Laboratory Detection of Enterobacteriaceae That Produce Carbapenemases

Diana Doyle,a Gisele Peirano,a,b Christine Lascols,d Tracie Lloyd,a Deirdre L. Church,a,b and Johann D. D. Pitouta,b,c
Division of Microbiology, Calgary Laboratory Services, Calgary, Alberta, Canada; Departments of Pathology and Laboratory Medicinea and Microbiology, Immunology and Infectious Diseases, University of Calgary, Calgary, Alberta, Canada, and International Health Management Associates Inc, Schaumburg, Illinois, USAa

A study was designed to evaluate the modified Hodge test (MHT), Mastdiscs ID inhibitor combination disks (MDI), Rosco Diagnostica Neo-Sensitabs (RDS), metallo-β-lactamase (MBL) Etest, and in-house multiplex PCR for the detection of well-characterized carbapenemase-producing Enterobacteriaceae. One hundred forty-two nonrepeat clinical isolates of carbapenemase-producing Enterobacteriaceae (including Klebsiella spp., Escherichia coli, Citrobacter freundii, and Enterobacter spp.) obtained from the SMART worldwide surveillance program during 2008 to 2009 were included. These included 49 KPCs, 27 NDMs, 19 VIMs, 14 OXA-48-like enzymes, and 5 IMP-producing isolates and 28 carbapenem-resistant, carbapenemase-negative isolates. The manufacturer’s instructions were followed for MDI, RDS, and MBL Etest and CLSI guidelines for MHT. A multiplex PCR was designed to detect KPC, NDM, VIM, IMP, and OXA-48-like carbapenemases. Overall, the sensitivity and specificity were 78% and 93% for MDI, 80% and 93% for RDS, 58% and 93% for MHT, and 55% and 100% for MBL Etest, respectively. The PCR had 100% sensitivity and specificity. MDI and RDS performed well for the detection of KPCs and NDMs but poorly for VIMs, IMPS, and OXA-48-like enzymes. MHT performed well for KPCs and OXA-48-like enzymes but poorly for NDMs, VIMs, and IMPS. MDI and RDS were easy to perform and interpret but lacked sensitivity for OXA-48-like enzymes, VIMs, and IMPS. MHT and MBL Etest were often difficult to interpret. We recommend using molecular tests for the optimal detection of carbapenemase-producing Enterobacteriaceae.

The Enterobacteriaceae, most notably Escherichia coli and Klebsiella pneumoniae, are among the most important causes of serious hospital-acquired and community-onset bacterial infections in humans (11). Since β-lactam antibiotics are a major drug class used to treat serious community-onset or hospital-acquired infections caused by Enterobacteriaceae, resistance to these agents will continue to challenge clinical therapeutic choices. Of special concern is the development of resistance to the carbapenems, since these agents are often the last line of effective therapy available for the treatment of infections caused by multiresistant Enterobacteriaceae (7).

Most importantly within the Enterobacteriaceae is the increasing recognition of isolates producing carbapenemases that cause resistance to the carbapenems. These enzymes include the class A carbapenemases (KPC types), the class B or metallo-β-lactamases (MBLs) (VIM, IPM, and NDM types), and the class D oxacillinases (e.g., OXA-48-like enzymes) (10).

Current recommendations for the detection of Enterobacteriaceae that produce carbapenemases from the Clinical and Laboratory Standards Institute (CLSI) can be summarized as follows (2). The carbapenem breakpoints (i.e., 0.5 µg/ml for ertapenem and 1 µg/ml for meropenem, imipenem, and doripenem) for Enterobacteriaceae will detect all clinically important resistance mechanisms, including the majority of carbapenemases. Some isolates that produce carbapenemases are categorized as susceptible with these breakpoints and should be reported as tested; i.e., the presence or absence of a carbapenemase does not in itself influence the categorization of susceptibility. In many areas, carbapenemase detection and characterization are recommended or mandatory for infection control purposes.

The presence of carbapenemases in Klebsiella spp. and E. coli in the majority of hospitalized patients is considered an infection control emergency; therefore, clinical microbiology laboratories should be able to rapidly detect these enzymes among members of the Enterobacteriaceae (10). Recently Mast Diagnostics and Rosco Diagnostica released commercial disks and Sensitabs containing meropenem with different inhibitors designed for the detection of Enterobacteriaceae that produce different types of carbapenemases. However, to our knowledge, these commercial inhibitor-based methods have not yet been evaluated.

A study was designed to evaluate the following phenotypic confirmatory tests for the presence of well-characterized carbapenemases among Enterobacteriaceae: the modified Hodge test (MHT), Mastdiscs ID inhibitor combination disks (MDI), Rosco Diagnostica Neo-Sensitabs (RDS), and the metallo-β-lactamase Etest. An in-house multiplex PCR was also designed for the detection of blakPC, blakNDM, blakOXA-48-like, blakIMP, and blakIMP.

MATERIALS AND METHODS

Bacteria. One hundred forty-two nonrepeat previously characterized clinical isolates of carbapenemase-producing Enterobacteriaceae were included in the study. They were obtained from the SMART worldwide surveillance program, Canada, and the United States (5, 6, 12–14). These included Klebsiella spp., E. coli, Citrobacter freundii, and Enterobacter spp. that produce KPCs (n = 49), NDMs (n = 27), VIMs (n = 19), OXA-48-like enzymes (n = 14), and IMPS (n = 5). An additional 28 carbapenem-resistant but carbapenemase-negative isolates were also included as negative controls. The carbapenem-resistant, carbapenemase-negative

Received 8 August 2012 Returned for modification 31 August 2012 Accepted 10 September 2012 Published ahead of print 19 September 2012
Address correspondence to Johann D. D. Pitout, johann.pitout@cls.ab.ca.
Copyright © 2012, American Society for Microbiology. All Rights Reserved.
doi:10.1128/JCM.02117-12
isolation included CTX-M, SHV, and AmpC producers with altered permeabilities (5). Isolates from the SMART surveillance program were obtained from Europe (20 sites), Asia (27 sites), North America (20 sites), Latin America (12 sites), the South Pacific (6 sites), and the Middle East (2 sites) (6). The Canadian isolates were obtained from Calgary, Medicine Hat, and Toronto, while the U.S. isolate originated from Chicago, IL (12–14). All of the isolates included in the study tested nonsusceptible (i.e., intermediate or resistant) to the carbapenems (i.e., MICs were >0.5 μg/ml for ertapenem and >1 μg/ml for meropenem and imipenem) as determined by using dehydrated broth microdilution MicroScan panels (Siemens Healthcare Diagnostics, Deerfield, IL).

**Phenotypic confirmation tests.** The manufacturer’s instructions were followed for Mastdiscs ID inhibitor combination disks (MDI) (Mast Diagnostica Neo-Sensitabs (RDS), and the metallo-β-lactamase (MBL) Etest (18), and the CLSI guidelines were used for the modified Hodge test (MHT) (2). Meropenem was used as the substrate for the MHT.

The Mastdiscs ID inhibitor combination disk method consists of 4 disks: disk A, containing a carbapenem (meropenem, 10 μg); disk B, consisting of meropenem (10 μg) and an MBL inhibitor; disk C, consisting of meropenem (10 μg) with a KPC inhibitor; and disk D, containing meropenem (10 μg) with an AmpC inhibitor. The interpretation of the test is as follows. The zone of inhibition of disk A is compared to the zones of inhibition of each of disks B, C, and D. If disk B shows a zone difference of ≥5 mm from disk A, the organism is recorded as demonstrating MBL activity. If disk C shows a zone difference of ≥4 mm from disk A, the organism is recorded as demonstrating KPC activity. If disk C and disk D both show a zone difference of ≥5 mm from disk A, the organism is recorded as demonstrating AmpC activity coupled with porin loss (impermeability).

The Rosco Diagnostica Neo-Sensitabs KPC and MBL confirmation kit consists of 4 tablets: tablet A contains meropenem, tablet B contains meropenem and diclofenac acid (MBL inhibitor), tablet C contains meropenem and cloxacillin (AmpC inhibitor), and tablet D contains meropenem and boronic acid (KPC inhibitor). The interpretation of the test is as follows. The zone of inhibition of tablet A is compared to the zones of inhibition of each of the carbapenem-plus-inhibitor tablets (B, C, and D). If tablet B shows a zone difference of ≥5 mm from tablet A, the organism is recorded as demonstrating MBL activity. If tablet D shows a zone difference of ≥5 mm from tablet A, the organism is recorded as demonstrating AmpC activity coupled with porin loss (impermeability).

**Multiplex PCR for detection of** bla<KPC>, bla<NDM>, bla<OXA-48-like>, bla<IMP> and bla<ampC> β-lactamase genes was carried out on a Veriti 96-well thermal cycler instrument (Applied Biosystems at Life Technologies, Foster City, CA) with the AmpliTaq Gold PCR master mix (Applied Biosystems at Life Technologies, Hammondtown, NJ). A total of 1 μl of sample lysate was added to the reaction mixture. The PCR program consisted of an initial denaturation step at 95°C for 5 min, followed by 35 cycles of DNA denaturation at 95°C for 45 s, primer annealing at 60°C for 45 s, and primer extension at 72°C for 1 min, followed by a final extension at 72°C for 8 min. After the last cycle, the products were stored at 4°C. The PCR products were analyzed by electrophoresis with 1.5% agarose gels in 0.5× Tris-borate-EDTA (TBE) buffer. The gels were stained with SYBR Safe DNA gel stain (Invitrogen, Portland, OR), and the PCR products were visualized with UV light.

The primers were used at concentrations of 0.3 μM each for bla<KPC>, bla<NDM>, and bla<ampC>, 0.4 μM for bla<OXA-48-like> and 0.5 μM for bla<OXA-48-like>. The primer sequences and amplicon sizes are shown in Table 1.

**RESULTS AND DISCUSSION**

Several phenotypic confirmation tests have been described for the detection of carbapenemase-producing *Enterobacteriaceae*. These include bioassays that detect the ability of these enzymes to hydrolyze the carbapenems (e.g., modified Hodge test [MHT]) and inhibitor-based methods using metal chelators for MBLs (e.g., MBL Etest), boronic acid for KPCs (9), and the commercial systems such as the Mastdiscs ID inhibitor combination disks and the Rosco Diagnostica Neo-Sensitabs KPC and MBL confirmation kit.

We report a study that evaluated different phenotypic confirmation tests for the detection of *Enterobacteriaceae* that produce carbapenemases. The results obtained with the different phenotypic tests and multiplex PCR are illustrated in Table 2. Overall, Mastdiscs ID inhibitor combination disks had a sensitivity of 78% and specificity of 93%, the Rosco Diagnostica Neo-Sensitabs had a sensitivity of 80% and specificity of 93%, and the modified Hodge test had a sensitivity of 61% and specificity of 93% (Table 2). The results obtained with the different species were identical.

Previous evaluations of the MHT have shown that this method reliably detects KPC- and OXA-48-producing isolates (3). Unfortunately, the MHT performs poorly in the detection of MBL-producing isolates (10). Our results support the findings of these previous studies; the MHT in our study had a sensitivity of 98% for detecting KPC producers and 93% for OXA-48-like enzyme producers but was less than optimal for detecting MBLs (i.e., sensitivity of only 12%). The specificity of the MHT when testing the carbapenemase-negative isolates was 93% (Table 2).

The MBL Etest was designed to detect the presence of MBLs in

---

**Table 1: Primers for the detection of Enterobacteriaceae that produce carbapenemases**

<table>
<thead>
<tr>
<th>Carbanemase gene</th>
<th>Amplicon size (bp)</th>
<th>Primer sequences</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>bla&lt;KPC&gt;</td>
<td>900</td>
<td>5'-TTGTCACGTCGAGGATTGC-3'</td>
<td>19</td>
</tr>
<tr>
<td>bla&lt;NDM&gt;</td>
<td>587</td>
<td>5'-CTCATGTCGACCCACCAAGAA-3'</td>
<td>15</td>
</tr>
<tr>
<td>bla&lt;ampC&gt;</td>
<td>389</td>
<td>5'-GAAGGGTTTATGTTTACAT-3'</td>
<td>15</td>
</tr>
<tr>
<td>bla&lt;ampC&gt;</td>
<td>782</td>
<td>5'-GTGAGTTCAGAAGGTGTC-3'</td>
<td>15</td>
</tr>
<tr>
<td>bla&lt;ampC&gt;</td>
<td>438</td>
<td>5'-TTGGTGTCGACCCACCAAGAA-3'</td>
<td>12</td>
</tr>
</tbody>
</table>

*The first and second primers for each gene are forward and reverse primers, respectively.*
TABLE 2 Laboratory detection of Enterobacteriaceae that produce carbapenemases

<table>
<thead>
<tr>
<th>Carbapenemase producing</th>
<th>MDI</th>
<th>RDS</th>
<th>MBL Etest</th>
<th>MHT</th>
<th>Multiplex PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>KPC (49)</td>
<td>48 (98)</td>
<td>49b (100)</td>
<td>0</td>
<td>48 (98)</td>
<td>49 (100)</td>
</tr>
<tr>
<td>KPC-2 (25)</td>
<td>24 (96)</td>
<td>25 (100)</td>
<td>0</td>
<td>24 (96)</td>
<td>25 (100)</td>
</tr>
<tr>
<td>KPC-3 (18)</td>
<td>18 (100)</td>
<td>18 (100)</td>
<td>0</td>
<td>18 (100)</td>
<td>18 (100)</td>
</tr>
<tr>
<td>KPC-11 (6)</td>
<td>6 (100)</td>
<td>6 (100)</td>
<td>0</td>
<td>6 (100)</td>
<td>6 (100)</td>
</tr>
<tr>
<td>NDM-1 (27)</td>
<td>27 (100)</td>
<td>27b (100)</td>
<td>20 (74)</td>
<td>3 (11)</td>
<td>27 (100)</td>
</tr>
<tr>
<td>VIM (19)</td>
<td>10 (53)</td>
<td>12 (63)</td>
<td>10 (53)</td>
<td>2 (11)</td>
<td>19 (100)</td>
</tr>
<tr>
<td>VIM-1 (16)</td>
<td>8 (42)</td>
<td>10 (53)</td>
<td>8 (42)</td>
<td>0</td>
<td>16 (100)</td>
</tr>
<tr>
<td>VIM-5 (1)</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td>0</td>
<td>1 (100)</td>
</tr>
<tr>
<td>VIM-27 (1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1 (100)</td>
</tr>
<tr>
<td>VIM-33 (1)</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td>1 (100)</td>
<td>0</td>
<td>1 (100)</td>
</tr>
<tr>
<td>IMP-26 (5)</td>
<td>2 (40)</td>
<td>2 (40)</td>
<td>2 (40)</td>
<td>1 (20)</td>
<td>5 (100)</td>
</tr>
<tr>
<td>OXA-48-like enzyme (14)</td>
<td>2c (14)</td>
<td>1c (7)</td>
<td>0</td>
<td>13 (93)</td>
<td>14 (100)</td>
</tr>
<tr>
<td>OXA-48 (7)</td>
<td>1 (14)</td>
<td>0</td>
<td>6 (86)</td>
<td>7 (100)</td>
<td></td>
</tr>
<tr>
<td>OXA-163 (2)</td>
<td>0</td>
<td>0</td>
<td>2 (100)</td>
<td>2 (100)</td>
<td></td>
</tr>
<tr>
<td>OXA-181 (5)</td>
<td>1 (20)</td>
<td>0</td>
<td>5 (100)</td>
<td>5 (100)</td>
<td></td>
</tr>
<tr>
<td>Carbapenem resistant, carbapenemase negative (28)</td>
<td>2c (8)</td>
<td>2c (8)</td>
<td>0</td>
<td>2 (8)</td>
<td>0</td>
</tr>
</tbody>
</table>

Total (142) | 91 (64) | 93 (65) | 69 (49) | 142 (100) |

a MDI, Mastdiscs ID inhibitor combination disks; RDS, Rosco Diagnostica Neo-Sensitabs; MBL, metallo-β-lactamase; MHT, modified Hodge test.

b RDS identified 3 KPC producers as both KPC and NDM positive, while 8 NDM producers were identified as both NDM and KPC positive.

c The 2 isolates were AmpC producers with altered permeability and were identified by MDI and RDS as AmpC and KPC positive.

d The OXA-48-like enzyme producers were identified by MDI and RDS as MBL positive.

Pseudomonas aeruginosa (18). This Etest is often difficult to interpret when investigating the presence of MBLs in Enterobacteriaceae (9). This is due to the fact that the MICs of imipenem (i.e., the substrate used in the MBL Etest) are often low in Enterobacteriaceae that produce MBLs. Our outcome was similar when using the MBL Etest, and only 32/51 (63%) of NDM-, VIM-, and IMP-producing Enterobacteriaceae gave positive results with this method. However, the specificity of the MBL Etest was 100% (i.e., none of the KPC-producing, OXA-48-like enzyme-producing, or carbapenemase-negative isolates gave positive results) (Table 2).

The Mastdiscs ID inhibitor combination disks had a sensitivity of 98% and specificity of 93% for detecting KPC producers and 100% sensitivity and specificity for NDM producers, but only 12/24 (50%) of VIM and IMP-producers were positive with this method (Table 2). The Rosco Diagnostica Neo-Sensitabs gave similar results; a sensitivity of 100% and specificity of 93% for detecting KPC-producing, 100% sensitivity and specificity for NDM-producers while 14/24 (58%) of VIM and IMP producers were positive with this method (Table 2). The Rosco Diagnostica Neo-Sensitabs had an additional problem in that the method identified 3 KPC producers as being both KPC and NDM positive, while 8 NDM producers were identified as being both NDM and KPC positive. The commercial systems performed well for the detection of KPCs and NDMs but poorly for VIM-, IMP-, and OXA-48-like enzyme-producing isolates. However, both methods were easy to perform and interpret and could easily be introduced into the work flow of the clinical microbiology laboratory.

The Mastdiscs ID inhibitor combination disks and Rosco Diagnostica Neo-Sensitabs were not specifically designed to detect Enterobacteriaceae that produce OXA-48-like β-lactamases. The phenotypic detection of Enterobacteriaceae that produce these β-lactamases remains problematic (16). The β-lactamase inhibitor NXL104 inhibits the hydrolytic activity of OXA-48-like enzymes but unfortunately is not specific for these carbapenemases (1). The high-level resistance to the β-lactam temocillin might be presumptive evidence for the presence of OXA-48 (4).

Several in-house multiplex PCRs and commercial real-time PCR and DNA microarray methods performed well for the detection of different types of carbapenemases in Enterobacteriaceae (8, 9). The in-house multiplex PCR from our study included primers for the detection of blakp, blanmd, blaoxa-48-like, blavim, and blaimp and had a sensitivity and specificity of 100% (Table 2).

The clinical laboratory acts as an early warning system, alerting the medical community to new resistance mechanisms present in clinically important bacteria. We believe that the presence of carbapenemases among Enterobacteriaceae is an infection control emergency and that the detection of these bacteria in clinical laboratories is a critical step required for appropriate management of patients and infection prevention and control efforts. Clinical microbiology laboratories should be able to rapidly detect these enzymes among members of the Enterobacteriaceae, especially when these enzymes are first introduced into the local bacterial population. We recommend using such molecular tests for the optimal detection of these isolates and feel that initially it is important to know what type of carbapenemase is present. Unfortunately, these tests are expensive and often are available only in large referral or research laboratories.

Phenotypic methods for the detection of antimicrobial resistance among Gram-negative bacteria, in general terms, are easy to perform, interpret, and introduce into the work flow of a clinical...
laboratory. We recommend an initial screening for Enterobacteriaceae by using >0.5 μg/ml of ertapenem and >1 μg/ml of meropenem, imipenem, or doripenem. If a clinical laboratory does not have access to or cannot afford to perform molecular tests, phenotypic tests such as the Mastdiscs ID inhibitor combination disks or Rosco Diagnostica Neo-Sensitabs and the modified Hodge test can be used to confirm the presence of carbapenemases. The commercial inhibitor disk methods will be able to identify KPC and NDM producers, while the modified Hodge test will ensure that OXA-48-like enzyme producers are detected. This will provide a cost-effective and rapid approach for the detection of carbapenemases in Enterobacteriaceae.

ACKNOWLEDGMENTS
J.D.D.P. has previously received research funds from Merck and Astra Zeneca.

This work was supported by a research grant from the Calgary Laboratory Services (73-6350).

REFERENCES