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

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Abstract

Enterobacteriaceae producing carbapenemases, such as KPC or metallo-β-lactamases (MBLs), have emerged on several continents. Phenotypic tests are urgently needed for their rapid and accurate detection. A novel carbapenemase detection test, comprising a meropenem disk, and meropenem disks supplemented with 730 μg of EDTA, 1000 μg of dipicolinic acid (DPA), 600 μg of aminophenylboronic acid (APBA), or 750 μg of cloxacillin, was evaluated against *Klebsiella pneumoniae* isolates with KPC (*n* = 34), VIM (*n* = 21), IMP (*n* = 4) or OXA-48 (*n* = 9) carbapenemases, and carbapenem-resistant *Enterobacteriaceae* with porin loss in combination with an extended-spectrum β-lactamase (ESBL) (*n* = 9) or AmpC hyperproduction (*n* = 5). Commercially available diagnostics tablets from Rosco containing meropenem and the same inhibitors as described above (except EDTA) were also evaluated. An increased meropenem inhibition zone was sought in the presence of each added β-lactamase inhibitor. APBA had excellent sensitivity for detecting *K. pneumoniae* with KPC enzymes. Isolates with combined AmpC hyperproduction and porin loss were also positive in the APBA test but, unlike KPC producers, showed cloxacillin synergy. Both DPA and EDTA had excellent sensitivity for detection of MBL-producing *K. pneumoniae*. However, EDTA showed poor specificity, with positive results noted for 1/9 ESBL-producing isolates, for 4/34 KPC-producing isolates, and for 4/9 OXA-48-producing isolates, whereas all of these were negative when DPA was used. The in-house test distinguished accurately between several different mechanisms mediating reduced susceptibility to carbapenems in *Enterobacteriaceae*. The commercial combination tablets from Rosco performed similarly to the in-house test, with the exception of one false-positive MBL result and one false-positive KPC result among the OXA-48 producers.

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Introduction

The rapid international spread of *Klebsiella pneumoniae* strains that produce KPC class A carbapenemases, recently also classified as extended-spectrum β-lactamase $(ESBL)_{CARBA-A}$ [1], is a major concern. Treatment options are severely limited, owing to linked multiresistance, and the association of both KPC-2 and KPC-3 with the successful ST258 *K. pneumoniae* clone [2] is a great challenge for infection control. Several tests have been described for phenotypic detection of KPC β-lactamases. A modified version of the cloverleaf (Hodge) test, originally established for detection of penicillinases [3], accurately detects KPC β-lactamases [4], but is not able to discriminate between these and other carbapenemases. Moreover, performance of this test requires experience, as the interpretation may not be straightforward with all carbapenemase-producing *Enterobacteriaceae* (A. Vatopoulos,

personal communication). Recently, a boronic acid disk test for the detection of KPC-producing *K. pneumoniae* was described [5], but it was not evaluated against *Enterobacteriaceae* with carbapenem resistance arising from a combination of porin loss and hyperproduction of chromosomal AmpC/plasmid-mediated AmpC, which might also be predicted to give positive results. Thus, the specificity of this test has not been fully evaluated.

Moreover, the emergence of metallo-β-lactamases (MBLs) in *K. pneumoniae* [6] and other *Enterobacteriaceae*, and their predominance among carbapenemase producers in, for example, Greece, has created a need for a reliable phenotypic test that can detect and distinguish clinically important carbapenemases. The rapid emergence of KPC-producing *Enterobacteriaceae* on several continents [7] emphasizes that such an assay is urgently needed from an infection control perspective.

The aim of this study was to determine whether meropenem disks supplemented with four β-lactamase inhibitors (dipicolinic acid (DPA), EDTA, aminophenylboronic acid (APBA) or cloxacillin) would be able to discriminate between various carbapenemase-producing *Enterobacteriaceae*, and carbapenem-non-susceptible isolates with ESBL/AmpC hyperproduction in combination with porin changes. Also, we evaluated the performance of commercially available diagnostic tablets containing meropenem in combination with DPA, APBA and cloxacillin.

Materials and Methods

Bacterial isolates

Well-characterized strains from several countries producing various β-lactamases were included in this study. The panel consisted of clinical isolates of KPC-2-producing (n = 9) and KPC-3-producing (n = 12) *K. pneumoniae* from the CDC [8], KPC-producing ST258 *K. pneumoniae* from the Health Protection Agency (HPA), London (n = 6) [9], and KPC-2-producing (n = 6) and KPC-3-producing (n = 1) ST258 *K. pneumoniae* from Norway and Sweden [2]. Isolates of VIM-1-producing *K. pneumoniae* from Greece (n = 12), VIM-1-producing *K. pneumoniae* from Norway and Sweden (n = 6), VIM-1-producing (n = 3) and IMP-producing (n = 3) *K. pneumoniae* and *Escherichia coli* (n = 1) from the HPA represented MBL-producing *Enterobacteriaceae*. *K. pneumoniae* isolates producing OXA-48 carbapenemase were obtained from the HPA (n = 9) (Zhang, 19th ECCMID, 2009, Abstract L249). Carbapenem-resistant *K. pneumoniae* isolates with CTX-M-15-type and/or SHV-type ESBLs in combination with loss of porins OmpK35 and/or OmpK36 (n = 9), as well as *Enterobacter cloacae* and *Enterobacter aerogenes* isolates hyperproducing AmpC and with complete or partial loss of OmpF and/or OmpC (n = 5) [10], were included to investigate the specificity of the various tests. Finally, three isolates of *E. coli* and one isolate of *Enterobacter cloacae* with a combination of plasmid-mediated (CMY-2) or chromosomal AmpC, ESBLs (CTX-M-15 or SHV-12) and porin loss were included.

Preparation of combination disks

Meropenem disks (Oxoid, Basingstoke, UK) were supplemented with 10 μL of three different β-lactamase inhibitors: 100 mg/mL DPA (Sigma, St Louis, MO, USA) (modified from Kimura *et al.*) [11], 0.2 M EDTA (Sigma) [12], 60 mg/mL APBA (Sigma) and 75 mg/mL cloxacillin (Sigma). Hence, the final amounts of β-lactamase inhibitor in the disks were 1000 μg of DPA, 730 μg of EDTA, 600 μg of APBA and 750 μg of cloxacillin. DPA was dissolved in dimethylsulphoxide (Sigma), whereas EDTA, APBA and cloxacillin were dissolved in sterile water. Disks were left to dry at room temperature for 30 min before they were used.

Test procedure and interpretation of the combination disk assay

A 0.5 McFarland inoculum was prepared and spread on cation-adjusted Mueller–Hinton II agar plates (Becton-Dickinson, Cockeysville, MD, USA). Five disks were placed on each plate: meropenem 10 μg, meropenem 10 μg + DPA, meropenem 10 μg + EDTA, meropenem 10 μg + APBA and meropenem 10 μg + cloxacillin. An increase ≥5 mm in zone diameter around disks containing β-lactamase inhibitors, as compared with the disk with meropenem alone, was considered to be a positive result for DPA, EDTA and cloxacillin, whereas an increase ≥ 4 mm was considered to be a positive result for APBA. An increase ≥4 mm for APBA was selected because 4/34 KPC-producing *K. pneumoniae* isolates only had an increase of 4 mm and were thus not detected with a cut-off ≥5 mm.

Commercial diagnostic tablets from Rosco (Rosco Diagnostica A/S, Taastrup, Denmark) were evaluated against the same isolates. Four tablets were placed on each plate: meropenem, meropenem + DPA, meropenem + APBA and meropenem + cloxacillin. The procedure was identical to the one used for applying in-house disks. An increase ≥5 mm in zone diameter around tablets containing β-lactamase inhibitors, as compared with the tablet with meropenem alone, was considered a positive result, according to the manufacturer's instruction.

Modified cloverleaf (Hodge) test)

The modified cloverleaf test was performed according to Anderson *et al.* [4]. In brief, cation-adjusted Mueller-Hinton II