i) In the double-DD synergy tests (DDST), a set of disks with IPM (10 µg), MEM (10 µg), or ETP (10 µg) were placed at several distances (10, 15, 20, 25, or 30 mm, center to center) from a blank disk containing 300 µg of APB (Sigma Chemicals, St. Louis, MO) (35) on a Muller-Hinton agar plate inoculated with a sample of the tested strain, adjusted to a 0.5 McFarland turbidity standard. APB disks were prepared before the DDST (by the addition of 10 µl of 30-mg/ml APB solution to a filter disk) and stored at -20°C until use. Enhancement of the zone of inhibition in the area between the carbapenems and the inhibitor-containing disk (demonstrating synergism) was considered to be a positive result. (ii) In the BA combined-disk (BA-CD) tests, sets of two disks each containing IPM (10 µg), MEM (10 µg), or ETP (10 µg) were placed onto a Muller-Hinton agar plate inoculated with a sample of the tested strain, adjusted to a 0.5 McFarland turbidity standard. The BA-CD plate was inoculated with the same bacterial suspension used for the DDST (see above) to avoid differences in the inoculum of bacteria used. Immediately after the disks had been placed onto the surface of the agar plate, 10 µl of a 30-mg/ml APB solution (300 µg of APB per disk) was added to one of the two carbapenem disks in each set. A filter disk containing 300 µg of APB only was placed onto the BA-CD plate and was assessed simultaneously to confirm the lack of an inhibitory effect of APB by itself (35).

Carbapenem MICs were determined to confirm the DD test results. Agar dilution (Mueller-Hinton agar was from Difco, BBL) MIC tests were carried out in duplicate according to CLSI recommendations by using a Steers device and a final inoculum of ca. 10⁴ CFU/spot (3). MICs of IPM and ETP (MSD, NJ) and MEM (AstraZeneca) in the absence and in the presence of 0.3 mg/ml of APB (35) were determined. Inoculated agar plates were incubated overnight at 35°C.

Escherichia coli ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were included as reference quality control strains for all methods.

Hodge test. The modified Hodge method was performed as described previously (5, 11). Briefly, a 1/10 dilution of 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 (diameter, 145 mm; Greiner Laboratory, Germany) of Mueller-Hinton agar (Difco, BBL) by swabbing. After the plate was allowed to stand for 10 min at room temperature, three disks (Difco, BBL), one with IPM (10 µg), one with MEM (10 µg), and one with ETP (10 µg), were placed onto the agar plate. Subsequently, by using 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 another. The presence of growth of the indicator strain toward the carbapenem disks was interpreted as a positive result for carbapenem hydrolysis screening.

Spectrophotometric assays. The precise identification of carbapenemase activities in crude extracts of bacterial strains was performed by a spectrophotometric assay using IPM as the substrate. In short, the cells were disrupted by the mechanic treatment described previously (17), and the carbapenemase activities of the crude extracts in a solution of 10 mM HEPES (pH 7.5; Sigma-Aldrich) and 0.4 mM IPM at 30°C were measured by monitoring IPM hydrolysis with a SmartSpec 3000 spectrophotometer (Bio-Rad) at 300 nm. For the testing of metallo-β-lactamases (MBLs), 0.01 mM ZnSO₄ (ICN) was added to the reaction mixture (17). Phenotypic APB inhibition of the imipenemase activities of extracts in the spectrophotometric assay was confirmed by adding 3 mM APB 10 min before the assay was performed (25).

PCR amplification and DNA sequencing. PCR analyses of all isolates were performed to confirm the carbapenemase status. The isolation of total DNA was performed as described previously (18). Strains were analyzed for *bla*_{IM}, *bla*_{IMP}, *bla*_{SPM}, *bla*_{KPC}, *bla*_{Sme}, and *bla*_{OXA} genes (subgroups I, II, and III) by using the primers described previously (18, 21, 25, 26). For *bla*_{IMI} and *bla*_{NMC-A}, specific primers were designed (forward, 5'-CAGAGCAAATGAACGATTTTC-3'; reverse, 5'-TGGTACGCTAGCACGAATAC-3'). Additionally, strains were tested for *bla*_{CTX-M}, *bla*_{TEM}, *bla*_{PER}, *bla*_{SHV}, and *bla*_{AmpC} genes (18, 28). The TGradient thermal cycler and MgCl₂, deoxynucleoside triphosphate, specific primers, and *Taq* polymerase were supplied by Biometra, Göttingen, Germany, and Invitrogen, respectively. PCR elongation times and temperatures were adjusted for the expected sizes of the PCR products and for the nucleotide sequences of the primers, respectively. Nucleotide sequencing of the GES amplicon with an ABI Prism system (Applied Biosystems, Foster City, CA) confirmed the carbapenemase allelic variants involved in this work. BLAST program searches were performed by using the National Center for Biotechnology Information website (<http://www.ncbi.nlm.nih.gov>).

SN and SP. Sensitivity (SN) and specificity (SP) calculations were done essentially as described in reference 13. 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 2f carbapenemases by the tested assay and c represents the number of true 2f 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 2f carbapenemases and b is the number of strains that were incorrectly identified as 2f carbapenemase producers.

RESULTS AND DISCUSSION

Screening tests for class A enzymes. First, we evaluated the performances of carbapenem susceptibility screenings for the identification of isolates suspected of producing class A carbapenemases.

(i) **DD tests.** Screening with IPM provided higher-level efficacy in distinguishing class A carbapenemase-producing isolates than screening with the other carbapenems. It was possible to differentiate class A carbapenemase-producing bacteria from non-carbapenemase-producing bacteria solely by the size of the IPM disk zone of inhibition by using ≤20 mm as the breakpoint. At this cutoff, IPM screening showed an SN of 1.0 and an SP of 0.95 (Fig. 1a). To date, there are no recommendations for the use of IPM disks for carbapenemase screening of *Enterobacteriaceae*. The CLSI criteria for screening with ETP and MEM (e.g., zones of inhibition of ≤21 mm in diameter) yielded less SN than our breakpoint for screening with IPM in detecting some class A carbapenemase-producing strains: several Sme producers were misdetected by screening with ETP, and representative producers of all class A carbap-

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

Strain ^a	Bacterial species ^b	β-Lactamase(s)	MIC (μg/ml) of:			Hodge test result	Reference or source
			IPM	MEM	ETP		
KPC producers (n = 12)							
9169	<i>Cfr</i>	KPC-2, TEM-1	1	1	2	+	25
9888	<i>Cfr</i>	KPC-like β-lactamase, inducible AmpC	1	2	1	+	This work
9171	<i>Kpn</i>	KPC-2, PER, TEM-1, SHV-1	64	32	128	+	25
D05/07	<i>Kpn</i>	KPC-2	2	2	16	+	CAP QC Assurance ^e
9885	<i>Kpn</i>	KPC-like β-lactamase	16	16	16	+	This work
9190 ^c	<i>Sal</i>	KPC-like β-lactamase	1	1	1	+	25
9204 ^c	<i>Sal</i>	KPC-like β-lactamase	1	0.5	1	+	25
9847	<i>Ecl</i>	KPC-like β-lactamase, hyperproduced AmpC	8	16	32	+	This work
9884	<i>Eco</i>	KPC-like β-lactamase	2	2	2	+	This work
10007	<i>Eco</i>	KPC-like β-lactamase, TEM-1	2	1	2	+	This work
10154	<i>Pmi</i>	KPC-like β-lactamase, TEM-1	4	2	4	+	This work
10155	<i>Pmi</i>	KPC-like β-lactamase, TEM-1	4	2	4	+	This work
Sme producers (n = 10)							
3981	<i>Sma</i>	Sme-1b	128	0.5	4	ND ^d	22
3989	<i>Sma</i>	Sme-1b, CTX-M-2, OXA-2	4	2	0.5	ND	22
3991	<i>Sma</i>	Sme-1b, OXA-2	256	2	2	+	22
5050	<i>Sma</i>	Sme-1b, CTX-M-2, OXA-2, SHV-1	16	0.15	4	+	22
5061	<i>Sma</i>	Sme-1b	1	0.5	0.5	+	22
5634	<i>Sma</i>	Sme-like β-lactamase	16	4	4	+	22
5635	<i>Sma</i>	Sme-like β-lactamase	16	8	4	+	22
5636	<i>Sma</i>	Sme-like β-lactamase	16	8	4	+	22
7596	<i>Sma</i>	Sme-like β-lactamase	16	8	4	+	This work
9247	<i>Sma</i>	Sme-like β-lactamase	64	2	4	+	This work
IMI or NMC-A producers (n = 2)							
3202	<i>Ecl</i>	IMI-1	4	8	1	+	A. Medeiros
9967	<i>Ecl</i>	NMC-A	4	4	0.25	+	This work
GES producers (n = 4)							
5401	<i>Ent</i>	GES-5	32	8	8	+	This work
5825	<i>Kpn</i>	GES-3, CTX-M-2	32	64	128	+	This work
5476 ^c	<i>Sal</i>	GES-5	16	16	4	+	This work
5490 ^c	<i>Sal</i>	GES-3	32	32	8	+	This work
MBL producers (n = 2)							
7647	<i>Kpn</i>	VIM-like β-lactamase, CTX-M-2, TEM-1, SHV-1	0.5	1	4	+	16
7527	<i>Kpn</i>	VIM-like β-lactamase, TEM-1, SHV-1	32	8	32	+	16
Producers of AmpC (derepressed or plasmid mediated) (n = 12)							
C2	<i>Kpn</i>	FOX-5	0.12	0.06	0.06	-	29
9234	<i>Eco</i>	AAC-1	0.12	0.03	0.03	-	L. Martinez-Martinez
9235	<i>Eco</i>	CMY-2	0.05	0.06	0.03	-	L. Martinez-Martinez
9236	<i>Kpn</i>	DHA	0.03	0.03	0.5	-	L. Martinez-Martinez
7896	<i>Sfl</i>	CMY-2	0.06	0.03	0.06	-	31
9324	<i>Pmi</i>	CMY-like β-lactamase	0.12	0.03	0.06	-	31
9140	<i>Kpn</i>	CMY-like β-lactamase	0.12	0.03	1	-	31
POS9	<i>Mmo</i>	Hyperproduced AmpC	2	0.12	0.12	-	24
POS15	<i>Cfr</i>	Hyperproduced AmpC	0.12	0.06	0.12	-	24
3986	<i>Cfr</i>	Hyperproduced AmpC	0.25	0.03	0.03	-	24
9251	<i>Ecl</i>	Hyperproduced AmpC	2	2	4	+	24
5485	<i>Ecl</i>	Hyperproduced AmpC, porins	4	16	64	+	6
ESBL producers (n = 7)							
1803	<i>Kpn</i>	PER-2, CTX-M-2, SHV-1, TEM-1	0.5	0.5	2	-	18
9375	<i>Kpn</i>	CTX-M-2, SHV-1, TEM-1	0.06	0.06	0.12	-	9

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TABLE 1—Continued

Strain ^a	Bacterial species ^b	β-Lactamase(s)	MIC (μg/ml) of:			Hodge test result	Reference or source
			IPM	MEM	ETP		
9163	<i>Kpn</i>	CTX-M-2, SHV-1, TEM-1, porins	1	2	16	+	9
9391	<i>Kpn</i>	CTX-M-2, SHV-1, TEM-1, porins	0.25	8	16	+	9
9310	<i>Kpn</i>	CTX-M-2, SHV-1, TEM-1, porins	0.12	8	16	+	9
700603	<i>Kpn</i>	SHV-18, porin	0.06	0.12	0.12	–	ATCC
Cos15	<i>Eco</i>	PER, TEM	0.03	0.03	0.12	–	8
OSBL producer strain 35218	<i>Eco</i>	TEM-1	0.12	0.015	0.015	–	ATCC
Producers of low levels of K1 or penicillinase or cefuroximase (<i>n</i> = 3)							
7522	<i>Cko</i>	Penicillinase	0.015	0.015	0.008	–	6
HMC22	<i>Ppe</i>	Cefuroximase	0.06	0.06	0.015	–	23
FLE17	<i>Kox</i>	Low-level K1	0.12	0.03	0.008	–	6
Producers of low basal levels of AmpC (inducible) (<i>n</i> = 5)							
GUT 8	<i>Cfr</i>	Inducible AmpC	0.25	0.03	0.015	–	24
CEN31	<i>Sma</i>	Inducible AmpC	0.12	0.03	0.06	–	24
HMI23	<i>Ecl</i>	Inducible AmpC	0.5	0.25	0.5	–	24
POS B	<i>Mmo</i>	Inducible AmpC	1	0.12	0.015	–	24
9113	<i>Pst</i>	Inducible AmpC	0.06	0.06	0.03	–	6
Producers of ESBL(s) plus inducible AmpC (<i>n</i> = 4)							
9291	<i>Sma</i>	CTX-M-2, TEM-1, inducible AmpC, porins	2	4	64	+	24
9383	<i>Sma</i>	CTX-M-2, inducible AmpC, porins	2	1	32	+	24
9339	<i>Ecl</i>	PER-2, inducible AmpC	0.12	0.25	0.5	–	24
3972	<i>Pst</i>	CTX-M-2, inducible AmpC	0.03	0.06	0.015	–	24
Strains with wild-type and/or marginal or low-level β-lactamase activities (<i>n</i> = 4)							
1744	<i>Sal</i>	None	0.12	0.015	0.008	–	18
27508	<i>Ecl</i>	None (AmpC [–] mutant)	0.06	0.03	0.015	–	ATCC
HMI13	<i>Pmi</i>	None	0.25	0.06	0.008	–	23
25922	<i>Eco</i>	Basal-level AmpC	0.06	0.015	0.015	–	ATCC

^a ESBL, extended-spectrum β-lactamase; OSBL, “older-spectrum” β-lactamase.

^b *Cfr*, *Citrobacter freundii*; *Kpn*, *K. pneumoniae*; *Sal*, *Salmonella* sp.; *Eco*, *E. coli*; *Pmi*, *Proteus mirabilis*; *Sma*, *S. marcescens*; *Ecl*, *E. cloacae*; *Ent*, *Enterobacter* sp.; *Sfl*, *Shigella flexneri*; *Mmo*, *Morganella morganii*; *Cko*, *Citrobacter koseri*; *Ppe*, *Proteus penneri*; *Kox*, *Klebsiella oxytoca*; *Pst*, *Providencia stuartii*.

^c Transconjugant strain.

^d ND, not determined.

^e CAP QC, College of American Pathologists Quality Control.

enemases were misdetected by screening with MEM (Fig. 1a). Because of the high proportion of strains with basal ETP/MEM resistance in the panel used, the ETP and MEM screening tests had significantly large proportions of false-positive results, corresponding to about a quarter of the control group (Fig. 1a). Improvements for MEM screening (SN values of >0.8 were prioritized) could be obtained by using a modified cutoff point of ≤27 mm, while for ETP screening, the CLSI cutoff of ≤21 mm gave optimal results (Fig. 1a).

(ii) **MIC tests.** Similar to DD testing, MIC testing made it possible to differentiate class A carbapenemase-producing bacteria from non-carbapenemase-producing bacteria exclusively by the IPM result obtained, using a MIC of ≥1 μg/ml as the breakpoint. At this proposed cutoff, IPM screening showed an SN of 1.0 and an SP of 0.79 (Fig. 1b). Most of the strains with false-positive IPM screening results had AmpC hyperproduction or a combination of CTX-M and porin mutations. Minor

interferences in the IPM screening of members of the tribe *Proteaeae* were observed. The CLSI guidelines currently proposed for IPM screening, with a cutoff one dilution higher (≥2 μg/ml) than that used in the present study, would not permit the detection of many carbapenemase-producing strains (mostly KPC producers) for which the IPM MIC is 1.0 μg/ml (Fig. 1b). Furthermore, the use of CLSI criteria for screening with ETP and MEM (e.g., MICs of ≥2 μg/ml) failed to detect various group 2f members, in addition to providing large proportions of false-positive results (Fig. 1b). Improvements of the performances of ETP and MEM screening could be obtained by using modified cutoff points of ≥0.5 μg/ml for both carbapenems (Fig. 1b).

Phenotypic confirmatory tests for class A enzymes. (i) **Hodge tests.** The Hodge test, a procedure proposed for the confirmation of suspected carbapenemase production, detects the presence of carbapenem-hydrolyzing activities in the unbroken

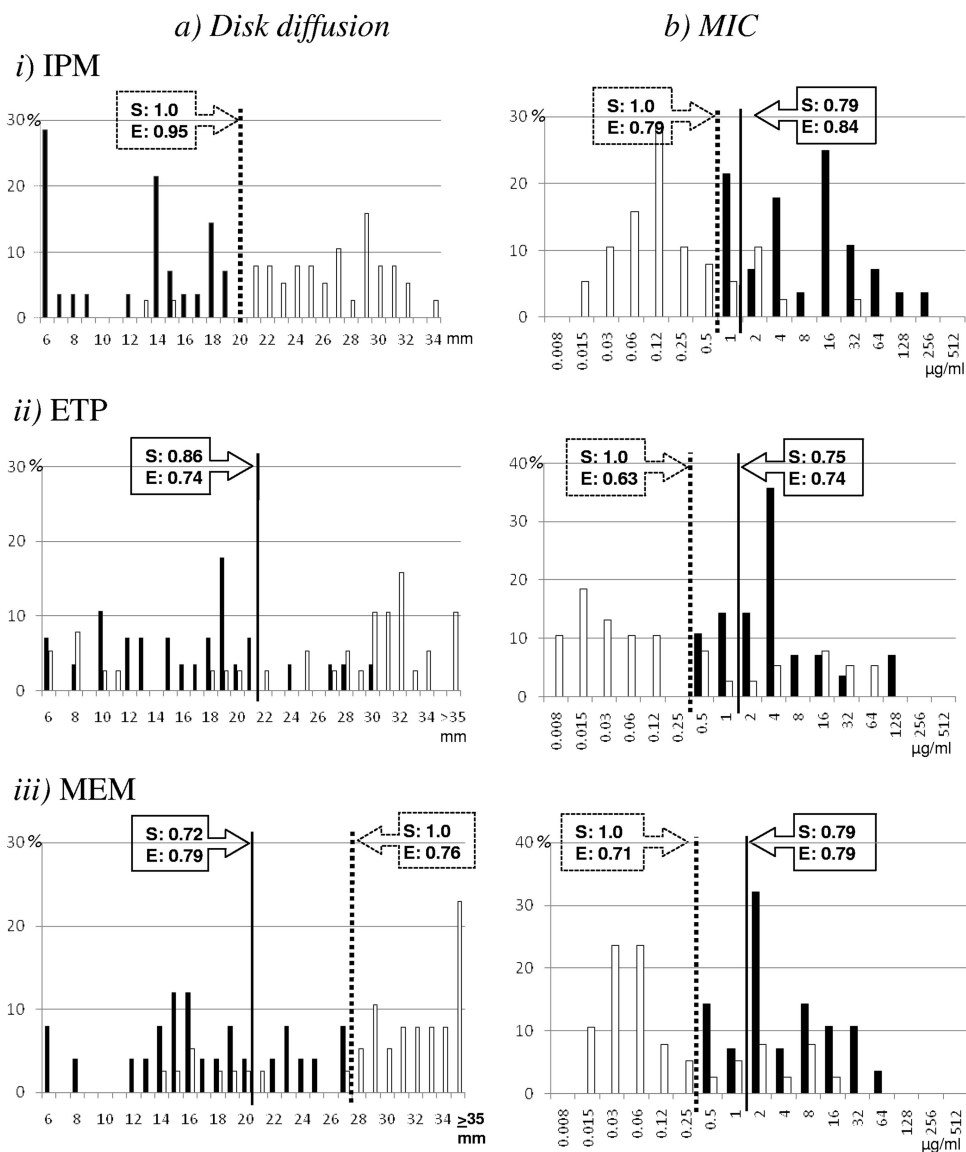
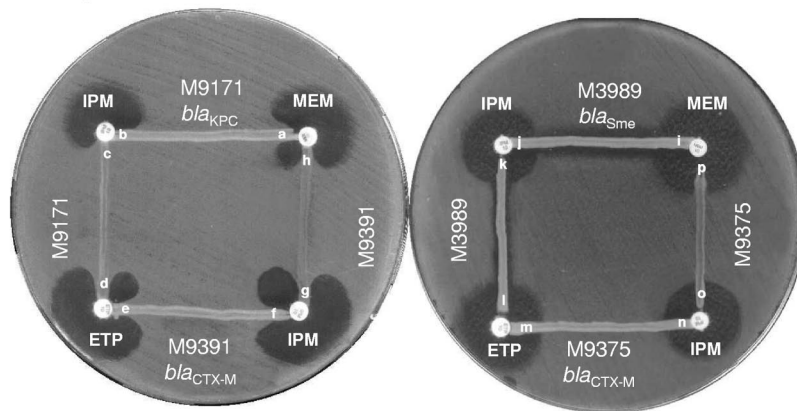


FIG. 1. Results of screening tests: frequencies of distribution of IPM, ETP, and MEM inhibition zones (a) or MICs (b) for strains included in the panel. Solid black bars indicate class A carbapenemase producers, whereas hollow bars correspond to the control group (group 2f carbapenemase nonproducers). Dashed vertical lines represent the cutoffs that permitted the best SN and SP for 2f carbapenemase detection, while solid vertical lines indicate current recommended CLSI cutoffs for carbapenemase screening (not available for the IPM DD test). Values within the arrows correspond to the SN (S) and SP (E) for the indicated cutoffs.

cells of the analyzed bacteria. Tested substrates recommended by the CLSI (ETP and MEM) were assessed; nevertheless, we also evaluated IPM performance. The Hodge test results for each strain in the panel are given in Table 1. We found that the Hodge method displayed high efficiency for the detection of carbapenemases, regardless of the substrate employed (the three carbapenems gave equivalent results). Class A carbapenemase-producing strains were detected with a high SN (0.93; results for two *bla*_{Sme}-positive strains remained undetermined because these strains inhibited the growth of the indicator strain). However, false-positive results were observed: besides MBL producers, as expected, seven carbapenemase nonproducers showed a positive Hodge test result with discernible carbapenemase-like patterns (Table 1 and Fig. 2A). Overall, the SP was as low as

0.76. False-positive results corresponded mainly to strains producing CTX-M and, to a lesser extent, those with hyperproduction of AmpC (Table 1). These unexpected outcomes were observed among isolates with characteristic carbapenem susceptibility patterns: an ETP MIC of ≥ 4.0 $\mu\text{g/ml}$ was associated with a false-positive Hodge result (Table 1). These false-positive results may occur due to minor carbapenem hydrolysis by CTX-M or AmpC enzymes (10, 14), enhanced by the prolonged incubation period for these tests, as the strains did not display measurable carbapenemase activities when examined spectrophotometrically (see below). As it is critically important to have confirmatory tests associated with high levels of confidence, we explored the capabilities of several APB methods.

A) Hodge Test



B)

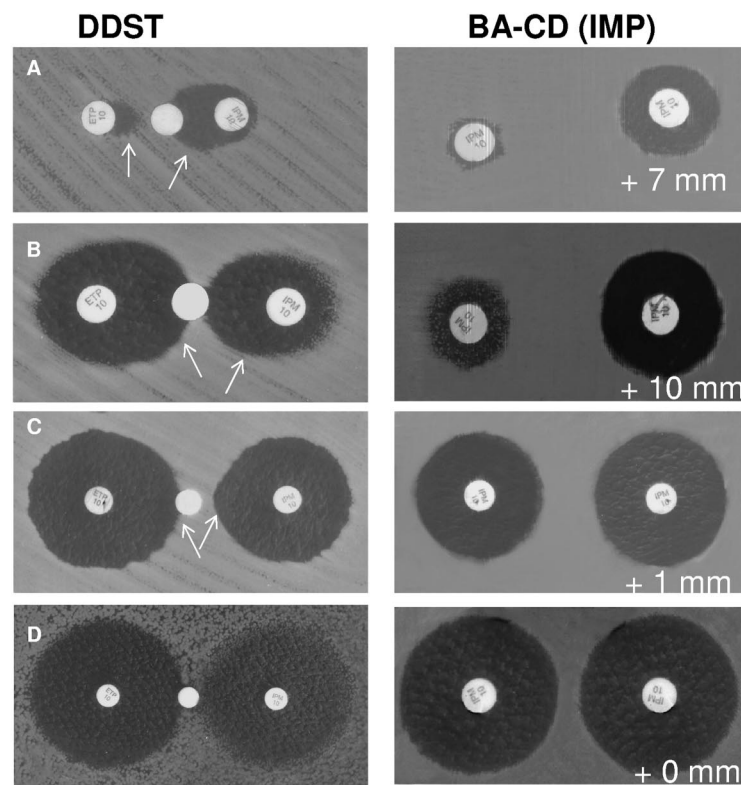


FIG. 2. Results of confirmatory tests. (A) Results of the modified Hodge test for representative isolates (*K. pneumoniae* M9171 [producing KPC-2], *K. pneumoniae* M9391 [producing CTX-M-2], *Serratia marcescens* M3989 [producing Sme-1b], and *K. pneumoniae* M9375 [producing CTX-M-2]). Letters *a* to *h* indicate positive results, as IPM, MEM, and ETP were hydrolyzed by the streaked cells; letters *i* to *l* indicate indeterminate results due to the inhibition of growth of the indicator strain; letters *m* to *p* indicate negative results. (B) Results of DDST with ETP (left disks), APB (300 μ g per disk; blank middle disks), and IPM (right disks) and results of BA-CD assay with IPM (left disks) and IPM plus APB (300 μ g per disk; right disks) for representative isolates. Arrows indicate evidence of synergism, and numbers indicate the increase in the diameter of the zone of inhibition produced by a carbapenem-inhibitor combination disk compared with that produced by a nonsupplemented carbapenem disk. Panels: A, *K. pneumoniae* KPC-2; B, *S. marcescens* Sme-1b; C, *Enterobacter cloacae* wild type; and D, *E. coli* ATCC 25922.

(ii) **DDST.** We developed novel DDST, which were similar to the test recommended by Yagi et al. for plasmidic AmpC, with the difference that we observed the enlargement of the growth-inhibitory zone diameter (indicating synergism) around the carbapenem disk (35). We used disks with the same APB concentrations used previously (25, 35). Several distances between the APB disks and the carbapenem disks were evaluated, and we found that the best results were obtained when

disks were placed at 20 mm from center to center (Fig. 2B). At this distance, IPM-APB was the most sensitive combination, and synergism against all of the 2f carbapenemase-producing controls except one (a GES producer) was observed (Table 2). ETP and MEM provided SN values inferior to those provided by IPM (Table 2). The occurrence of false-positive results (weak synergism) was observed exclusively for strains with AmpC β -lactamases (Table 2 and Fig. 2B).

TABLE 2. Confirmatory test analysis^a

Assay	SN	SP
Hodge test using: IPM, ETP, or MEM	0.93	0.76
DDST (with disks at 20-mm distance ^b) for:		
IPM-APB synergism	0.96 ^c	0.92 ^f
MEM-APB synergism	0.80	0.84
ETP-APB synergism	0.82	0.79
CD test for increase of ≥ 4 mm ^c using:		
IPM-APB	1.0	1.0
MEM-APB	0.86	0.95
ETP-APB	0.89	0.97
BA-based test for ≥ 3 -fold reduction in MIC ^d of:		
IPM-APB	1.0	1.0
MEM-APB	0.89	1.0
ETP-APB	0.86	0.92

^a The levels of performance of and the most favorable conditions for the phenotypic confirmatory tests described in this study for the detection of 2f carbapenemases among species of *Enterobacteriaceae* are summarized.

^b Center-to-center distance.

^c Increase in the diameter of the zone of inhibition compared to that achieved with a carbapenem alone.

^d Reduction in the MIC of the combination compared to that of the carbapenem alone.

^e A false-negative result for *K. pneumoniae* M5825 (producing GES-3 and CTX-M-2) was obtained.

^f False-positive results for *E. cloacae* M9251 and HMI23 (both hyperproducing AmpC) and *K. pneumoniae* M9236 (producing DHA) were obtained.

(iii) **BA-CD tests.** As faint synergism was frequently observed among strains with false-positive results from the DDST, we planned a quantitative strategy in order to avoid this interference: CD tests with carbapenem disks supplemented with APB solution to achieve an inhibitor burden similar to that reported previously (35). IPM-APB was the most sensitive combination: it was possible to differentiate class A carbapenemase-producing bacteria from non-carbapenemase-producing bacteria based solely on a ≥ 4 -mm increase in the inhibition zone diameter, with the SN and SP both being 1.0 (Table 2 and Fig. 2B). The use of the previously reported breakpoint of a ≥ 5 -mm increase for the IPM-APB disk (7) resulted, for our panel, in the loss of detection of GES-producing strains. ETP-APB and MEM-APB did not achieve the performance of IPM-APB (the ≥ 4 -mm-increment cutoff was also the better breakpoint for these combinations) (Table 2). In the present study, the BA-CD assay performed with slightly greater SN and SP values than the DDST (Table 2). However, for the BA-CD assay, the APB solutions had to be loaded directly onto carbapenem disks already placed on the agar plate (preliminary data on *E. coli* ATCC 25922 indicated the deterioration of the carbapenem content when disks were prepared previously). Thus, for a voluminous work burden, we find the DDST method more practical than the BA-CD assay for routine clinical laboratories because the disks can be prepared prior to the test and remain stable for more than 30 weeks at 4°C.

(iv) **BA-based MIC tests.** The addition of APB to the carbapenem MIC tests confirmed the BA-CD results. IPM-APB showed the highest level of performance: by using a ≥ 3 -fold

reduction in the MIC as the cutoff, it was possible to differentiate class A carbapenemase-producing bacteria from non-carbapenemase-producing bacteria with maximum SN and SP values, both being 1.0 (Table 2). The ≥ 3 -fold MIC reduction cutoff also proved to be a better breakpoint for ETP- and MEM-APB, but these combinations had lower levels of performance than IPM-APB (Table 2).

Spectrophotometric assays. Crude extracts of only the class A and MBL carbapenemase-producing groups displayed measurable imipenemase activities. The broad inhibition properties of APB for the entire 2f family were confirmed by the spectrophotometric method: inhibition of class A carbapenemase producers but not of MBL producers tested in parallel was observed after the addition of APB at 0.3 mM.

Strategic screening for 2f carbapenemases. On the basis of the results of this work, we recommend a flow chart for the rational and hierarchical use of the routine information; in scenarios with high proportions of strains expressing non-carbapenemase-mediated resistance (such as that generated by endemic CTX-M), this approach enables the optimization of the resources available in clinical microbiology laboratories for carbapenemase detection with high levels of confidence (Fig. 3). (i) On the basis of the observation in this work that screening with IPM provides better performance than screening with ETP or MEM for class A carbapenemase detection, screening starts solely with the IPM results of the routine antimicrobial susceptibility tests: this strategy would improve initial carbapenem screening. Either the IPM MIC or the DD test inhibition zone diameter may be used, although the latter gave better results: a diameter of ≤ 20 mm or a MIC of ≥ 1 $\mu\text{g/ml}$ categorically selects isolates suspected of producing class A carbapenemase. The microbiologist should be aware that ETP/MEM-based screening schemes may misdetect some carbapenemases (especially nonmobile ones, but with potentially large clinical impact) due to low-level resistance to ETP or MEM (20). Minor interference in IPM results (mostly MICs) by members of the tribe *Proteeae* was observed, even though a considerable number of such strains (constituting about 18% of all strains analyzed) were included in the panel. When a species of *Proteeae* had an IPM MIC of ≥ 1 $\mu\text{g/ml}$, screening could be improved by the combined use of two carbapenem results, such as those for IPM plus ETP or MEM (using the optimized breakpoints): this approach resulted in strengthened SP for members of the tribe *Proteeae* without the loss of SN (SN value, 1.0; SP value, 0.84). (ii) Given the clinical and public health impact involved in the discovery of a strain carrying carbapenemase, phenotypic confirmatory assays for isolates with a positive screening test result play a key role in the detection of carbapenemases. The modified Hodge method should be used carefully as a confirmatory test in countries in which CTX-M producers are endemic: in this scenario, only a negative test result is reliable (with a predictive negative value of 1.0). Because of the occurrence of false-positive results, the Hodge test was not included in the proposed flow chart. In its place, we propose BA-based methods as confirmatory assays for isolates with a positive screening test result. Accordingly, DDST (to detect synergism), BA-CD tests (to detect an increase of ≥ 4 mm in the inhibition zone diameter), or BA-based MIC tests (to detect a decrease of ≥ 3 dilutions in the MIC), using IPM as the substrate, could identify not only those isolates with very high

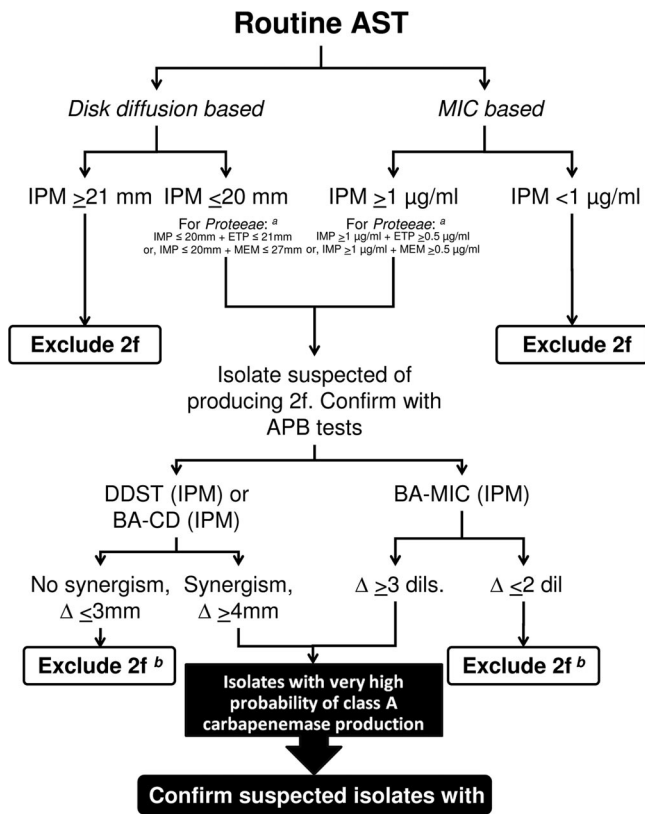


FIG. 3. Proposed flow chart for screening of suspected carbapenemase-producing strains of *Enterobacteriaceae*. The substrate for the BA-based methods is indicated in parentheses. ^a, for a member of the *Proteoae* for which the IPM MIC is ≥ 1 $\mu\text{g/ml}$ or the zone of inhibition is ≤ 20 mm, use two carbapenem results (at the optimized breakpoints) simultaneously to screen for class A carbapenemases. ^b, MBL screening is recommended for these isolates. AST, antibiotic susceptibility test; dil, dilution.

probabilities of producing KPC (irrespective of the bacterial species), but also the isolates likely to produce any member of the class A carbapenemases, for which the results should be confirmed by reference molecular methods (Fig. 3). Furthermore, the performances of these tests were comparable to those considered to be “gold standard” methods (PCR and spectrophotometric assays).

Concluding remarks. This is the first study to assess the accuracy of screening and confirmatory phenotypic methods, suitable to the hospital level, in detecting all major types of mobile and nonmobile 2f carbapenemases described, including KPC, NMC-A, IMI, Sme, and GES, produced by diverse species of *Enterobacteriaceae* with distinct carbapenem susceptibility patterns. We propose here an alternative approach to screening tests for suspected carbapenemase production by species of *Enterobacteriaceae* for countries in which the ETP/MEM baseline resistance level is high or is increasing due to endemic CTX-M producers. In addition, we propose BA-based confirmatory tests to circumvent the Hodge test carbapenemase-like susceptibility patterns associated with CTX-M enzymes. Nowadays, BA derivatives with broad inhibition spectrums that include the entire class A carbapenemase family should be considered. The proposed scheme represents a sat-

isfactory and inexpensive methodology for the detection of class A carbapenemase-producing bacteria in such circumstances.

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