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# Simple Disk-Based Method for Detection of *Klebsiella pneumoniae* Carbapenemase-Type β-Lactamase by Use of a Boronic Acid Compound[▽]

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**A disk potentiation method using carbapenems as substrates and 3-aminophenyl boronic acid as an inhibitor was evaluated for the detection of *Klebsiella pneumoniae* carbapenemase (KPC)-type β-lactamases. When combined with nonsusceptibility to ertapenem, the method was easy to perform and reliably differentiated isolates producing KPC-type β-lactamases from those producing other types of β-lactamases.**

Since the initial report in 2001 (24), carbapenem resistance mediated by the production of *Klebsiella pneumoniae* carbapenemase (KPC)-type β-lactamases is increasingly identified in the United States and more recently worldwide (3, 22). Initially confined mostly to *K. pneumoniae*, these enzymes are now reported in various species of *Enterobacteriaceae* as well as *Pseudomonas aeruginosa* (7, 16, 21). Clinically, infections due to KPC-producing strains of *Enterobacter* spp. have been associated with increased mortality in affected patients (14). From the infection control standpoint, KPC-producing *K. pneumoniae* and *Escherichia coli* have caused outbreaks in acute-care hospitals and long-term-care facilities, respectively (4, 19, 22). However, laboratory detection of these organisms is difficult (5, 20). Several methods have been developed specifically for the detection of KPC-producing *Enterobacteriaceae*. A method that utilizes nonsusceptibility to ertapenem for screening followed by confirmation using the modified Hodge test has been proposed (1). In the modified Hodge test, a carbapenem disk is placed on a Mueller-Hinton agar plate inoculated with *E. coli* ATCC 25922. The test strain is then streaked radially from the edge of the disk to the periphery of the plate. After an overnight incubation, the presence of a distorted inhibition zone indicates the carbapenem-hydrolyzing activity of the test strain. This method is relatively easy to perform and feasible in clinical laboratories but requires some experience in interpreting the results. A method measuring the hydrolysis of carbapenems by cell extracts has been reported as well, though this method is technically demanding and requires specialized laboratory equipment (15).

Boronic acid compounds are known to be excellent inhibitors of class C β-lactamases (2, 13). One such compound, 3-aminophenyl boronic acid (APB), has recently been used successfully in detecting the production of plasmid-mediated class C β-lactamases in *Enterobacteriaceae* (6, 10, 12, 23). We have subsequently undertaken a study investigating the effects of APB on zone diameters of carbapenem-containing disks in a set of isolates producing KPC-type β-lactamases as well as other non-KPC broad-spectrum β-lactamases.

A total of 23 epidemiologically unrelated *K. pneumoniae* and *E. coli* clinical isolates (10 producing KPC-type β-lactamase, 3 ertapenem-resistant isolates without KPC-type β-lactamase, 5 producing extended-spectrum β-lactamase [ESBLs], and 5 producing plasmid-mediated class C β-lactamase) were included in the study. Specifically, the KPC-producing isolates originated from five hospitals in three states. We also included *E. coli* DH10B strains carrying recombinant plasmids that bear the genes for the metallo-β-lactamases (MBLs) IMP-1 and VIM-2. The β-lactamase types were determined by PCR analysis and nucleotide sequencing as appropriate. For the detection and sequencing of the KPC gene, primers KPC-1-F (5′-GGC TTG CCG CTC GGT GAT ATT-3′) and KPC-1-R (5′-TAT CTG TGA GGG CGA AGG TTA-3′) were used at an annealing temperature of 62°C. ESBL genes and plasmid-mediated class C β-lactamase genes were amplified as described previously (11, 18). The isolates were suspended in and diluted with normal saline to $10^8$ CFU/ml by comparison with a McFarland 0.5 turbidity standard and spread on a Mueller-Hinton agar plate (BD Microbiology Systems, Sparks, MD) as recommended by the Clinical and Laboratory Standards Institute (CLSI) (8). The following disks (BD Microbiology Systems) were tested: ertapenem (10 μg), ertapenem (10 μg) with APB (300 μg), imipenem (10 μg), imipenem (10 μg) with APB (300 μg), meropenem (10 μg), meropenem (10 μg) with APB (300 μg), ceftazidime (30 μg), and ceftazi-

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dime (30 μg) with APB (300 μg). APB (3-aminophenyl boronic acid hydrochloride; Sigma-Aldrich, St. Louis, MO) was dissolved in water at 50 mg/ml, and 6 μl was applied per disk. The amount of APB to be applied to the disks was determined based on the following observation: when the inhibitory effects of APB on representative KPC-positive and -negative isolates were examined at 100, 200, 300, 450, and 600 μg per disk, 300 μg was found to provide optimal sensitivity and specificity in detecting the presence of KPC-type β-lactamase when combined with ertapenem or meropenem, and a cutoff of a 5-mm difference in zone diameter was used. The zone diameters were read by at least two microbiologists. The modified Hodge test was performed to confirm the production of carbapenem-hydrolyzing β-lactamase as described previously (1).

The results are summarized in Table 1. The modified Hodge test was positive for all 10 KPC-producing clinical isolates and 2 MBL-producing laboratory strains, confirming the presence of significant carbapenem-hydrolyzing activity. None of the other isolates had positive results with the modified Hodge test. All 10 KPC-producing isolates were resistant to ertapenem, intermediate or resistant to meropenem, and variably resistant to imipenem. Ertapenem and meropenem were both sensitive substrates for potentiation by APB. When APB was added to ertapenem or meropenem disks, an increase in zone diameter of ≥5 mm was observed for all KPC-producing isolates (Table 1; Fig. 1). Potentiation of ≥5 mm was observed in 6 of the 10 isolates for imipenem. On the other hand, none of the three ertapenem-resistant isolates without KPC-type β-lactamase or the two MBL-producing strains produced a ≥5-mm increase in zone diameter with any of the carbapenems when combined with APB. Of note, all five isolates producing plasmid-mediated class C β-lactamase gave a ≥5-mm increase with ertapenem when combined with APB but not with imipenem or meropenem. However, they were all susceptible to ertapenem and were thus unlikely to be screened for the presence of KPC-type β-lactamase.

The mechanism for inhibition of KPC-type β-lactamase by APB is not known. A recent report from Argentina described the inhibition of the carbapenem-hydrolyzing activity of crude extracts from KPC-producing *K. pneumoniae* by APB (17). The authors cautioned that synergism between APB and KPC could lead to a false assumption of class C β-lactamase production. However, our results suggest that APB may have a role in the phenotypic confirmation of KPC production. Nonsusceptibility to ertapenem has been reported to be an excellent indicator of the presence of KPC-type β-lactamase with sensitivity exceeding 90% (1). Therefore, a two-tiered detection method, i.e., screening for ertapenem nonsusceptibility followed by confirmation with a disk potentiation test between ertapenem or meropenem and APB, appears to be a viable approach in identifying KPC-producing *K. pneumoniae* and *E. coli*. Our study is limited by the relatively small number of isolates assessed, especially ertapenem-resistant isolates without KPC-type β-lactamase. However, if replicated on large, diverse samples of KPC producers and nonproducers, this method holds promise for the busy clinical microbiology laboratory since the algorithm is analogous to the ESBL detection method using disk diffusion as defined by the CLSI (9), thus

TABLE 1. Zone diameters of the carbapenem disks tested against the study isolates

| Study strains | Zone diameter (mm)[a] | | | | | | | | | | Hodge test |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | ETP | ETP +APB | Δ | IPM | IPM + APB | Δ | MEM | MEM + APB | Δ | CAZ | CAZ +APB | Δ | |
| KPC positive (8 *K. pneumoniae*, 2 *E. coli*)[b] | 6–14 (9) | 12–22 (17) | 6–10 (8) | 12–24 (17) | 19–26 (22) | 1–7 (5) | 6–15 (9) | 12–21 (16) | 6–10 (8) | 6–14 (8) | 6–21 (10) | 0–9 (2) | All positive |
| ETP resistant, KPC-negative (2 *K. pneumoniae*, 1 *E. coli*)[c] | 8–13 (11) | 12–15 (14) | 2–4 (3) | 20–26 (24) | 20–26 (24) | 0–1 (0) | 12–20 (16) | 15–20 (18) | 0–3 (2) | 6–18 (11) | 6–19 (14) | 0–8 (3) | All negative |
| ESBL positive (2 *K. pneumoniae*, 3 *E. coli*)[d] | 20–33 (27) | 21–34 (28) | −3–4 (1) | 26–33 (29) | 26–32 (29) | −1–2 (0) | 24–32 (28) | 19–32 (25) | −9–0 (−3) | 6–17 (13) | 6–19 (14) | −1–3 (1) | All negative |
| Class C positive (1 *K. pneumoniae*, 4 *E. coli*)[e] | 20–26 (23) | 26–32 (29) | 4–9 (6) | 26–28 (27) | 27–28 (28) | 0–2 (1) | 25–31 (27) | 22–31 (25) | −3–0 (−2) | 9–13 (10) | 20–27 (22) | 11–14 (12) | All negative |
| MBL positive (2 *E. coli*)[f] | 19 (19) | 19 (19) | 0 (0) | 16–19 (18) | 15–20 (18) | −1–1 (0) | 19 (19) | 18–20 (19) | −1–1 (0) | 6 (6) | 6 (6) | 0 (0) | All positive |

[a] Values are given as ranges (means). ETP, ertapenem; IPM, imipenem; MEM, meropenem; CAZ, ceftazidime. The CLSI breakpoints for *Enterobacteriaceae* are as follows: for ertapenem, susceptible, ≤15 mm, intermediate, 16 to 18 mm, and resistant, ≥19 mm; for imipenem and meropenem, susceptible, ≤13 mm, intermediate, 14 to 15 mm, and resistant, ≥16 mm; and for ceftazidime, susceptible, ≤14 mm, intermediate, 15 to 17 mm, and resistant, ≥18 mm.

[b] Five KPC-2 and five KPC-3.

[c] One SHV and two unknown types.

[d] Two SHV and three CTX-M types.

[e] Four CMY types and one FOX type.

[f] One IMP-1 and one VIM-2 cloned and expressed in *E. coli* DH10B.

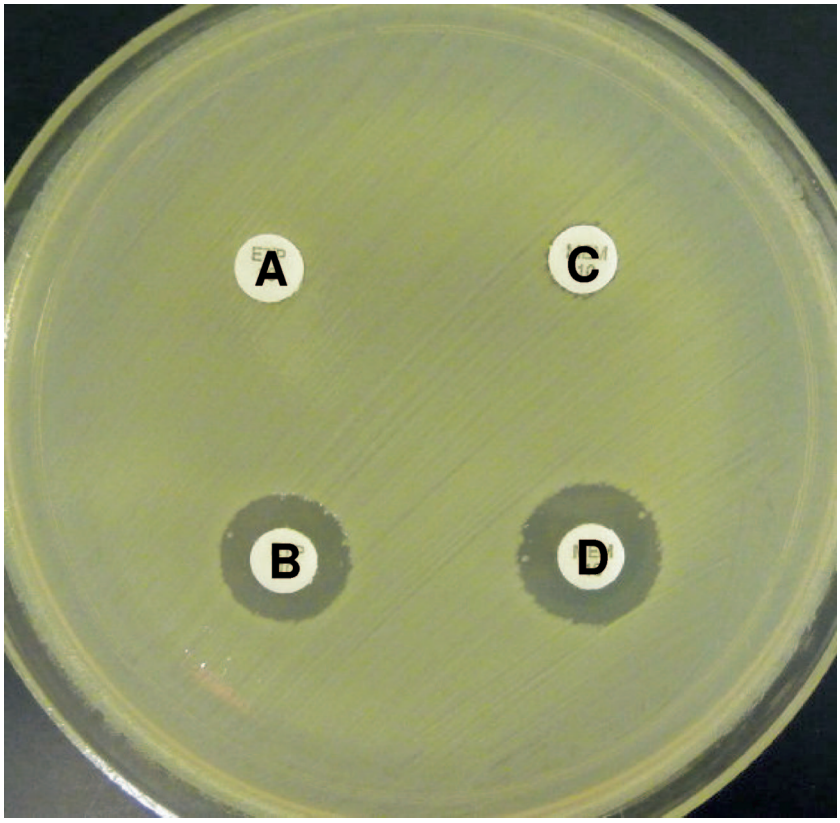


FIG. 1. Potentiation of carbapenems by APB in *K. pneumoniae* producing KPC-2. (A) Ertapenem (10 μg); (B) ertapenem plus APB (300 μg); (C) meropenem (10 μg); (D) meropenem plus APB (300 μg).

making it easy for the laboratory staff to incorporate it into daily practice.

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