Modified Hodge test using Mueller–Hinton agar supplemented with cloxacillin improves screening for carbapenemase-producing clinical isolates of Enterobacteriaceae

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Increasing numbers of clinical isolates of Enterobacteriaceae that produce carbapenemase are now being detected, with the most common carbapenemase found among Enterobacteriaceae in Japan being IMP-1-type metallo-β-lactamase. Clinical isolates of Enterobacteriaceae harbouring carbapenemases may be resistant to carbapenem antimicrobial agents, despite apparent in vitro susceptibility when tested according to Clinical and Laboratory Standards Institute criteria. We evaluated the prevalence of carbapenemase producers among isolates of Enterobacteriaceae at our hospital and assessed the performance of the modified Hodge test (MHT) for correctly identifying the phenotype. We studied 47 clinical isolates obtained between 2006 and 2010 for which the MIC of imipenem was 2 or 4 μg imipenem ml⁻¹. Antibacterial susceptibility testing was done for cephalosporins and carbapenems, the MHT was performed with meropenem and detection of the genes encoding IMP-1, VIM-2, KPC-2 and NDM-1-type metallo-β-lactamases was performed by PCR. Twelve isolates showed a positive result in the MHT with meropenem and were classified as carbapenemase producers. Of these 12 isolates, seven carried the gene for IMP-1 type, but not for VIM-2, KPC-2 or NDM-1 types. None of the carbapenemase genes tested were detected in the other five isolates. All five isolates were Enterobacter cloacae showing high resistance to ceftazidime and aztreonam. False-positive results were inhibited when Mueller–Hinton agar supplemented with 200 mg cloxacillin ml⁻¹ was used for the MHT. Five of 12 MHT-positive isolates were shown to have no carbapenemase genes and these isolates were high AmpC producers. Adding cloxacillin when performing the MHT prevented such false-positive results. The MHT with cloxacillin can overcome most problems related to detection of carbapenemases.

INTRODUCTION

Bacterial resistance mediated by β-lactamase is increasingly associated with plasmid-encoded carbapenemases, including the Klebsiella pneumonia carbapenemase (KPC) family of serine carbapenemases (mainly in the USA), as well as Verona integron-encoded metallo-β-lactamase (VIM) (mainly in the Mediterranean region), imipenemase metallo-β-lactamase (IMP)-1 (mainly in Asia, including Japan) and New Delhi metallo-β-lactamase (NDM)-1 and oxacillinase (OXA)-48 (metallo-β-lactamase carbapenemases that are now endemic or rare) (Oteo et al., 2014). However, carbapenemase-producing Enterobacteriaceae may have imipenem MICs as low as 0.125 μg imipenem ml⁻¹ (Psichogiou et al., 2008).

Abbreviation: MHT, modified Hodge test.
This suggests that several carbapenem screening tests may be needed to identify all strains requiring confirmation. Also, resistance of *Enterobacteriaceae* to β-lactams and other antibiotics is frequently associated with plasmid-mediated resistance determinants that are easily transferred among species.

The modified Hodge test (MHT) (http://www.cdc.gov/hai/) can detect carbapenemase production in isolates of *Enterobacteriaceae*. The Clinical and Laboratory Standards Institute (CLSI) has recommended that carbapenem-susceptible *Enterobacteriaceae* with elevated MICs or reduced disc diffusion inhibition zones should be tested for production of carbapenemases (CLSI, 2009). However, some isolates show a slight indentation in the MHT, but do not produce carbapenemase (Carvalhaes et al., 2010; Thomson, 2010; Giske et al., 2011). In this study, we evaluated the performance of the MHT for correctly identifying carbapenemase production by *Enterobacteriaceae*.

**RESULTS**

The susceptibility rates for imipenem, ceftazidime and aztreonam were 70.2% (33/47), 66.0% (31/47) and 83.0% (39/47), respectively (data not shown). Twelve of the 47 isolates (25.5%) were classified as carbapenemase producers based on a positive MHT with meropenem. Among these 12 isolates with a positive MHT, PCR detected the gene for IMP-1 in seven isolates (58.3%), but not genes for VIM-2, KPC-2 or NDM-1. None of the carbapenemase genes tested were detected in the other five isolates (41.7%) (Table 1). The five isolates that tested negative for carbapenemase genes were found to be high-level AmpC producers with a strongly positive P/Case test result and were highly resistant to aztreonam. These isolates were then retested using a MHT method that was unaffected by AmpC β-lactamases, which involved adding 200 μg cloxacillin ml⁻¹ (an AmpC inhibitor) to the culture medium. When we compared the common MHT to this revised MHT, we found that adding cloxacillin inhibited indentation by a high-level AmpC-producing strain without any carbapenemase genes (Fig. 1a, b), but not a strain bearing the IMP-type carbapenemase gene (Fig. 1c, d).

Among the 12 strains that were positive in the common MHT, five strains (*E. cloacae* KY842, 825, 867, 855 and 856) that did not possess carbapenemase genes were negative in the revised MHT with 200 μg cloxacillin ml⁻¹, while the remaining seven strains with IMP-1 genes were still positive in the revised MHT. Thus, false-positive results induced by high-level AmpC production could be corrected by performing the MHT with cloxacillin.

**DISCUSSION**

Currently, the breakpoints for carbapenems have been lowered in the US (CLSI) and European (The European Committee on Antimicrobial Susceptibility Testing: EUCAST) standards to permit better detection of carbapenem-resistant isolates. According to the CLSI (2012), the breakpoints of imipenem and meropenem are ≤ 1 for susceptible and ≥ 4 μg ml⁻¹ for resistant, while EUCAST still defines imipenem susceptibility as up to 2 μg imipenem ml⁻¹ (EUCAST, 2015). In our study, the MIC of imipenem was ≥ 2 μg ml⁻¹ for 14 strains, but IPM-1, VIM-2, KPC-2 and NDM-1 were not detected in eight of these. Therefore, detection of carbapenemase producers based only on MIC values may lack sensitivity. Twelve strains with a positive MHT all showed ceftazidime resistance (Table 1). For this reason, ceftazidime is used for daily examination of *Enterobacteriaceae* and can be suitable for screening MBL-producing strains. Cloxacillin is a cephalosporinase inhibitor that can be used to prevent growth of *Enterobacteriaceae* isolates with high expression of cephalosporinases (Carvalhaes et al., 2010).

As shown in Fig. 1, inhibition of AmpC activity by plating test strains on cloxacillin-containing agar allowed differentiation between strains that would recover susceptibility to carbapenems and those exhibiting low-level carbapenem resistance owing to carbapenem production. Moland...
et al. (2008) reported that false-positive MHT results can occur with high-level AmpC producers. In addition, Bartolini et al. (2014) suggested that if negative results are obtained for carbapenem-non-susceptible strains, further analysis by the cloxacillin inhibition test will allow unambiguous detection of AmpC enzyme production. Meropenem discs supplemented with dipicolinic acid, EDTA, aminophenylboronic acid, CHROMagar KPC and chromID CARBA medium (Samra et al., 2008; Giske et al., 2011; Papadimitriou-Olivgeris et al., 2014) would be able to discriminate among Enterobacteriaceae with various carbapenemases. Recent studies have assessed the frequency of falsely detecting carbapenemase production in Enterobacteriaceae with the MHT in the routine clinical microbiology setting by employing zinc sulfate (SUPERCARBA medium) (Nordmann et al., 2012) and the CarbaNP test (Vasoo et al., 2013). Mathers et al. (2013) demonstrated the indirect carbapenemase test was superior to the MHT for non-Klebsiella Enterobacteriaceae.

Galani et al. (2008) highlighted one limitation of the MHT, which is that imipenem-susceptible isolates with a positive MHT that are negative by all other methods produce AmpC from chromosomal or plasmid-encoded genes. Therefore, equivocal MHT isolates must be confirmed by another method. There have been a few published surveys on the detection of KPC and metallo-β-lactamase carbapenemase in Enterobacteriaceae using meropenem discs supplemented with cefoxitin (Giske et al., 2011; Pasteran et al., 2011), but none of these reports covered the MHT using agar supplemented with cefoxitin. Therefore, our method is the first to be used for detection of

### Table 1. Characteristics of the 12 MHT-positive isolates of Enterobacteriaceae

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC (µg ml⁻¹)</th>
<th>Carbapenemase gene*</th>
<th>AmpC production†</th>
<th>MHT Without cloxacillin</th>
<th>MHT With cloxacillin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Imipenem</td>
<td>Ceftazidime</td>
<td>Aztreonam</td>
<td>IMP-1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>&gt;128</td>
<td>0.5</td>
<td>IMP-1</td>
<td>+</td>
</tr>
<tr>
<td>K. pneumoniae KY869</td>
<td>2</td>
<td>128</td>
<td>0.25</td>
<td>IMP-1</td>
<td>+</td>
</tr>
<tr>
<td>E. cloacae KY831</td>
<td>2</td>
<td>128</td>
<td>0.12</td>
<td>IMP-1</td>
<td>+</td>
</tr>
<tr>
<td>E. cloacae KY835</td>
<td>8</td>
<td>&gt;128</td>
<td>0.5</td>
<td>IMP-1</td>
<td>+</td>
</tr>
<tr>
<td>E. cloacae KY849</td>
<td>0.5</td>
<td>&gt;128</td>
<td>32</td>
<td>IMP-1</td>
<td>+</td>
</tr>
<tr>
<td>E. cloacae KY824</td>
<td>2</td>
<td>&gt;128</td>
<td>4</td>
<td>IMP-1</td>
<td>+</td>
</tr>
<tr>
<td>E. cloacae KY842</td>
<td>0.5</td>
<td>64</td>
<td>64</td>
<td>negative</td>
<td>+</td>
</tr>
<tr>
<td>E. cloacae KY825</td>
<td>1</td>
<td>64</td>
<td>32</td>
<td>negative</td>
<td>+</td>
</tr>
<tr>
<td>E. cloacae KY867</td>
<td>2</td>
<td>64</td>
<td>64</td>
<td>negative</td>
<td>+</td>
</tr>
<tr>
<td>E. cloacae KY855</td>
<td>2</td>
<td>128</td>
<td>64</td>
<td>negative</td>
<td>+</td>
</tr>
<tr>
<td>E. cloacae KY856</td>
<td>4</td>
<td>&gt;128</td>
<td>128</td>
<td>negative</td>
<td>+</td>
</tr>
</tbody>
</table>

*IMP-1, VIM-2, KPC-2 and NDM-1 genes were tested by PCR.
†Assayed by the P/Case test.
+ , yellow in 30 mins; ++, yellow in 10 mins; +++ yellow in immediately.

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**Fig. 1.** Improved performance of the MHT supplemented with cloxacillin. In the common MHT, *E. cloacae* KY867 (a) with high-level AmpC production and no detectable carbapenemase genes and *E. cloacae* KY831 (c) possessing the IMP-1 gene both show indentation of the inhibition zone. In the revised MHT with cloxacillin, indentation of the zone no longer occurred with *E. cloacae* KY867 (b), but was still observed with *E. cloacae* KY831 (d).
carbapenemase production by *Enterobacteriaceae* which is not influenced by AmpC.

In conclusion, the common MHT can incorrectly report a positive result for carbapenemase production by *Enterobacteriaceae*, possibly due to high AmpC production. PCR-based techniques remain the reference standard for identification and differentiation of carbapenemases, but have the disadvantages of higher cost and the requirement for trained technicians. Detecting carbapenemases by the revised MHT with cloxacillin is a rapid and cost-effective test that can identify resistant pathogens in any laboratory.

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**REFERENCES**


