JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 2011, p. 2804–2809 Vol. 49, No. 8
0095-1137/11/$12.00 doi:10.1128/JCM.00666-11

Comparative Evaluation of Combined-Disk Tests Using Different Boronic Acid Compounds for Detection of *Klebsiella pneumoniae* Carbapenemase-Producing *Enterobacteriaceae* Clinical Isolates▽

Athanassios Tsakris,[1]* Katerina Themeli-Digalaki,[2] Aggeliki Poulou,[1,3] Georgia Vrioni,[1] Evangelia Voulgari,[1] Vasiliki Koumaki,[1] Antonella Agodi,[4] Spyros Pournaras,[5] and Danai Sofianou[6]

Department of Microbiology, Medical School, University of Athens, Athens,[1] Department of Microbiology, Tzaneion General Hospital, Piraeus,[2] and Department of Microbiology, Serres General Hospital, Serres,[3] Greece; Department of Biomedical Sciences, University of Catania, Catania, Italy[4]; and Department of Microbiology, Medical School, University of Thessaly, Larissa,[5] and Department of Microbiology, Hippokration University Hospital, Thessaloniki,[6] Greece

Received 4 April 2011/Returned for modification 22 April 2011/Accepted 19 May 2011

The accurate phenotypic detection of *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Enterobacteriaceae* is an increasing necessity worldwide. We evaluated the performance of boronic acid combined-disk tests using as substrate imipenem or meropenem and as inhibitor of KPC production 300 μg aminophenylboronic acid (APBA), 600 μg APBA, or 400 μg phenylboronic acid (PBA). Tests were considered positive when an increase in the growth-inhibitory zone around a carbapenem disk with KPC inhibitor was 5 mm or greater of the growth-inhibitory zone diameter around the disk containing carbapenem alone. The comparison of the combined-disk tests was performed with 112 genotypically confirmed KPC-possessing *Enterobacteriaceae* isolates. To measure the specificity of the tests, 127 genotypically confirmed KPC-negative *Enterobacteriaceae* isolates that were nonsusceptible to at least one carbapenem were chosen for testing. Using disks containing imipenem without and with 300 μg APBA, 600 μg APBA, or 400 μg PBA, 72, 92, and 112 of the KPC producers, respectively, gave positive results (sensitivities, 64.3%, 82.1%, and 100%, respectively). Using disks containing meropenem without and with 300 μg APBA, 600 μg APBA, or 400 μg PBA, 87, 108, and 112 of the KPC producers, respectively, gave positive results (sensitivities, 77.7%, 96.4%, and 100%, respectively). Among KPC producers, the disk potentiation tests using meropenem and PBA demonstrated the largest differences in inhibition zones ($P < 0.001$). All combined-disk tests correctly identified 124 of the 127 non-KPC producers (specificity, 97.6%). This comparative study showed that PBA is the most effective inhibitor of KPC enzymes, and its use in combined-disk tests with meropenem may give the most easily interpreted results.

Carbapenem resistance among *Enterobacteriaceae* clinical isolates had been infrequent. However, during the last decade, carbapenem resistance has emerged among *Enterobacteriaceae* in health care settings and is increasingly attributed to the production of β-lactamases capable of hydrolyzing carbapenems (4). Among these enzymes, the class A *Klebsiella pneumoniae* carbapenemases (KPCs) have a rapid international spread, being harbored predominantly by *K. pneumoniae* and less frequently by other *Enterobacteriaceae* genera (23). The KPC-producing pathogens have become increasingly prevalent in the northeastern regions of the United States since the beginning of the 2000s (5, 19) and have subsequently disseminated and caused outbreaks in Israel and Greece, as well as in large geographic areas such as South America and China (6, 20, 28, 31).

The current wide spread of KPC enzymes makes them a potential threat to currently available antibiotic-based treatments (23). It is also of note that KPCs are mostly plasmid-encoded determinants that can easily disseminate to other enterobacterial strains and species (9, 30, 36). Therefore, the accurate phenotypic detection of this carbapenem resistance mechanism is crucial for controlling the spread of KPC enzymes among *Enterobacteriaceae*. The phenotypic detection of KPC-producing organisms was originally based on reduced susceptibility to carbapenems, which has to be confirmed by the modified Hodge test (MHT) (1). While the MHT was found to be useful for the phenotypic detection of KPC enzymes in hospitals where these β-lactamases are endemic (1), the test cannot discriminate between KPCs and other carbapenemases, and obviously, its positive predictive value for KPC detection is low in regions where other carbapenem-hydrolyzing enzymes, like metallo-β-lactamases (MBLs), are also prevailing.

In that regard, boronic acid compounds have recently been proposed to be inhibitors that can be used in disk potentiation tests and efficiently differentiate KPC producers from those producing MBLs or other broad-spectrum β-lactamases (12, 16, 24, 34). However, the described combined-disk methods are often based on different substrates and boronic acid compounds or concentrations, as well as on different interpretation criteria, and therefore, their reliability for KPC detection may vary considerably. In the present study, under the same conditions, we compared the performance of the proposed bo-

* Corresponding author. Mailing address: Department of Microbiology, Medical School, University of Athens, 11527 Athens, Greece. Phone: 30-210-7462011. Fax: 30-210-7462210. E-mail: atsakris@med.uoa.gr.

▽ Published ahead of print on 1 June 2011.

TABLE 1. Strain groups and species of *Enterobacteriaceae* clinical isolates used in this study

Group	No. of isolates					
	Klebsiella pneumoniae	*Klebsiella oxytoca*	*Escherichia coli*	*Enterobacter* spp.	*Serratia marcescens*	Total
KPC and KPC/ESBL[a] producers (n = 112)	89	3	7	5	8	112
VIM and VIM/ESBL[b] producers (n = 58)	39	4	3	12		58
AmpC and AmpC/ESBL[c] producers (n = 47)	20		19	8		47
ESBL[d] producers (n = 22)	7		15			22
Total	155	7	44	25	8	239

[a] $bla_{CTX\text{-}M\text{-}3}$ (n = 2), $bla_{CTX\text{-}M\text{-}15}$ (n = 5), $bla_{SHV\text{-}5}$ (n = 3), and $bla_{SHV\text{-}12}$ (n = 63).
[b] $bla_{CTX\text{-}M\text{-}15}$ (n = 6) and $bla_{SHV\text{-}5}$ (n = 27).
[c] $bla_{CTX\text{-}M\text{-}3}$ (n = 3), $bla_{CTX\text{-}M\text{-}15}$ (n = 6), and $bla_{shv\text{-}5}$ (n = 18).
[d] $bla_{CTX\text{-}M\text{-}3}$ (n = 3), $bla_{CTX\text{-}M\text{-}15}$ (n = 17), and $bla_{SHV\text{-}5}$ plus $bla_{CTX\text{-}M\text{-}3}$ (n = 2).

ronic acid combined-disk tests concerning their ability to detect effectively KPC producers in the clinical laboratory.

MATERIALS AND METHODS

Clinical isolates. The evaluation of the boronic acid disk tests for the detection of KPC was performed with 112 genotypically confirmed KPC-possessing *Enterobacteriaceae* isolates that belonged to different species. The collection contained clinical isolates of *K. pneumoniae* (n = 89), *Serratia marcescens* (n = 8), *Escherichia coli* (n = 7), *Enterobacter* spp. (n = 5), and *Klebsiella oxytoca* (n = 3) (Table 1). The isolates were recovered from 2007 to 2010 from separate patients who were hospitalized in six tertiary care hospitals located in four distinct Greek regions (two hospitals in the broad region of Athens and one hospital each in Thessaloniki, Larissa, and Serres) and in one Italian region (Catania).

To measure the specificity of methods to detect KPC-mediated resistance, 127 genotypically confirmed KPC-negative *Enterobacteriaceae* clinical isolates were chosen for testing (Table 1). Non-KPC-possessing isolates were randomly selected from among isolates that possessed MBLs, AmpCs, and/or extended-spectrum β-lactamases (ESBLs) and were nonsusceptible to at least one of the carbapenems (imipenem, meropenem, or ertapenem; imipenem and meropenem MICs > 1 μg/ml; ertapenem MICs > 0.25 μg/ml) (7). These 127 isolates came from collections held at the clinical laboratories providing KPC-positive isolates for the present study. The identification of all isolates was confirmed by using the API 20E system (bioMérieux, Marcy l'Etoile, France). In addition, the reference strains *K. pneumoniae* NCTC 13442 and *E. coli* NCTC 13476, carrying OXA-48 and IMP-type carbapenemases, respectively, were included in the study.

Antimicrobial susceptibility testing and phenotypic screening. Detailed susceptibility analysis was carried out by the agar dilution method following the CLSI guidelines and interpretative criteria (7). For tigecycline, the U.S. Food and Drug Administration recommendation was used (susceptible, ≤2 μg/ml; resistant, ≥8 μg/ml), and for colistin, the CLSI recommendation for *Acinetobacter* spp. was used (susceptible, ≤2 μg/ml; resistant, ≥4 μg/ml). The MHT was applied to detect carbapenemase production (1). The MBL Etest (AB Biodisk, Solna, Sweden) and the combined-disk test with imipenem and EDTA (15) were used to screen for the production of class B carbapenemases. ESBL production was tested using the CLSI confirmatory test and a modification which uses clavulanate in combination with boronic acid (35).

Molecular testing for β-lactamase genes. β-Lactamase genes were amplified in single PCRs for each gene using a panel of primers for detection of all types of MBLs (18), KPCs (22), OXA-type carbapenemases (27), plasmid-mediated AmpCs (26), and ESBLs, including SHV, TEM, CTX-M, and GES/IBC enzymes (37). Among *Enterobacter aerogenes* and *Enterobacter cloacae* isolates, total RNA from logarithmic-phase-grown cultures was extracted with TRI reagent (Ambion, Austin, TX), and reverse transcription (RT) of 1 μg of total RNA was performed with a ThermoScript RT-PCR system (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Derepressed AmpC-hyperproducing *E. aerogenes* and *E. cloacae* isolates were identified with quantitative real-time PCR using the Quanti test SYBR green (Qiagen, Hilden, Germany) and primers described previously (2, 29). The positive controls used were previously characterized isolates from our collection carrying all types of tested β-lactamases.

The PCR products were subjected to direct sequencing. PCR products were purified using ExoSAP-IT reagent (USB Corporation, Cleveland, OH) and used as templates for sequencing on both strands with an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA).

Phenotypic methods for detection of KPCs. The phenotypic detection of KPC-possessing *Enterobacteriaceae* isolates was evaluated employing boronic acid combined-disk tests that use as antibiotic substrate imipenem or meropenem and as inhibitor of KPC production 300 μg of aminophenylboronic acid (APBA), 600 μg of APBA, or 400 μg of phenylboronic acid (PBA).

The stock solution of PBA was prepared as previously recommended (8, 34) by dissolving PBA (benzeneboronic acid; Sigma-Aldrich, Steinheim, Germany) in dimethyl sulfoxide and water at a concentration of 40 mg/ml. From this solution, 10 μl (containing 400 μg of PBA) was dispensed onto commercially available antibiotic disks. The stock solutions of APBA compounds at 300 μg and 600 μg were prepared by dissolving APBA (Sigma-Aldrich) in water at concentrations of 30 mg/ml and 60 mg/ml, respectively. From these solutions, 10 μl (containing 300 μg and 600 μg of PBA, respectively) was dispensed onto commercially available antibiotic disks.

The combined-disk tests were performed by inoculating two Mueller-Hinton agar plates with a sample of the tested strain. The inoculum was adjusted to a 0.5 McFarland turbidity standard (7) and was placed in one plate with disks of imipenem (10 μg; Becton Dickinson, Sparks, MD) without and with 300 μg of APBA, 600 μg of APBA, and 400 μg of PBA and in the other plate with disks of meropenem (10 μg; Becton Dickinson) without and with 300 μg of APBA, 600 μg of APBA, and 400 μg of PBA. The diameter of the growth-inhibitory zone around a carbapenem disk with boronic acid was compared with that around the carbapenem disk without boronic acid. The test was considered positive for the detection of KPC enzyme production when the growth-inhibitory zone diameter around a carbapenem disk with boronic acid was 5 mm or greater of the growth-inhibitory zone diameter around the disk containing the carbapenem alone. Interpretation of all tests was performed by two microbiologists after incubation of agar plates at 37°C for 18 h.

Sensitivity, specificity, and statistical analysis. The performance of the various combined-disk tests for the detection of KPC-possessing *Enterobacteriaceae* was evaluated by using PCR as the "gold standard." For each test, the sensitivity was calculated from the number of KPC-possessing organisms that were correctly determined, while the specificity was calculated from the number of non-KPC-possessing organisms that were correctly determined. Positive and negative predictive values were also calculated.

All available data were entered into a database using SPSS statistical software (SPSS, version 15.0, for Windows; SPSS Inc., Chicago, IL). Numerical data were analyzed using the one-way analysis of variance (ANOVA) in order to compare the mean values. A probability equal to or less than 5% was considered statistically significant.

RESULTS

Antimicrobial susceptibilities. Carbapenem MICs varied substantially among the 112 KPC-positive isolates and ranged from 2 to 64 μg/ml, 1 to 64 μg/ml, and 4 to >128 μg/ml for imipenem, meropenem, and ertapenem, respectively (Table 2). All KPC-positive isolates exhibited resistance to penicillins (ampicillin and piperacillin), β-lactam–β-lactamase–inhibitor

TABLE 2. Distribution of carbapenem MICs for the carbapenemase-positive and carbapenemase-negative clinical isolates of the study

PCR-confirmed strain group/antimicrobial	No. of isolates with an MIC (mg/liter) of:										
	<0.5	0.5	1	2	4	8	16	32	64	128	>128
KPC and KPC/ESBL positive (n = 112)											
Imipenem				6	17	25	31	23	10		
Meropenem			2	9	21	33	23	18	6		
Ertapenem					8	16	27	38	17	4	2
VIM and VIM/ESBL positive (n = 58)											
Imipenem				2	4	13	16	17	5	1	
Meropenem				4	6	15	18	13	2		
Ertapenem					3	7	12	21	11	3	1
AmpC and AmpC/ESBL positive (n = 47)											
Imipenem	8	13	11	7	5	3					
Meropenem	16	12	9	6	4						
Ertapenem		14	10	7	5	4	4	3			
ESBL positive (n = 22)											
Imipenem	6	11	4	1							
Meropenem	10	8	4								
Ertapenem		8	9	3	2						

combinations (amoxicillin-clavulanate, piperacillin-tazobactam), cefoxitin, expanded-spectrum cephalosporins (ceftazidime, cefotaxime, cefepime), and aztreonam. Additionally, 101 KPC producers showed resistance to ciprofloxacin, 92 to trimethoprim, 85 to amikacin, 23 to colistin, 7 to gentamicin, and 5 to tigecycline.

Among the 127 KPC-negative isolates, 74 were nonsusceptible to imipenem and/or meropenem (MIC > 1 μg/ml) (Table 2). Additionally, as many as 108, 125, and 127 were nonsusceptible to cefoxitin (MIC > 8 μg/ml), ceftazidime (MIC > 4 μg/ml), and cefotaxime (MIC > 1 μg/ml), respectively.

Phenotypic and molecular screening. PCR and sequencing results showed that all 112 KPC-positive isolates contained the KPC-2 variant, except 8 *K. pneumoniae* isolates that contained the KPC-3 variant. Phenotypic testing for ESBL production was in accordance with molecular testing and revealed that 73 of the 112 KPC-bearing isolates were additionally ESBL producers, while the remaining 39 isolates were negative for ESBL production. In 66 cases an SHV-type ESBL was detected, and in 7 cases a CTX-M type ESBL was detected (Table 1). PCR testing for other groups of ESBL genes (TEM, GES/IBC), as well as for plasmid-mediated AmpC genes, was consistently negative. Quantitative real-time PCR showed that compared to control *Enterobacter* sp. strains containing inducible chromosomal AmpCs, all 5 *Enterobacter* sp. KPC-positive isolates contained stably derepressed AmpCs. It is also of note that 85 of the 112 KPC producers harbored the TEM-1 β-lactamase.

Molecular testing in combination with phenotypic testing of the 127 non-KPC-possessing isolates showed that 58 harbored VIM-type MBLs, 82 harbored ESBLs (45 harbored SHV types, 35 harbored CTX-M types, and 2 harbored SHV and CTX-M types), and 47 harbored AmpC β-lactamases which belonged in two of the six plasmid-mediated AmpC clusters (28 belonged to the cluster MOX-1, MOX-2, CMY-1, and CMY-8 to CMY-11, and 19 belonged to the cluster LAT-1 to LAT-4, CMY-2 to CMY-7, and BIL-1) (Table 1). In addition, quantitative real-time PCR showed that compared to control *Enterobacter* sp. strains containing inducible chromosomal AmpCs, all *E. aerogenes* and *E. cloacae* non-KPC-possessing isolates produced high levels of AmpCs, which were therefore specified to be stably derepressed AmpCs.

Boronic acid combined-disk tests. Detailed results of the boronic acid disk tests regarding the increases in the inhibition zone diameters of imipenem and meropenem (in mm) in the presence of 300 μg APBA, 600 μg APBA, and 400 μg PBA for KPC-positive and KPC-negative isolates are presented in Tables 3 and 4, respectively, while their sensitivities, specificities, positive predictive values, and negative predictive values are shown in Table 5. Using disks containing imipenem without and with 300 μg APBA, 600 μg APBA, or 400 μg PBA, as many as 72, 92, and 112 of the KPC producers, respectively, gave positive results (sensitivities, 64.3%, 82.1%, and 100%, respectively). PBA enhanced remarkably (mean increase, 9 mm) the activity of imipenem in all 112 KPC producers, irrespective of the carbapenem MICs, clearly indicating the inhibitory activity of PBA against KPC enzymes. Considerably lower mean increases in the inhibition zones were obtained using as inhibitor 300 μg or 600 μg of APBA (5 and 6 mm, respectively), resulting in several false-negative results (Table 3).

Using disks containing meropenem without and with 300 μg APBA, 600 μg APBA, or 400 μg PBA, as many as 87, 108, and 112 of the KPC producers, respectively, gave positive results (sensitivities, 77.7%, 96.4%, and 100%, respectively). Although few false-positive results were obtained using meropenem disks and 600 μg APBA as inhibitor, the mean increase in the inhibition zone differed considerably between 400 μg of PBA (mean increase, 10 mm) and 600 μg of APBA (mean increase, 8 mm) (Table 4). Eventually, the comparative study showed that the meropenem duplicate disk with and without 400 μg PBA exhibits the largest differences in inhibition zone diameters among KPC producers (Tables 3 and 4).

For KPC producers, the mean diameter increase in the inhibition zone of imipenem as well as meropenem among the three different combined-disk tests (using as inhibitor 300 μg

TABLE 3. Detailed results of the three combined-disk tests employing imipenem with 300 μg APBA, 600 μg APBA, or 400 μg PBA against the 112 KPC-positive and 127 non-KPC-producing isolates

β-Lactamase	Imipenem + 300 μg APBA (≥5 mm)				Imipenem + 600 μg APBA (≥5 mm)				Imipenem + 400 μg PBA (≥5 mm)			
	Increase in inhibition zone diam (mm)[a]		No. of isolates		Increase in inhibition zone diam (mm)		No. of isolates		Increase in inhibition zone diam (mm)		No. of isolates	
	Range	Mean increase	Positive	Negative	Range	Mean increase	Positive	Negative	Range	Mean increase	Positive	Negative
KPC (n = 112)	2 to 8	5	72	40	3 to 9	6	92	20	5 to 12	9	112	0
VIM (n = 58)	0 to 2	0	0	58	0 to 3	0	0	58	0 to 3	1	0	58
AmpC (n = 47)	−2 to 5	1	3	44	−2 to 6	0	3	44	0 to 7	2	3	44
ESBL (n = 22)	−3 to 1	−1	0	22	−2 to 1	−1	0	22	0 to 3	2	0	22
Total non-KPC (n = 127)	−3 to 5	0	3	124	−2 to 6	0	3	124	0 to 7	2	3	124

[a] Increase in inhibition zone diameter around imipenem disks containing β-lactamase inhibitors.

APBA, 600 μg APBA, or 400 μg PBA) was shown to be significantly different (ANOVA test; $P < 0.001$ both for imipenem and meropenem). Also, the impact of 400 μg PBA on the increase of the inhibition zone was greater than that of the other two inhibitors ($P < 0.001$ for both imipenem and meropenem). Furthermore, the use of 600 μg APBA as an inhibitor resulted in a greater increase in the inhibition zone than that given by 300 μg APBA ($P < 0.001$ for both imipenem and meropenem).

All combined-disk tests employing imipenem or meropenem correctly identified 124 of the 127 non-KPC producers (specificity, 97.6%; Tables 3 and 4). Three stably derepressed *E. aerogenes* isolates harboring SHV-type ESBLs gave false-positive results with all boronic acid combined-disk tests. The MHT has been applied in these three *E. aerogenes* isolates and gave results difficult to interpret. Using MHT and meropenem as the substrate, one of these isolates was considered weakly positive and the remaining two isolates were considered negative, while using MHT and ertapenem as the substrate, two *E. aerogenes* isolates were considered weakly positive and the remaining one was considered negative.

Finally, the two reference strains carrying IMP-type and OXA-48 carbapenemases showed clearly negative results for KPC production, with all substrate-boronic acid derivative combinations having increases in the inhibition zone ranging from 0 to 2 mm.

DISCUSSION

Carbapenem-nonsusceptible KPC-producing *Enterobacteriaceae* strains are rapidly disseminating in several countries and over large geographic areas (9, 19, 23). However, *Enterobacteriaceae* that produce carbapenemases of MBL types are still widespread worldwide (4), while broad-spectrum β-lactamases, such as ESBLs and plasmidic AmpCs, may also contribute to reduced levels of susceptibility to carbapenems (13, 21, 38). Thus, in regions where carbapenem resistance might also be caused by other resistance mechanisms, competent and easily interpreted tests are needed to differentiate KPC producers (32). Indeed, the application of an accurate and simple phenotypic assay is the crucial first step to implement infection control measures and ensure optimal therapeutic schemes in regions where KPC enzymes are endemic (17).

Current studies have proposed that KPC-producing *Enterobacteriaceae* can be detected by implementing an imipenem disk-diffusion test or testing carbapenem MICs according to the 2010 CLSI updated criteria (3, 10). However, these methods obviously lack specificity in regions where other carbap-

TABLE 4. Detailed results of the three combined-disk tests employing meropenem with 300 μg APBA, 600 μg APBA, and 400 μg PBA against the 112 KPC-positive and 127 non-KPC-producing isolates

β-Lactamase	Meropenem + 300 μg APBA (≥5 mm)				Meropenem + 600 μg APBA (≥5 mm)				Meropenem + 400 μg PBA (≥5 mm)			
	Increase in inhibition zone diam (mm)[a]		No. of isolates		Increase in inhibition zone diam (mm)		No. of isolates		Increase in inhibition zone diam (mm)		No. of isolates	
	Range	Mean increase	Positive	Negative	Range	Mean increase	Positive	Negative	Range	Mean increase	Positive	Negative
KPC (n = 112)	3 to 10	6	87	25	4 to 12	8	108	4	6 to 14	10	112	0
VIM (n = 58)	0 to 2	0	0	58	0 to 3	1	0	58	0 to 3	1	0	58
AmpC (n = 47)	−1 to 6	1	3	44	0 to 6	2	3	44	0 to 7	2	3	44
ESBL (n = 22)	−1 to 3	1	0	22	−1 to 3	1	0	22	0 to 3	2	0	22
Total non-KPC (n = 127)	−1 to 6	1	3	124	−1 to 6	1	3	124	0 to 7	2	3	124

[a] Increase in inhibition zone diameter around imipenem disks containing β-lactamase inhibitors.

TABLE 5. Summary of sensitivities, specificities, positive predictive values, and negative predictive values of the combined-disk tests using different boronic acid compounds and different antibiotic substrates in the phenotypic detection of KPC-producing isolates

Antibiotic substrate and boronic acid	Sensitivity (%)	Specificity (%)	PPV[a] (%)	NPV[b] (%)
Imipenem				
APBA, 300 μg	64.3	97.6	96.0	75.6
APBA, 600 μg	82.1	97.6	96.8	86.1
PBA, 400 μg	100	97.6	97.4	100
Meropenem				
APBA, 300 μg	77.7	97.6	96.7	83.2
APBA, 600 μg	96.4	97.6	97.3	96.9
PBA, 400 μg	100	97.6	97.4	100

[a] PPV, positive predictive value.
[b] NPV, negative predictive value.

enem resistance mechanisms may also exist. MHT is also a phenotypic method that has been recommended for the detection of KPC-producing organisms (7). While the latter assay has been found to be useful for KPC detection (1), its interpretation is often difficult and subjective (3, 10, 16) and does not distinguish between carbapenemase types (32). There is also increasing evidence that the MHT may give false-positive results among CTX-M ESBL-positive or AmpC-hyperproducing *Enterobacteriaceae* (14, 25).

Recently, there has been increasing interest in the use of boronic acid compounds as KPC inhibitors for the phenotypic detection of KPCs. Disk tests based on the inhibitory activity of boronic acid have originally been described for the detection of plasmid-mediated AmpC enzymes using as substrates various cephamycins and oxyimino-cephalosporins (8, 11). The initial testing of boronic acid compounds as inhibitors of KPC enzymes has revealed that KPC production is associated with positive combined-disk tests using boronic acid and cefepime and carbapenems as substrates (33). A further evaluation with a large number of KPC-producing isolates revealed that combined-disk tests using 400 μg of PBA with and without cefepime, imipenem, or meropenem are highly sensitive and specific for the detection of KPC enzymes (34). Combined carbapenem disk tests using different APBA concentrations have also been employed for the detection of KPC production. In a collection of KPC-producing *K. pneumoniae* isolates, various APBA concentrations were tested, and promising results were found using as inhibitor 300 μg of APBA and disks of ertapenem or meropenem (12). However, in this report the rate of KPC detection was low when APBA was combined with imipenem. Another study used the same concentration of APBA and concluded that the required augmentation of the inhibition zone to detect production of class A carbapenemase should be equal to or greater than 4 mm (24). This study has shown that when imipenem was used as substrate, the highest sensitivity and specificity for KPC detection were obtained. Furthermore, combined-disk tests using meropenem and 600 μg of APBA were successfully employed for the detection of KPCs (16). The latter study has shown some false-positive results among AmpC hyperproducers when the required augmentation of the inhibition zone of KPC detection was equal to or greater than 4 mm.

The discrepancies in the literature regarding the substrates, the boronic acid compounds, and the required augmentation of the inhibition zones prompted the design of the present study. A large collection of KPC-producing and non-KPC-producing *Enterobacteriaceae* isolates with reduced susceptibility to carbapenems was used to compare combined-disk tests based on different carbapenems and different boronic acid compounds and concentrations. Ertapenem was not used as substrate in the present evaluation, since ertapenem was previously found to exhibit some false-positive results among AmpC-producing isolates which were not observed with imipenem or meropenem (12, 34).

The current study has shown that meropenem is a more sensitive substrate than imipenem for the detection of KPCs, irrespective of the boronic acid compound or concentration. Similar findings have been observed in some of the previous phenotypic studies employing as KPC inhibitor PBA or APBA (12, 34). The present evaluation has also shown that 600 μg of APBA may identify more KPC producers than 300 μg of APBA without compromising the specificity of the method. Moreover, it was found that the combined-disk tests using PBA exhibit the greatest sensitivities for KPC detection, while their specificities are equal to those of potentiation tests using APBA at various concentrations.

It should be noted that using 400 μg PBA, the combined-disk tests accurately detected KPC producers even in cases of strains that exhibited very low carbapenem MICs (imipenem and/or meropenem MIC range, 1 to 2 μg/ml). Moreover, the PBA potentiation tests were able to identify all KPC-2- and KPC-3-possessing *Enterobacteriaceae* and successfully detected KPCs in *Enterobacter* sp. isolates with derepressed AmpCs that coproduced ESBLs. It is also noteworthy that the use of PBA was associated not only with higher sensitivities but also with very significantly higher mean values in the increase of inhibition zones, allowing the easy interpretation of KPC-positive samples. It should also be pointed out that in the present evaluation, surprisingly, in some AmpC and/or ESBL producers, the inhibition zones around the disks containing carbapenem with 300 or 600 μg of APBA were lower than those containing only carbapenem. Similar observations have previously been noticed in phenotypic studies using APBA for KPC detection (16).

In the microbiological laboratory, it is vital to promptly report any isolate with carbapenemase activity to the hospital infection control team to allow the implementation of appropriate infection control measures (17). In that respect, the combined-disk test using meropenem and PBA is a very convenient procedure that may be effectively used for the detection of KPCs. Further studies will be needed to establish the efficiency of boronic acid-based tests in detecting KPC enzymes among nonfermenting Gram-negative bacteria. Efforts also have to be applied to the identification of boronic acid compounds that more specifically inhibit the acitivity of KPCs. The mapping of the KPC binding site may suggest alterations to enhance the affinity of boronic acid derivatives for KPCs.

REFERENCES

1. **Anderson, K. F., et al.** 2007. Evaluation of methods to identify the *Klebsiella pneumoniae* carbapenemase in *Enterobacteriaceae*. J. Clin. Microbiol. **45:** 2723–2725.
2. **Barnaud, G., et al.** 2001. Extension of resistance to cefepime and cefpirome associated to a six amino acid deletion in the H-10 helix of the cephalosporinase of an *Enterobacter cloacae* clinical isolate. FEMS Microbiol. Lett. **195:**185–190.
3. **Benenson, S., et al.** 2011. Imipenem disc for detection of KPC carbapenemase-producing *Enterobacteriaceae* in clinical practice. J. Clin. Microbiol. **49:**1617–1620.
4. **Bilavsky, E., M. J. Schwaber, and Y. Carmeli.** 2010. How to stem the tide of carbapenemase-producing Enterobacteriaceae?: proactive versus reactive strategies. Curr. Opin. Infect. Dis. **23:**327–331.
5. **Bratu, S., et al.** 2005. Rapid spread of carbapenem-resistant *Klebsiella pneumoniae* in New York City: a new threat to our antibiotic armamentarium. Arch. Intern. Med. **165:**1430–1435.
6. **Cai, J. C., H. W. Zhou, R. Zhang, and G. X. Chen.** 2008. Emergence of *Serratia marcescens*, *Klebsiella pneumoniae*, and *Escherichia coli* isolates possessing the plasmid-mediated carbapenem-hydrolyzing β-lactamase KPC-2 in intensive care units of a Chinese hospital. Antimicrob. Agents Chemother. **52:**2014–2018.
7. **Clinical and Laboratory Standards Institute.** 2010. Performance standards for antimicrobial susceptibility testing. Twentieth informational supplement update. Document M100-S20 U. Clinical and Laboratory Standards Institute, Wayne, PA.
8. **Coudron, P. E.** 2005. Inhibitor-based methods for detection of plasmid-mediated AmpC β-lactamases in *Klebsiella* spp., *Escherichia coli*, and *Proteus mirabilis*. J. Clin. Microbiol. **43:**4163–4167.
9. **Cuzon, G., et al.** 2010. Worldwide diversity of *Klebsiella pneumoniae* that produce β-lactamase bla_{KPC-2} gene. Emerg. Infect. Dis. **16:**1349–1356.
10. **Doern, C. D., W. M. Dunne, Jr., and C. A. Burnham.** 2011. Detection of *Klebsiella pneumoniae* carbapenemase (KPC) production in non-*Klebsiella pneumoniae Enterobacteriaceae* isolates by use of the Phoenix, Vitek 2, and disk diffusion methods. J. Clin. Microbiol. **49:**1143–1147.
11. **Doi, Y., and D. L. Paterson.** 2007. Detection of plasmid-mediated class C β-lactamases. Int. J. Infect. Dis. **11:**191–197.
12. **Doi, Y., et al.** 2008. Simple disk-based method for detection of *Klebsiella pneumoniae* carbapenemase-type β-lactamase by use of a boronic acid compound. J. Clin. Microbiol. **46:**4083–4086.
13. **Doumith, M., M. J. Ellington, D. M. Livermore, and N. Woodford.** 2009. Molecular mechanisms disrupting porin expression in ertapenem-resistant *Klebsiella* and *Enterobacter* spp. clinical isolates from the UK. J. Antimicrob. Chemother. **63:**659–667.
14. **Endimiani, A., et al.** 2010. Evaluation of updated interpretative criteria for categorizing *Klebsiella pneumoniae* with reduced carbapenem susceptibility. J. Clin. Microbiol. **48:**4417–4425.
15. **Franklin, C., L. Liolios, and A. Y. Peleg.** 2006. Phenotypic detection of carbapenem-susceptible metallo-β-lactamase-producing gram-negative bacilli in the clinical laboratory. J. Clin. Microbiol. **44:**3139–3144.
16. **Giske, C. G., et al.** 2011. A sensitive and specific phenotypic assay for detection of metallo-β-lactamases and KPC in *Klebsiella pneumoniae* with the use of meropenem disks supplemented with aminophenylboronic acid, dipicolinic acid and cloxacillin. Clin. Microbiol. Infect. **17:**552–556.
17. **Grundmann, H., et al.** 2010. Carbapenem-non-susceptible *Enterobacteriaceae* in Europe: conclusions from a meeting of national experts. Euro Surveill. **15**(46)**:**pii=19711.
18. **Ikonomidis, A., et al.** 2005. Outbreaks in distinct regions due to a single *Klebsiella pneumoniae* clone carrying a bla_{VIM-1} metallo-β-lactamase gene. J. Clin. Microbiol. **43:**5344–5347.
19. **Kitchel, B., et al.** 2009. Molecular epidemiology of KPC-producing *Klebsiella pneumoniae* in the United States: clonal expansion of MLST sequence type 258. Antimicrob. Agents Chemother. **53:**3365–3370.
20. **Leavitt, A., S. Navon-Venezia, I. Chmelnitsky, M. J. Schwaber, and Y. Carmeli.** 2007. Emergence of KPC-2 and KPC-3 in carbapenem-resistant *Klebsiella pneumoniae* strains in an Israeli hospital. Antimicrob. Agents Chemother. **51:**3026–3029.
21. **Leavitt, A., et al.** 2009. Ertapenem resistance among extended-spectrum-β-lactamase-producing *Klebsiella pneumoniae* isolates. J. Clin. Microbiol. **47:** 969–974.
22. **Moland, E. S., et al.** 2003. Plasmid-mediated, carbapenem-hydrolysing beta-lactamase, KPC-2, in *Klebsiella pneumoniae* isolates. J. Antimicrob. Chemother. **51:**711–714.
23. **Nordmann, P., G. Cuzon, and T. Naas.** 2009. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. Lancet Infect. Dis. **9:**228–236.
24. **Pasteran, F., T. Mendez, L. Guerriero, M. Rapoport, and A. Corso.** 2009. Sensitive screening tests for suspected class A carbapenemase production in species of *Enterobacteriaceae*. J. Clin. Microbiol. **47:**1631–1639.
25. **Pasteran, F., T. Mendez, M. Rapoport, L. Guerriero, and A. Corso.** 2010. 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. J. Clin. Microbiol. **48:**1323–1332.
26. **Pérez-Pérez, F. J., and N. D. Hanson.** 2002. Detection of plasmid-mediated AmpC β-lactamase genes in clinical isolates by using multiplex PCR. J. Clin. Microbiol. **40:**2153–2162.
27. **Poirel, L., C. Héritier, V. Tolün, and P. Nordmann.** 2004. Emergence of oxacillinase-mediated resistance to imipenem in *Klebsiella pneumoniae*. Antimicrob. Agents Chemother. **48:**15–22.
28. **Pournaras, S., et al.** 2009. Clonal spread of KPC-2 carbapenemase-producing *Klebsiella pneumoniae* strains in Greece. J. Antimicrob. Chemother. **64:**348–352.
29. **Preston, K. E., C. C. Radomski, and R. A. Venezia.** 2000. Nucleotide sequence of the chromosomal ampC gene of *Enterobacter aerogenes*. Antimicrob. Agents Chemother. **44:**3158–3162.
30. **Samuelsen, Ø., et al.** 2009. Emergence of clonally related *Klebsiella pneumoniae* isolates of sequence type 258 producing plasmid-mediated KPC carbapenemase in Norway and Sweden. J. Antimicrob. Chemother. **63:**654–658.
31. **Seki, L. M., et al.** 2011. Molecular epidemiology of KPC-2-producing *Klebsiella pneumoniae* isolates in Brazil: the predominance of sequence type 437. Diagn. Microbiol. Infect. Dis. **70:**274–277.
32. **Thomson, K. S.** 2010. Extended-spectrum-β-lactamase, AmpC, and carbapenemase issues. J. Clin. Microbiol. **48:**1019–1025.
33. **Tsakris, A., et al.** 2008. First occurrence of KPC-2-possessing *Klebsiella pneumoniae* in a Greek hospital and recommendation for detection with boronic acid disc tests. J. Antimicrob. Chemother. **62:**1257–1260.
34. **Tsakris, A., et al.** 2009. Evaluation of boronic acid disk tests for differentiating KPC-possessing *Klebsiella pneumoniae* isolates in the clinical laboratory. J. Clin. Microbiol. **47:**362–367.
35. **Tsakris, A., et al.** 2009. Use of boronic acid disk tests to detect extended-spectrum β-lactamases in clinical isolates of KPC carbapenemase-possessing *Enterobacteriaceae*. J. Clin. Microbiol. **47:**3420–3426.
36. **Tsakris, A., et al.** 2010. In vivo acquisition of a plasmid-mediated bla_{KPC-2} gene among clonal isolates of *Serratia marcescens*. J. Clin. Microbiol. **48:** 2546–2549.
37. **Tzelepi, E., et al.** 2003. Extended-spectrum β-lactamase types in *Klebsiella pneumoniae* and *Escherichia coli* in two Greek hospitals. Int. J. Antimicrob. Agents **21:**285–288.
38. **Yang, Q., et al.** 2010. Phenotypic and genotypic characterization of *Enterobacteriaceae* with decreased susceptibility to carbapenems: results from large hospital-based surveillance studies in China. Antimicrob. Agents Chemother. **54:**573–577.