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Evaluation of Boronic Acid Disk Tests for Differentiating KPC-Possessing *Klebsiella pneumoniae* Isolates in the Clinical Laboratory▽

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The worldwide increase in the occurrence and dissemination of KPC β-lactamases among gram-negative pathogens makes critical the early detection of these enzymes. Boronic acid disk tests using different antibiotic substrates were evaluated for detection of KPC-possessing *Klebsiella pneumoniae* isolates. A total of 57 genotypically confirmed KPC-possessing *K. pneumoniae* isolates with varying carbapenem MICs were examined. To measure the specificity of the tests, 106 non-KPC-possessing isolates (89 *K. pneumoniae* and 17 *Escherichia coli* isolates) were randomly selected among those exhibiting reduced susceptibility to cefoxitin, expanded-spectrum cephalosporins, or carbapenems. As many as 56, 53, and 40 of the non-KPC-possessing isolates harbored extended-spectrum β-lactamases, metallo-β-lactamases, and plasmid-mediated AmpC β-lactamases, respectively. By use of CLSI methodology and disks containing imipenem, meropenem, or cefepime, either alone or in combination with 400 μg of boronic acid, all 57 KPC producers gave positive results (sensitivity, 100%) whereas all 106 non-KPC producers were negative (specificity, 100%). The meropenem duplicate disk with or without boronic acid demonstrated the largest differences in inhibition zone diameters between KPC producers and non-KPC producers. By use of disks containing ertapenem, all isolates were correctly differentiated except for five AmpC producers that gave false-positive results (sensitivity, 100%; specificity, 95.3%). These practical and simple boronic acid disk tests promise to be very helpful for the accurate differentiation of KPC-possessing *K. pneumoniae* isolates, even in regions where different broad-spectrum β-lactamases are widespread.

Carbapenem resistance is one of the major threats to the antimicrobial treatment of infections with gram-negative organisms. In the past, it was distinctly unusual among *Klebsiella pneumoniae* isolates and was attributed mostly to porin loss in combination with an AmpC-type enzyme or extended-spectrum β-lactamase (ESBL) (5, 8). In recent years, however, carbapenem resistance has emerged among *K. pneumoniae* isolates in many geographical locations due to the acquisition of carbapenemases, which usually belong to Ambler class B metallo-β-lactamases (MBLs) or to Ambler class A KPC-type enzymes (25). MBLs have emerged among *K. pneumoniae* isolates in the Far East and southern Europe, while KPC-type enzymes spread first in the northeastern regions of United States and thereafter in several other regions (3, 18, 25).

Phenotypic detection of carbapenemase-producing *K. pneumoniae* isolates is critical for limiting the spread of the underlying resistance mechanisms (4, 18). In the clinical laboratory, MBL-producing *K. pneumoniae* isolates can be confidently predicted by using several double-disk synergy tests and a combined-disk test with imipenem and EDTA, which accurately identifies MBLs even among carbapenem-susceptible bacterial populations (10). However, detection of KPC-possessing *K. pneumoniae* still remains a challenging issue, and the strategies for laboratory identification of this resistance need to be reviewed and adjusted, because this mechanism is spreading further (1). In regions where KPC enzymes prevail among non-carbapenem-susceptible *K. pneumoniae* isolates, the accurate determination of levels of susceptibility to ertapenem and the modified Hodge test have been proposed to be sufficiently sensitive methods for the detection of these enzymes (1, 4). Nevertheless, assays that combine high sensitivity and high specificity in the detection of these enzymes do not exist. Tests with such characteristics are particularly needed in regions where MBLs or other resistance determinants are frequently detected among *K. pneumoniae* isolates with reduced susceptibility to carbapenems. In Greek hospitals, MBL-producing *K. pneumoniae* clinical isolates are endemic (12, 24), but recently the first KPC-possessing *K. pneumoniae* isolate has also been described (28). The latter isolate was phenotypically identified as a KPC producer by using boronic acid disk tests, and this prompted our laboratory to propose a sensitive but also highly specific phenotypic scheme based on the inhibitory effects of boronic acid. We report the evaluation of boronic acid tests using different antibiotic substrates for the phenotypic detection of KPC-possessing *K. pneumoniae* clinical isolates.

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MATERIALS AND METHODS

Clinical isolates. The boronic acid disk tests for the detection of KPC were evaluated with 57 genotypically confirmed KPC-possessing *K. pneumoniae* iso-

lates. The isolates were recovered from separate patients who were hospitalized in five 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). The presence of bla_{KPC} was determined using previously described oligonucleotide primers and cycling conditions (19). Reverse transcription-PCR using these primers and a previously described protocol (22) was performed to demonstrate the activity of the KPC enzyme. To measure the specificity of methods to detect KPC-mediated resistance, 89 *K. pneumoniae* isolates were chosen for testing. All 89 isolates were negative for bla_{KPC} by PCR. Non-KPC-possessing isolates were randomly selected from isolates that were considered to have reduced susceptibility to either cefoxitin (MIC, >8 μg/ml), expanded-spectrum cephalosporins (cefotaxime or ceftazidime; MIC, >8 μg/ml), or carbapenems (imipenem or meropenem; MIC, >4 μg/ml). In addition, 17 KPC-negative *Escherichia coli* isolates with the same characteristics were included. These 106 isolates possessed a variety of plasmid-mediated AmpC types, ESBLs, and MBLs and came from collections held at the clinical laboratories providing the KPC-positive isolates for the present study. The identification of all isolates was confirmed by using the API 20E system (bioMerieux, Marcy l'Etoile, France).

Antimicrobial susceptibility testing and phenotypic screening. Detailed susceptibility analysis was carried out by the agar dilution method according to CLSI guidelines and interpretative criteria (6). For tigecycline, the U.S. Food and Drug Administration recommendation was used (susceptibility, MIC of ≤2 μg/ml; resistance, MIC of ≥8 μg/ml), and for colistin, the CLSI recommendation for *Acinetobacter* spp. was used (susceptibility, MIC of ≤2 μg/ml; resistance, MIC of ≥4 μg/ml). Screening for the presence of a carbapenemase was performed with the modified Hodge test (16). The MBL Etest (AB Biodisk, Solna, Sweden) and the combined-disk test with imipenem and EDTA (10) 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 (26). Isolates were phenotypically tested for the presence of AmpC β-lactamase by determining cefoxitin and imipenem MICs in agar with and without 200 μg/ml cloxacillin (23) and by using the AmpC detection Etest strips (AB Biodisk), which contain cefotetan with or without cloxacillin.

Molecular testing for β-lactamase genes. β-Lactamase genes were amplified by using a panel of primers for the detection of all types of MBLs (12), KPCs (19), plasmid-mediated AmpC enzymes (with single PCRs for each gene) (21), and ESBLs, including SHV, TEM, CTX-M, and GES/IBC enzymes (29). The primers for KPC are located from nucleotide positions −39 to +68 of the total, 882-bp bla_{KPC} gene (GenBank accession no. EU176014) and produce a 989-bp amplicon. As positive controls we used previously characterized isolates from our collection carrying all types of tested β-lactamases. In some isolates, 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).

Boronic acid disk tests. The phenotypic detection of KPC-possessing *K. pneumoniae* isolates was evaluated with boronic acid disk tests. The stock solution was prepared as previously recommended (7) by dissolving phenylboronic acid (benzeneboronic acid; Sigma-Aldrich, Steinheim, Germany) in dimethyl sulfoxide at a concentration of 20 mg/ml. From this solution, 20 μl (containing 400 μg of boronic acid) was dispensed onto commercially available antibiotic disks. The disks were then dried and used within 60 min. The tests were performed by inoculating Mueller-Hinton agar by the standard diffusion method (6) and placing disks containing eight different β-lactams (imipenem, meropenem, ertapenem, cefepime, cefoxitin, cefotetan, cefotaxime, and ceftazidime) with or without boronic acid onto the agar. The agar plates were incubated at 37°C overnight. The diameter of the growth-inhibitory zone around a β-lactam disk with boronic acid was compared with that around the corresponding β-lactam disk without boronic acid. The test was considered positive for the detection of KPC enzyme production when the diameter of the growth-inhibitory zone around a β-lactam disk with boronic acid was ≥5 mm larger than that around a disk containing the β-lactam substrate alone. It should be noted that the concentration of boronic acid employed in the present study did not show any detectable effect on bacterial growth, since the boronic acid MIC exceeded 2,500 μg/ml.

Sensitivity and specificity. The performances of the various boronic acid disk tests for the detection of KPC enzymes were evaluated using PCR as the "gold standard." For each test, sensitivity was calculated from the number of KPC-possessing organisms that were correctly determined, while specificity was calculated from the number of non-KPC-possessing organisms that were correctly determined.

RESULTS

Clinical isolates. Of the 57 KPC-possessing *K. pneumoniae* isolates, 19 were from blood (33.3%), 18 from wound swabs (31.6%), 11 from urine (19.3%), 6 from respiratory tract specimens (10.5%), and 3 from vascular catheter tips (5.3%). Of the 106 non-KPC-possessing isolates, 32 were from blood (30.2%), 29 from urine (27.4%), 21 from respiratory tract specimens (19.8%), 19 from wound swabs (17.9%), 4 from vascular catheter tips (3.8%), and 1 from cerebrospinal fluid (0.9%).

Antimicrobial susceptibilities. Carbapenem MICs differed substantially among the 57 KPC-positive isolates, ranging from 4 to 64 μg/ml, 2 to 64 μg/ml, and 4 to >128 μg/ml for imipenem, meropenem, and ertapenem, respectively. Ten of the KPC producers were susceptible to meropenem (MICs, 2 to 4 μg/ml), susceptible or intermediate to imipenem (MICs, 4 to 8 μg/ml), and intermediate or resistant to ertapenem (MICs, 4 to 16 μg/ml), while the remaining 47 isolates were either intermediate or resistant to all three carbapenems (Table 1). All KPC-positive isolates exhibited resistance to penicillins (ampicillin and piperacillin), β-lactam–inhibitor combinations (amoxicillin-clavulanate, piperacillin-tazobactam), cefoxitin, expanded-spectrum cephalosporins (ceftazidime, cefotaxime, cefepime), and aztreonam. Additionally, all KPC producers showed resistance to ciprofloxacin, while 51 showed resistance to trimethoprim, 23 to amikacin, 6 to colistin, and 4 to gentamicin, but none were resistant to tigecycline (MICs, 0.5 to 2 μg/ml).

Among the 106 KPC-negative isolates, 61 were nonsusceptible (imipenem and meropenem MICs, >4 μg/ml; ertapenem MIC, >2 μg/ml) to at least one of the three carbapenems (Table 1). Additionally, as many as 93, 100, and 99 were nonsusceptible (MIC, >8 μg/ml) to cefoxitin, ceftazidime, and cefotaxime, respectively.

Phenotypic and molecular screening. All 57 KPC-positive isolates had positive results by the modified Hodge test, and reverse transcription-PCR experiments confirmed the expression of the KPC gene in all of them. Sequencing results for 10 randomly selected isolates identified the KPC-2 variant of the gene. Phenotypic testing for ESBL production in combination with molecular testing revealed that as many as 48 of the 57 KPC-bearing isolates were also ESBL producers. In all of these cases, an SHV-type ESBL was detected. Sequencing analysis was performed for all these amplicons; it detected SHV-11 in 2 cases and SHV-12 in the remaining 46 cases. PCR testing for other groups of ESBL genes (CTX-M, TEM, GES/IBC), as well as for MBL genes and all known clusters of plasmid-mediated AmpC genes, was consistently negative. In accordance with the latter results, all isolates gave negative results on the phenotypic tests for MBL detection and the cloxacillin inhibition tests, further indicating the absence of MBLs and plasmid-mediated AmpC enzymes. It is also noteworthy that 26 of the 57 KPC producers harbored the TEM-1 β-lactamase.

Molecular testing in combination with phenotypic testing of the 106 non-KPC-possessing isolates showed that 53 harbored VIM-type MBLs, 56 harbored ESBLs (33 harbored SHV-type ESBLs, 19 harbored CTX-M-type ESBLs, 2 harbored SHV- and TEM-type ESBLs, and 2 harbored GES/IBC-type ESBLs), and 40 harbored AmpC β-lactamases, which belonged to two

TABLE 1. Distribution of carbapenem MICs for the 57 KPC-possessing and the 106 non-KPC-possessing clinical isolates in this study

Characteristic of strain group (by PCR-confirmed β-lactamase content) and antimicrobial	No. of isolates with a MIC (μg/ml) of:									
	<1	1	2	4	8	16	32	64	128	>128
KPC and KPC/ESBL positive (n = 57)										
Imipenem				9	10	14	18	6		
Meropenem			2	8	11	13	15	8		
Ertapenem				1	10	13	17	11	3	2
VIM and VIM/ESBL positive (n = 53)										
Imipenem				3	8	14	19	7	2	
Meropenem			2	4	12	18	13	4		
Ertapenem				2	11	10	15	12	3	
AmpC and AmpC/ESBL positive (n = 40)										
Imipenem	22	11	2	3	2					
Meropenem	29	8	3							
Ertapenem	24	5	3	1	1	3	3			
ESBL positive (n = 13)										
Imipenem	9	4								
Meropenem	12	1								
Ertapenem	10	3								

of the six plasmid-mediated AmpC enzyme clusters (24 belonged to the cluster comprising MOX-1, MOX-2, CMY-1, and CMY-8 to CMY-11, and 16 belonged to the cluster comprising LAT-1 to LAT-4, CMY-2 to CMY-7, and BIL-1).

Boronic acid disk tests. Detailed results of the boronic acid disk tests are shown in Table 2, while their sensitivities, specificities, positive predictive values, and negative predictive values are shown in Table 3. All 57 KPC-possessing isolates showed increases of 5 mm or more in the zone diameters of the combined disks over those for cefotetan or cefepime alone (sensitivity, 100%). Boronic acid also enhanced remarkably (by ≥7 mm) the activities of all three carbapenems (imipenem, meropenem, and ertapenem) for all 57 KPC producers (sensitivity, 100%) irrespective of the carabapenem MICs, clearly indicating the inhibitory activity of boronic acid against KPC enzymes. Figure 1a and b show the growth patterns of a representative KPC-possessing *K. pneumoniae* isolate without or with boronic acid and with meropenem, ertapenem, cefotetan, and cefepime as antibiotic substrates. This method showed lower sensitivity when cefoxitin or cefotaxime was used as a substrate (96.5% or 66.7%, respectively). The inhibitor affected the activity of ceftazidime against the 9 ESBL-negative isolates as well as the 2 SHV-11 producers but not against the remaining 46 SHV-12 producers (sensitivity, 19.3%), most likely due to the presence of the SHV-12 ESBL, which is not restrained by boronic acid.

None of the 106 isolates negative for KPC by PCR gave positive results in boronic acid tests using disks of cefepime,

TABLE 2. Results of the boronic acid disk tests for isolates with different β-lactamase contents

Characteristic of strain group (by PCR-confirmed β-lactamase content)	No. (%) of isolates positive by the boronic acid test[a] with:							
	IPM	MER	ETP	FEP	CTT	FOX	CTX	CAZ
KPC positive (n = 9)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)	9 (100)
KPC/ESBL positive (n = 48)	48 (100)	48 (100)	48 (100)	48 (100)	48 (100)	46 (95.8)	29 (60.4)	2 (4.2)
Total KPC-possessing isolates (n = 57)	57 (100)	57 (100)	57 (100)	57 (100)	57 (100)	55 (96.5)	38 (66.7)	11 (19.3)
VIM positive (n = 41)	0 (0)	0 (0)	0 (0)	0 (0)	2 (4.9)	0 (0)	0 (0)	0 (0)
VIM/ESBL positive (n = 12)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
AmpC positive (n = 9)	0 (0)	0 (0)	0 (0)	0 (0)	9 (100)	8 (88.9)	8 (88.9)	7 (77.8)
AmpC/ESBL positive (n = 31)	0 (0)	0 (0)	5 (16.1)	0 (0)	31 (100)	24 (77.4)	11 (35.5)	6 (19.4)
ESBL positive (n = 13)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Total non-KPC-possessing isolates (n = 106)	0 (0)	0 (0)	5 (4.7)	0 (0)	42 (39.6)	32 (30.2)	19 (17.9)	13 (12.3)

[a] Abbreviations: IPM, imipenem; MER, meropenem; ETP, ertapenem, FEP, cefepime; CTT, cefotetan; FOX, cefoxitin; CTX, cefotaxime; CAZ, ceftazidime.

TABLE 3. Summary of sensitivities, specificities, positive predictive values, and negative predictive values of the boronic acid tests using different antibiotic substrates in the phenotypic detection of KPC-possessing isolates

Antibiotic used in boronic acid disk test	No. (%) of isolates confirmed by PCR as:		Test performance[a] (%)			
	Possessing KPC (n = 57)	Not possessing KPC (n = 106)	Sensitivity	Specificity	PPV	NPV
Imipenem	57 (100)	0 (0)	100	100	100	100
Meropenem	57 (100)	0 (0)	100	100	100	100
Ertapenem	57 (100)	5 (4.7)	100	95.3	91.9	100
Cefepime	57 (100)	0 (0)	100	100	100	100
Cefotetan	57 (100)	42 (39.6)	100	60.4	57.6	100
Cefoxitin	55 (96.5)	32 (30.2)	96.5	69.8	63.2	97.4
Cefotaxime	38 (66.7)	19 (17.9)	66.7	82.1	66.7	82.1
Ceftazidime	11 (19.3)	13 (12.3)	19.3	87.7	45.8	66.9

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

imipenem, or meropenem (specificity, 100%) (Tables 2 and 3). When disks containing ertapenem were used, all isolates negative for KPC by PCR were correctly identified as KPC negative except for five AmpC producers that gave false-positive results (specificity, 95.3%) (Tables 2 and 3). Boronic acid tests using cefotetan, cefoxitin, cefotaxime, and ceftazidime as antibiotic substrates were positive for 42, 32, 19, and 13 isolates, respectively (specificities for KPC detection ranged from 60.4% to 87.7%) (Table 3). Figure 1c to h show the growth patterns of three representative non-KPC-possessing *K. pneumoniae* isolates (harboring a VIM enzyme, plasmid-mediated AmpC, and ESBL, respectively) without or with boronic acid and with meropenem, ertapenem, cefotetan, and cefepime as antibiotic substrates. All these results were consistent for two different batches of Mueller-Hinton agar.

It is noteworthy that among the three antibiotic compounds (imipenem, meropenem, and cefepime) that correctly differentiated all 163 isolates in the study, meropenem demonstrated the largest differences in inhibition zone diameters between KPC producers and non-KPC producers (Fig. 2).

DISCUSSION

KPC enzymes have become increasingly prevalent among *K. pneumoniae* isolates on the East Coast of the United States since the beginning of the last decade. They have also caused outbreaks in Israel and recently have become emerging public health concerns in several regions worldwide (3, 15, 18, 25). Given the limited therapeutic options available, the accurate detection of KPC-possessing *K. pneumoniae* strains will be the crucial first step in controlling their spread and ensuring an optimal clinical outcome (3). Real-time PCR or multiplex PCR analyses may provide accurate results in the identification of KPC enzymes (11, 14). Molecular assays are increasingly utilized to detect antimicrobial resistance genes, but their accessibility is often limited to medical institutions, such as university hospitals. Thus, the current guidelines for phenotypic differentiation of KPC-possessing organisms in U.S. hospitals are based on an indirect indicator, reduced susceptibility to ertapenem or meropenem (1, 4, 19), as well as performance of the modified Hodge test (1). However, nonsusceptibility to

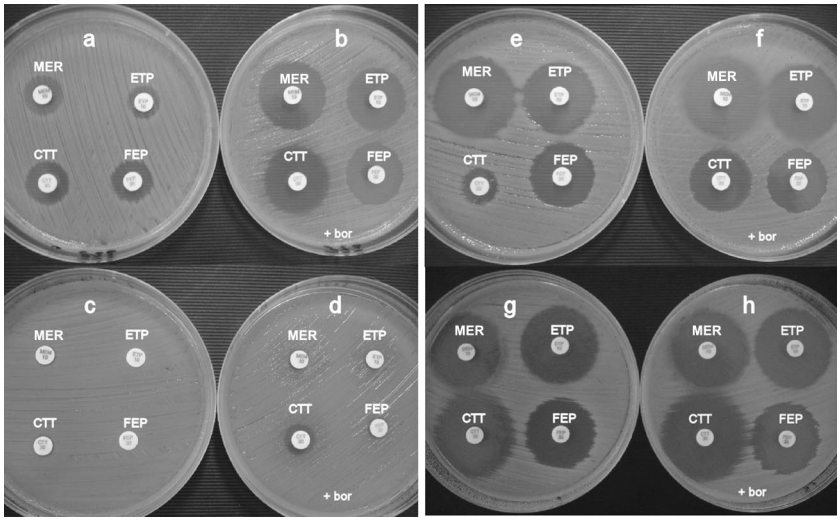

FIG. 1. Representative results using the boronic acid-based method without or with boronic acid (bor) for isolates possessing KPC/ESBL (a and b), VIM (c and d), AmpC/ESBL (e and f), or ESBL (g and h). MER, meropenem; ETP, ertapenem; CTT, cefotetan; FEP, cefepime.

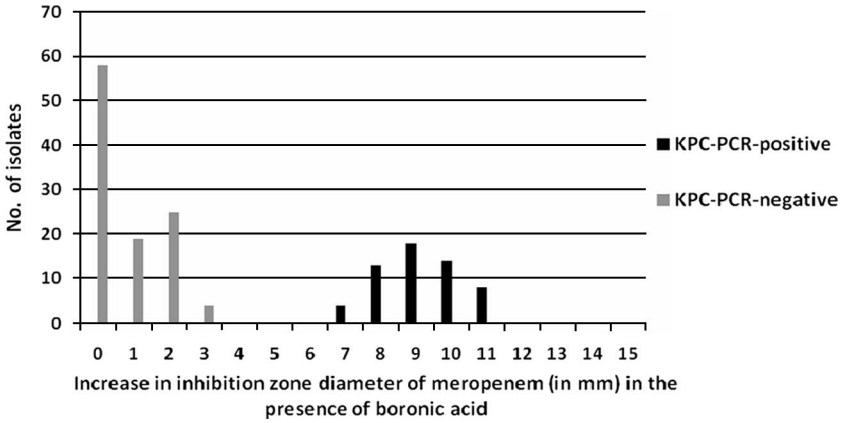

FIG. 2. Increases in the inhibition zone diameters of meropenem (in millimeters) in the presence of boronic acid for 57 isolates positive for KPC by PCR and 106 isolates negative for KPC by PCR.

ertapenem is not specific for carbapenemase production, especially when carbapenemase production is uncommon in a carbapenem-resistant bacterial environment, and obviously, a confirmation test is required. The modified Hodge test has been found to be an accurate assay for the phenotypic detection of KPC enzymes in hospitals where these enzymes predominate among non-carbapenem-susceptible *K. pneumoniae* isolates (1). This technique is less costly but may give positive results when any enzyme with carbapenemase activity is present. Thus, practical, highly sensitive, and highly specific methods for the detection of KPC-possessing isolates are needed for regions where other carbapenem-resistant determinants are also prevalent.

Boronic acid compounds are known class C enzyme inhibitors, which are not based on a β-lactam structure (2). Disk tests based on their inhibitory activities were originally reported for the detection of plasmid-mediated AmpC enzymes among enterobacterial pathogens (7, 9). These tests were found to considerably increase the growth-inhibitory zones around disks of cefotetan, allowing the accurate differentiation of plasmid-mediated AmpC-producing isolates (7). Subsequently, boronic acid tests using disks of cefoxitin, cefotaxime, and ceftazidime were found to be successful at the detection of AmpC enzymes (27, 30).

It has been shown previously that boronic acid compounds also have inhibitory activity against other serine β-lactamases, such as the class A β-lactamase I from *Bacillus cereus* (13). However, no bacteriological data have been reported for any of these compounds. In the present study, we evaluate for the first time the use of boronic acid disk tests for the phenotypic detection of class A KPC-possessing *K. pneumoniae* isolates in the clinical laboratory. The inhibitory activity of phenylboronic acid with several antibiotic substrates was tested against a large collection of clinical isolates, some positive and some negative for KPC by PCR. Our results are compatible with an enhanced affinity of the phenylboronic acid moiety with the active-site serine residue of class A KPC β-lactamase. Boronic acid tests using cefepime, imipenem, or meropenem as an antibiotic substrate demonstrated an excellent ability to differentiate KPC enzymes. The increase in the growth-inhibitory zone diameters was so great that it permitted easy recognition of KPC producers. Moreover, these tests allowed the accurate detection of KPC producers that exhibited low carbapenem MICs or were ESBL carriers. Boronic acid tests using disks of cefotetan or ertapenem were also highly sensitive in the detection of KPC producers. However, the cefotetan test, as expected, had low specificity for KPC detection, while the ertapenem test also gave false-positive results for a few AmpC-positive but KPC-negative isolates.

In our region, carbapenem resistance among *K. pneumoniae* clinical isolates is alarming and is attributed mostly to the production of VIM-type MBLs (12, 24). Also, plasmid-mediated AmpC-producing *K. pneumoniae* isolates are frequently detected and in several cases contribute to reduced susceptibility to carbapenems (unpublished data), which was observed among AmpC producers in the present study. In addition, carbapenem resistance due to a KPC-type carbapenemase was recently reported from a northern Greek hospital (28), and during the past year, KPC-possessing *K. pneumoniae* isolates have become established and have been increasingly detected in several tertiary-care Greek hospitals (unpublished data). Thus, in our region, where carbapenem resistance might also be caused by other resistance mechanisms, highly specific tests are needed to differentiate KPC producers. A recent study has shown that by use of benzo-thiophene-2-boronic acid as an inhibitor of AmpC enzymes, isolates with KPCs were wrongly inferred to have AmpC enzymes (17). Also, in two KPC-2-possessing clinical isolates from Argentina, synergism was detected between 3-aminophenylboronic acid disks and cefotaxime, ceftazidime, and carbapenem disks (20). It was assumed that the inhibitory activity of boronic acid was associated with the sole presence of the KPC enzyme, since no AmpC enzyme was detectable. In accordance with these observations, our study demonstrates that screening with boronic acid disks may accurately detect KPC producers among *K. pneumoniae* isolates that exhibit reduced susceptibility to either carbapenems, cefoxitin, or expanded-spectrum cephalosporins. According to the present results, boronic acid assays using cefepime or carbapenem disks are very simple, highly sensitive, and highly specific for the differentiation of KPC-possessing *K. pneumoniae* isolates and may be fully applicable for routine use in clinical microbiology laboratories. The tests

also seem to provide some useful information on the possible coproduction of an SHV-type ESBL, since the boronic acid test using ceftazidime was constantly negative for KPC producers that harbored SHV-12 ESBL. This test was positive for the two KPC producers that harbored SHV-11, most likely due to the weak activity of SHV-11 against expanded-spectrum cephalosporins.

Further studies will be needed to establish the reliability of boronic acid disk tests in detecting KPC enzymes among other pathogens, such as *E. coli*, *Enterobacter cloacae*, and *Proteus mirabilis*, as well as among pathogens possibly coproducing other broad-spectrum β-lactamases. It would also be of interest to test the specificity of boronic acid disk tests against *K. pneumoniae* isolates containing class D oxacillinases with considerable carbapenemase activity.

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