Microbiology, Nijmegen), Steven F.T. Thijsen MD PhD (Diakonessenhuis, Department of Medical Microbiology, Utrecht), Kees Verduin MD PhD (Stichting PAMM, Laboratory of Medical Microbiology, Veldhoven), Liesbeth Verhoef MD PhD (SALTRO, Primary Health Care Laboratory, Department of Medical Microbiology, Utrecht), Bart J.M. Vlaminckx MD PhD (St Antonius Hospital, Department of Medical Microbiology, Nieuwegein), Guido M. Voets (University Medical Center Utrecht, Department of Medical Microbiology, Utrecht), Willem H.M. Vogels MD (Laboratory for Infectious Diseases Groningen, Groningen), Rolf W. Vreede MD PhD (Diagnostic Center SSDZ, Department of Medical Microbiology, Delft), Karola Waar MD PhD (Izore, Center of Infectious Diseases Friesland, Leeuwarden), Hans H.T. Wagenvoort MD PhD (Atrium Medisch Centrum Parkstad, Department of Medical Microbiology, Heerlen), Peter C. Wever MD PhD (Jeroen Bosch Hospital, Regional Laboratory of Medical Microbiology and Infectious Control, Den Bosch), Rob G.F. Wintermans MD (Franciscus Hospital, Laboratory of Medical Microbiology, Roosendaal), Maurice J.H.M. Wolfhagen MD PhD (Isala Clinics, Laboratory of Medical Microbiology and Infectious Diseases, Zwolle).

References

1. Pitout JD, Laupland KB. Extended-spectrum beta-lactamase-producing Enterobacteriaceae: an emerging public-health concern. *Lancet Infect Dis* 2008; 8: 159–166.
2. Cohen Stuart J, Dierikx C, Al Naiemi N *et al.* Rapid detection of TEM, SHV and CTX-M extended-spectrum beta-lactamases in Enterobacteriaceae using ligation-mediated amplification with microarray analysis. *J Antimicrob Chemother* 2010; 65: 1377–1381.
3. Endimiani A, Hujer AM, Hujer KM *et al.* Evaluation of a commercial microarray system for detection of SHV-, TEM-, CTX-M-, and KPC-type beta-lactamase genes in gram-negative isolates. *J Clin Microbiol* 2010; 48: 2618–2622.
4. Naas T, Cuzon G, Truong H, Bernabeu S, Nordmann P. Evaluation of a DNA microarray, the check-points ESBL/KPC array, for rapid detection of TEM, SHV, and CTX-M extended-spectrum beta-lactamases and KPC carbapenemases. *Antimicrob Agents Chemother* 2010; 54: 3086–3092.
5. Voets GM, Fluit AC, Scharringa J, Cohen Stuart JW, Leverstein-van Hall MA. A set of multiplex PCRs for genotypic detection of extended-spectrum beta-lactamases, carbapenemases, plasmid-mediated AmpC beta-lactamases and OXA beta-lactamases. *Int J Antimicrob Agents* 2011; 27: 356–359.
6. Oteo J, Perez-Vazquez M, Campos J. Extended-spectrum [Beta]-lactamase producing *Escherichia Coli*: changing epidemiology and clinical impact. *Curr Opin Infect Dis* 2010; 23: 320–326.

A simple test for the detection of KPC and metallo-β-lactamase carbapenemase-producing *Pseudomonas aeruginosa* isolates with the use of meropenem disks supplemented with aminophenylboronic acid, dipicolinic acid and cloxacillin

F. Pasteran, O. Veliz, D. Faccone, L. Guerriero, M. Rapoport, T. Mendez and A. Corso

Servicio Antimicrobianos, Departamento Bacteriología, Instituto Nacional de Enfermedades Infecciosas (INEI) – ANLIS 'Dr Carlos G. Malbrán', Ministerio de Salud y Ambiente, Ciudad Autónoma de Buenos Aires, Argentina

Abstract

We evaluated the ability of the combination disk test (CDT) and the Modified Hodge Test (MHT) to discriminate between various carbapenemase-producing *Pseudomonas aeruginosa* isolates (KPC, n = 36; metallo-β-lactamase (MBL), n = 38) and carbapenemase non-producers (n = 75). For the CDT, the optimal inhibitor concentrations and cut-off values were: 600 μg of 3-aminophenylboronic acid (APB) per disk (an increment of ≥4 mm), 1000 μg of dipicolinic acid (DPA) per disk (an increment of ≥5 mm) and 3000 μg of cloxacillin per disk (an increment of ≥3 mm). APB had excellent sensitivity (97%) and specificity (97%) for the detection of KPC enzymes. DPA detected MBL enzymes with a sensitivity and specificity of 97% and 81%, respectively. The MHT resulted in a low sensitivity (78%) and specificity (57%). The CDT could be very useful in daily practice to provide fast and reliable detection of KPC and MBL carbapenemases among *P. aeruginosa* isolates.

Keywords: 3-Aminophenylboronic acid, carbapenemase, cloxacillin, dipicolinic acid, *Pseudomonas aeruginosa*

Original Submission: 7 February 2011;
Revised Submission: 1 May 2011; **Accepted:** 15 May 2011
Editor: R. Cantón
Article published online: 24 May 2011

Clin Microbiol Infect 2011; **17:** 1438–1441
10.1111/j.1469-0691.2011.03585.x

Corresponding author: A. Corso, Servicio Antimicrobianos, Instituto Nacional de Enfermedades Infecciosas (INEI) – ANLIS 'Dr Carlos G. Malbrán', Velez Sarsfield 563 Ave. (C1282AFF), Buenos Aires, Argentina
E-mail: acorso@anlis.gov.ar

Detection of carbapenemase producers in the clinical laboratory is of major importance for the determination of appropriate therapeutic schemes and the implementation of infection control measures [1,2]. Several inhibitor-based tests have been developed for the detection of carbapenemase producers. Recently, a combination disk test (CDT) for the detection of metallo-β-lactamase (MBLs) and KPC in *Enterobacteriaceae* with the use of meropenem disks supplemented with 3-aminophenylboronic acid (APB), dipicolinic acid (DPA) or cloxacillin was described [3]. Typically, KPC-producing *Enterobacteriaceae* showed an increased meropenem inhibition zone (ZI) in the presence of APB, a class A carbapenemase and class C β-lactamase inhibitor. Unlike KPC-producing isolates, those with combined AmpC overproduction and porin loss gave positive results in the APB test, but also showed cloxacillin (a class C β-lactamase inhibitor) synergy. MBL producers showed an increased meropenem zone diameter in the presence of DPA, a class B β-lactamase inhibitor [3]. The Modified Hodge test (MHT) has been widely used for carbapenemase screening in *Enterobacteriaceae* [4], but it has not been further explored in *Pseudomonas aeruginosa*. The aim of this study was to determine whether the CDT and the MHT would be able to discriminate between various carbapenemase-producing *P. aeruginosa* isolates and carbapenemase non-producers.

A panel of *P. aeruginosa* isolates (n = 149) were included. The carbapenemases represented were (n): KPC (36), VIM-2 (16), SPM-1 (8), VIM-11 (5), IMP-13 (5), and IMP-16 (4) (Pasteran *et al.*, 49th ICAAC, 2009, Abstract 1003). The resistance mechanisms among carbapenemase non-producers were (n): wild type (6), narrow-spectrum-β-lactamases (2), extended-spectrum β-lactamases (7), cephalosporinase overproduction (7), efflux overproduction (15) and dual mechanisms (efflux plus cephalosporinase overproduction, 38). The isolates were from clinical sources. All strains corresponded to different pulsed-field gel electrophoresis types [5], with the exception of KPC producers, which were divided into two pulsed-field gel electrophoresis types. KPC producers belonged to two multilocus sequence types (STs): ST654 and ST162 (http://pubmlst.org/paeruginosa/). β-Lactamases were characterized by PCR and DNA sequencing [6,7]. AmpC-type cephalosporinase production was determined with spectrophotometric assays [8,9]. Efflux estimation was performed as described previously [10]. The CDT was performed with freshly prepared disks [3]. The MHT was performed with *Escherichia coli* ATCC 25922 as the indicator organism, as described previously [4].

First, we evaluated the performance of carbapenemase screening by using meropenem ZIs. Meropenem ranges (in mm) were 6–9, 6–23 and 6–34 for KPC producers, MBL producers and carbapenemase non-producers, respectively. Thus, all carbapenemase producers had a ZI ≤23 mm, coincident with a non-susceptible result as defined by EUCAST criteria (http://www.eucast.org/clinical_breakpoints/).

Subsequently, we tested the CDT by using the β-lactamase inhibitor concentration and the cut-off values reported for *Enterobacteriaceae*: 600 μg of APB per disk (Sigma, St. Louis, MO, USA), 750 μg of cloxacillin per disk (Hangzkou BM Chemical, Hangzkou, China) and 1000 μg of DPA per disk (Sigma). For APB, an increment ≥4 mm in zone diameter around disks containing the inhibitor, as compared with the disk with meropenem alone, was used as the cut-off; for DPA and cloxacillin, an increment ≥5 mm was considered to be a positive result [3]. When tested in *P. aeruginosa*, APB synergy detected all but one of the isolates producing KPC (average increments of 9 mm). None of these strains showed cloxacillin synergy (no increments observed), and thus were properly classified as KPC producers (Table 1). Cephalosporinase overproducers were also positive in the APB test. Unexpectedly, none of these strains showed cloxacillin synergy under the conditions described for *Enterobacteriaceae* (average increments of 2 mm), and were therefore incorrectly classified as KPC producers (specificity of 40%). To reduce the number of false-positive results, we tested disks with 1500 μg of cloxacillin: 85% of cephalosporinase overproducers still had average increments of 3 mm, and were overestimated as KPC producers (specificity of 49%). Subse-

TABLE 1. Sensitivities and specificities of dipicolinic acid (DPA), 3-aminophenylboronic acid (APB) and cloxacillin in the combination disk test for detecting various β-lactamases

Test(s)	Resistant mechanism sought by test	Sensitivity (%)	Specificity (%)
APB-positive plus cloxacillin-negative	KPC	97	97 (98)[a]
DPA-positive	MBL	97	81
APB-negative or APB-positive plus cloxacillin-positive	Carbapenemase non-producer	97 (98)[a]	97
Modified Hodge test	Carbapenemase producer	78	57

[a]Corrected values with the inclusion of strains with halos of meropenem ≤23 mm (a screening breakpoint defined in this study).

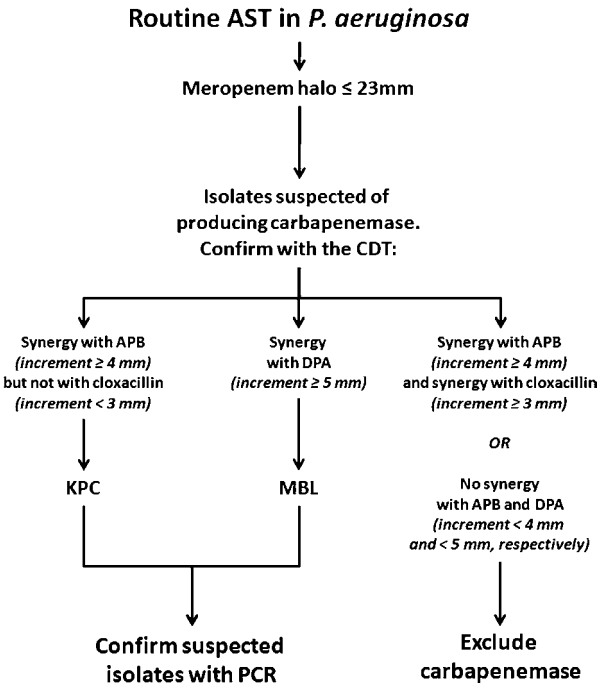

FIG. 1. Preliminary protocol for carbapenemase screening among *Pseudomonas aeruginosa* isolates. APB, 3-aminophenylboronic acid; AST, antimicrobial susceptibility test; CDT, combination disk test; DPA, dipicolinic acid; MBL, metallo-β-lactamase. The algorithm required a meropenem disk (10 μg) and meropenem disks supplemented with 600 μg of APB per disk, 1000 μg of DPA per disk and 3000 μg of cloxacillin per disk.

quently, we evaluated disks with 3000 μg of cloxacillin, and obtained average increments of 4 mm. Thus, we decided to use a less exigent cut-off value of ≥3 mm for a positive result with the 3000-μg cloxacillin disk. With these conditions, all but two cephalosporinase overproducers were classified as true producers of this mechanism (specificity of 97%; Table 1). With the test conditions described for *Enterobacteriaceae*, DPA detected all MBL producers except for one VIM-11 producer (average increments of 9 mm). About 17 MBL non-producers (distributed as follows: cephalosporinase overproducers (n = 3), efflux overproducers (n = 3), dual mechanisms (n = 8), and extended-spectrum β-lactamase producers (GES-1 (n = 2) and OXA-31 (n = 1)) were also positive with the DPA test (Table 1).

With the MHT, a high proportion of indeterminate results (inhibition of the growth of the indicator strain produced by the test isolate) was observed (22% and 43% of carbapenemase producers and non-producers, respectively). Thus, the sensitivity and specificity were 78% and 57%, respectively (Table 1).

We present here for the first time a method with high sensitivity and specificity for the detection of KPC-producing *P. aeruginosa* isolates. The proposed test is an improvement on the CDT recommended for *Enterobacteriaceae* [3]. The APB and DPA concentrations and cut-off values for screening in *P. aeruginosa* were coincident with those used for screening in *Enterobacteriaceae*. However, the cloxacillin concentration (750 μg per disk) and the respective cut-off value recommended for screening in *Enterobacteriaceae* overestimated the presence of KPC producers among *P. aeruginosa* isolates at a rate of 60%. Higher concentrations of cloxacillin (3000 μg per disk) and a lower cut-off value were required, probably because of the greater impermeability of the *P. aeruginosa* outer membrane.

On the basis of our results, we propose a preliminary scheme for phenotypic carbapenemase screening in *P. aeruginosa* (Fig. 1). Screening starts with the routine meropenem results: a ZI ≤23 mm (non-susceptible by EUCAST) categorically selects isolates suspected of producing carbapenemases. The actual number of *P. aeruginosa* strains that will be included with this cut-off value in any given laboratory will depend upon the local epidemiology: in Europe, it is estimated to be 14% of the strains (http://www.eucast.org/mic_distributions), and in Argentina 31% (WHONET-Argentina Network). Subsequently, the CDT should be performed. With this protocol, laboratories will detect >95% of carbapenemase producers. Finally, the assumptions of carbapenemase production made on the basis of the CDT should be confirmed by molecular methods. The MHT was not included, because of the unacceptable performance; indeterminate results occurred at a rate ten times higher than that for *Enterobacteriaceae* [7].

One limitation of the study is that KPC producers belong to a reduced number of clones. However, it should be noted that ST564, the most prevalent clone included in this study, has been reported in Singapore, where it is also endemic and has been associated with IMP-type MBLs (http://pubmlst.org/paeruginosa/), and it has also been reported in Sweden as a VIM producer, indicating that this is a successful, worldwide, multiresistant clone with the ability to acquire relevant carbapenemases [11]. Therefore, we believe that is highly likely that, at least, ST654 will have an important role in the global dissemination of KPC in *P. aeruginosa*, and the CDT has shown an excellent performance among strains belonging to this ST. Another limitation is that isolates producing both MBL and KPC could emerge simultaneously in the near future, and could pose another challenge to this protocol. As proposed for *Enterobacteriaceae*, such isolates could probably be identified with the addition of both DPA and APB to meropenem disks (Casals *et al.*, 21st ECCMID, 2011, Abstract 697).

In conclusion, we propose a phenotypic screening strategy for identification of carbapenemases among *P. aeruginosa* iso-

lates, based on the meropenem susceptibility test result and the use of meropenem disks supplemented with APB, cloxacillin or DPA. These tests will enable routine laboratories to identify, with high confidence levels, those *P. aeruginosa* isolates suspected of producing either KPC or MBL carbapenemases.

Transparency Declaration

The authors have no conflicts of interest to declare. This work was performed with the regular budget of the Ministry of Health of Argentina.

References

1. Miriagou V, Cornaglia G, Edelstein M *et al.* Acquired carbapenemases in gram-negative bacterial pathogens: detection and surveillance issues. *Clin Microbiol Infect* 2010; 16: 112–122.
2. Carmeli Y, Akova M, Cornaglia G *et al.* Controlling the spread of carbapenemase-producing gram-negatives: therapeutic approach and infection control. *Clin Microbiol Infect* 2010; 16: 102–111.
3. Giske CG, Gezelius L, Samuelsen O *et al.* A sensitive and specific phenotypic assay for detection of metallo-beta-lactamases and KPC in *Klebsiella pneumoniae* with the use of meropenem disks supplemented with aminophenylboronic acid, dipicolinic acid and cloxacillin. *Clin Microbiol Infect* 2011; 17: 552–556.
4. Clinical Laboratory Standards Institute. *Performance standards for antimicrobial susceptibility testing: 21st informational supplement*. CLSI document M100-S21. Wayne, PA: CLSI, 2011.
5. Tenover FC, Arbeit RD, Goering RV *et al.* Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. *J Clin Microbiol* 1995; 33: 2233–2239.
6. Pasteran F, Mendez T, Guerriero L *et al.* Sensitive screening tests for suspected class A carbapenemase production in species of Enterobacteriaceae. *J Clin Microbiol* 2009; 47: 1631–1639.
7. Pasteran F, Mendez T, Rapoport M *et al.* 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* 2010; 48: 1323–1332.
8. Juan C, Maciá MD, Gutiérrez O *et al.* Molecular mechanisms of beta-lactam resistance mediated by AmpC hyperproduction in *Pseudomonas aeruginosa* clinical strains. *Antimicrob Agents Chemother* 2005; 49: 4733–4738.
9. Papanicolaou GA, Medeiros A, Jacoby G. Novel plasmid-mediated beta-lactamase (MIR-1) conferring resistance to oxyimino- and alpha-methoxy beta-lactams in clinical isolates of *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 1990; 34: 2200–2209.
10. Jiang X, Zhang Z, Li M *et al.* Detection of extended-spectrum beta-lactamases in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 2006; 50: 2990–2995.
11. Samuelsen O, Toleman MA, Sundsfjord A *et al.* Molecular epidemiology of metallo-beta-lactamase-producing Pseudomonas aeruginosa isolates from Norway and Sweden shows import of international clones and local clonal expansion. *Antimicrob Agents Chemother* 2010; 54: 346–352.

Streptococcus pneumoniae serotype 1 causing invasive disease among children in Barcelona over a 20-year period (1989–2008)

C. Esteva[1], L. Selva[1], M. F. de Sevilla[2], J. J. Garcia-Garcia[2], R. Pallares[3] and C. Muñoz-Almagro[1]

1) *Department of Microbiology,* 2) *Department of Paediatrics, Hospital Sant Joan de Deu and University of Barcelona, Esplugues and* 3) *Infectious Diseases and Clinical Research Unit, Idibell, Ciberes, Bellvitge Hospital and University of Barcelona, L'Hospitalet, Barcelona, Spain*

Abstract

Fifty-six isolates of serotype 1 were identified during a 20-year prospective study (1989–2008), including all children with culture-proven invasive pneumococcal disease (IPD) admitted to a children's hospital in Barcelona. Forty-eight of them (85.7%) were in children aged >2 years. Complicated pneumonia (n = 28) and non-complicated pneumonia (n = 20) were the main clinical presentations. The frequency of serotype 1 IPD increased from 1999–2003 to 2004–2008: 1.2 to 4.4 episodes/100 000 children (p <0.001). The ST306 clone were identified in 70.4% of isolates. As IPD caused by serotype 1 is mainly detected in older children, a vaccination programme for children >2 years should be considered.

Keywords: Invasive pneumococcal disease, MLST, resistance, serotype 1, *Streptococcus pneumoniae*

Original Submission: 1 November 2010; **Revised Submission:** 16 February 2011; **Accepted:** 7 March 2011
Editor: J.-L. Mainardi
Article published online: 4 April 2011

Clin Microbiol Infect 2011; **17:** 1441–1444
10.1111/j.1469-0691.2011.03526.x

Corresponding author: C. Muñoz-Almagro, Molecular Microbiology Department, Hospital Sant Joan de Déu, University of Barcelona, Pº Sant Joan de Déu no. 2, 08950 Esplugues, Barcelona, Spain
E-mail: cma@hsjdbcn.org