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 Bacteriologia, Instituto Nacional de Enfermedades Infecciosas (INEI) – ANLIS ‘Dr Carlos G. Malbrán’, Min-isterio 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 carbapenem 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

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 over-production (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 $\leq$ 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 $\beta$-lactamase inhibitor concentration and the cut-off values reported for *Enterobacteriaceae*: 600 $\mu$g of APB per disk (Sigma, St. Louis, MO, USA), 750 $\mu$g of cloxacillin per disk (Hangzkou BM Chemical, Hangzkou, China) and 1000 $\mu$g of DPA per disk (Sigma). For APB, an increment $\geq$ 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 $\geq$ 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 $\mu$g of cloxacillin: 85% of cephalosporinase overproducers still had average increments of 3 mm, and were overestimated as KPC producers (specificity of 49%). Subsequently, we evaluated disks with 3000 $\mu$g of cloxacillin, and obtained average increments of 4 mm. Thus, we decided to use a less exigent cut-off value of $\geq$ 3 mm for a positive result with the 3000-$\mu$g cloxacillin disk. With these conditions, all but two cephalosporinase overproducers were considered 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 $\beta$-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 $\mu$g 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 $\mu$g 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 $\leq$ 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

<table>
<thead>
<tr>
<th>Test(s)</th>
<th>Resistant mechanism sought by test</th>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>APB-positive plus cloxacillin-negative</td>
<td>KPC</td>
<td>97</td>
<td>97 (98)*</td>
</tr>
<tr>
<td>DPA-positive</td>
<td>MBL</td>
<td>97</td>
<td>81</td>
</tr>
<tr>
<td>APB-negative or APB-positive plus cloxacillin-positive</td>
<td>Carbapenemase non-producer</td>
<td>97 (98)*</td>
<td>97</td>
</tr>
<tr>
<td>Modified Hodge test</td>
<td>Carbapenemase producer</td>
<td>78</td>
<td>57</td>
</tr>
</tbody>
</table>

*Corrected values with the inclusion of strains with halos of meropenem $\leq$ 23 mm (a screening breakpoint defined in this study).
Routine AST in \textit{P. aeruginosa}

\begin{itemize}
\item Meropenem halo ≤ 23mm
\item Isolates suspected of producing carbapenemase. Confirm with the CDT:
\begin{itemize}
\item Synergy with APB (increment ≥ 4 mm) but not with cloxacillin (increment < 3 mm)
\item Synergy with DPA (increment ≥ 5 mm) and synergy with cloxacillin (increment ≥ 3 mm)
\item Synergy with APB (increment ≥ 4 mm) and synergy with DPA (increment < 4 mm and < 5 mm, respectively)
\end{itemize}
\end{itemize}

\begin{itemize}
\item KPC
\item MBL
\item No synergy with APB and DPA
\end{itemize}

Confirm suspected isolates with PCR

Exclude carbapenemase

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

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 \textit{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 \textit{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 \textit{P. aeruginosa} isolates, 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 \textit{P. aeruginosa} isolates suspected of producing either KPC or MBL carbapenemases.

\textbf{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.

\textbf{References}