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Mădălina Maria Muntean, Andrei-Alexandru Muntean, François Guerin, Vincent Cattoir, Elodie Creton, Garance Cotellon, Saoussen Oueslati, Mircea Ioan Popa, Delphine Girlich, Bogdan Iorga, et al.

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**OPTIMISATION OF THE RAPID CARBAPENEM INACTIVATION METHOD FOR  
USE WITH AMPC HYPERPRODUCERS**

Mădălina-Maria MUNTEAN,<sup>1,2,3§</sup> Andrei-Alexandru MUNTEAN,<sup>1,2,3§</sup> François GUERIN,<sup>4,5</sup>  
Vincent CATTOIR,<sup>4,5,6</sup> Elodie CRETON,<sup>1,7,8,9</sup> Garance COTELLON,<sup>1,7,8,9</sup> Saoussen  
OUESLATI,<sup>1,7,8,9</sup> Mircea Ioan POPA,<sup>2,3</sup> Delphine GIRLICH,<sup>1,8,9</sup> Bogdan I. IORGA,<sup>10</sup> Rémy  
A. BONNIN,<sup>1,8,9</sup> and Thierry NAAS<sup>1,7,8,9</sup> \*

- 1 Team RESIST, INSERM U1184, School of Medicine, Université Paris-Saclay, LabEx LERMIT, Le Kremlin-Bicêtre, France
- 2 The “Carol Davila” University of Medicine and Pharmacy, Bucharest, Romania
- 3 The “Cantacuzino” National Medico-Military Institute for Research and Development, Bucharest, Romania
- 4 CHU de Rennes, Service de Bactériologie-Hygiène Hospitalière, Rennes, France.
- 5 CNR de la Résistance aux Antibiotiques (laboratoire associé 'Entérocoques'), Rennes, France.
- 6 Université de Rennes 1, Unité Inserm U1230, Rennes, France.
- 7 Bacteriology-Hygiene unit, Assistance Publique/Hôpitaux de Paris, Bicêtre Hospital, Le Kremlin-Bicêtre, France
- 8 French National Reference Center for Antibiotic Resistance: Carbapenemase-producing Enterobacteriaceae, Le Kremlin-Bicêtre, France
- 9 Joint research Unit EERA « Evolution and Ecology of Resistance to Antibiotics », Institut Pasteur – APHP - Université Paris Sud, Paris, France
- 10 Université Paris-Saclay, CNRS, Institut de Chimie des Substances Naturelles, Gif-sur-Yvette, France

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31 § Both authors contributed equally

32 \* Corresponding author: T. Naas, Service de Bactériologie-Hygiène, Hôpital Bicêtre, 78 rue  
33 du Général Leclerc, 94270 Le Kremlin-Bicêtre, France. Tel: +33 1 45 21 20 19. Fax: +33 1  
34 45 21 63 40. E-mail: [thierry.naas@aphp.fr](mailto:thierry.naas@aphp.fr)

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36

37 **Abstract (249)**

38 **Objectives:** Detection of carbapenemase-producing *Enterobacterales* (CPEs) is sometimes  
39 difficult with AmpC-hyperproducing *Enterobacterales* (AHE), as they may falsely be  
40 classified as CPEs. Here, we present a rapid Carbapenem Inactivation Method optimized for  
41 AmpC producers (rCIM-A) that allows rapid and easy discrimination between AHEs and  
42 CPEs.

43 **Methods:** *Enterobacterales* (n=249), including natural AmpC producers, AHEs, CPEs, and  
44 non-carbapenemase carbapenem-resistant control strains were evaluated, using the Carba NP,  
45 rCIM, and rCIM-A. The latter differs from rCIM by the addition of cloxacillin (400 µg/mL) to  
46 the initial antibiotic incubation step.

47 **Results:** The rCIM-A yielded a sensitivity and a specificity of 84.26% (95% CI: 76 - 90.55%)  
48 and 99.29% (95% CI: 96.11 - 99.98%), respectively, while those of the rCIM were 86.11%  
49 (95% CI: 78.13 - 92.01%) and 80.85% (95% CI: 73.38 - 86.99%), respectively, while those of  
50 the CarbaNP were lower 84.04% (95% CI: 75.05 - 90.78%) and 91.37% (95% CI: 85.41 -  
51 95.46%), respectively, due to indeterminate results. The rCIM-A was capable to discriminate  
52 between AHEs and true CPEs, but still fails to identify OXA-23 producing *P. mirabilis* isolates  
53 and remains only partially reliable to identify IMI-like- and a few metallo-β-lactamase- (2  
54 NDM-1, 1 LMB-1, 1 TMB-1 and 1 IMP-13) producers. One chromosomally-encoded AmpC  
55 variant, MIR-10, gave repeatedly positive results using the three tests and was thus considered  
56 a false positive.

57 **Conclusions:** Specificity for AHEs greatly improved with rCIM-A without altering the test  
58 performances for the other resistance mechanisms. It may replace rCIM as a cheap, easy, rapid  
59 and accurate CPE detection test.

60

## 61 **Introduction**

62           Strains belonging to the *Enterobacter cloacae* complex, *Citrobacter* spp., *Serratia* spp.,  
63 *Morganella morganii* or *Providencia* spp. are Gram-negative bacilli that naturally-produce an  
64 inducible chromosomally-encoded AmpC  $\beta$ -lactamase (cAmpC).<sup>1</sup> At the basal level of  
65 production, AmpC confers resistance to penicillins, first- and second-generation  
66 cephalosporins whereas when overproduced it also hydrolyses oxyimino-cephalosporins (such  
67 as ceftriaxone, cefotaxime, and ceftazidime) and monobactams but spares cefepime and  
68 carbapenems, and it is not inhibited by classical  $\beta$ -lactamase inhibitors such as clavulanic acid.  
69 Noteworthy, these enzymes are inhibited by cloxacillin and 3-aminophenylboronic acid.<sup>1</sup>  
70 Although not as widely distributed as other *Enterobacterales* species such as *Escherichia coli*  
71 or *Klebsiella pneumoniae*, AmpC-producers are responsible for a wide range of nosocomial  
72 infections such as bacteraemia in both neonatal and adult intensive care units, urinary tract  
73 infections, infections in orthopaedic or transplant patients.<sup>2-4</sup> The EPIC II (Extended Prevalence  
74 of Infection in Intensive Care) study, an international 1-day, prospective, point prevalence  
75 study conducted in 2007 by JL Vincent *et al.*, revealed that *Enterobacter* spp. infections  
76 accounted for 7% of the total Gram-negative bacterial (GNB) infections.<sup>5</sup> Carbapenem  
77 resistance mechanisms in AmpC-producers may be complicated to decipher, as it can be linked  
78 to AmpC hyperproduction associated with a decrease in outer membrane permeability.<sup>6</sup>

79           We have recently described the rapid Carbapenem Inactivation Method (rCIM), a  
80 variant of the CIM that takes only 3 hours versus an 8-hour to overnight incubation for the  
81 CIM.<sup>7</sup> While the performances of both of these tests were excellent, a few false positives were  
82 observed, especially with AmpC-producers. Indeed, Muntean *et al.* reported only two false  
83 positives, that were two *E. cloacae* strains.<sup>7</sup> In France, 70 - 90% of carbapenem-resistance in  
84 *E. cloacae* complex is not linked to carbapenemase production.<sup>8</sup>

85           The aim of this work was to optimize the rCIM test for species naturally producing  
86 AmpCs. The resulting optimized test (rCIM-A) was evaluated using well characterized AmpC-  
87 producing *Enterobacterales* (including AmpC hyperproducing *Enterobacterales* (AHEs)) and  
88 a collection of *Enterobacterales* with reduced susceptibility to at least one carbapenem  
89 received at the French National Reference Center for Carbapenem-Resistant *Enterobacterales*  
90 (F-NRC-CREs).

91

## 92 **Materials and Methods**

### 93 **Bacterial isolates tested**

94           Forty-eight strains of *Enterobacter* spp. were kindly provided by F. Guérin.<sup>9</sup> They  
95 represent a well characterized collection containing representative strains from the 13 clusters:  
96 3 strains from Clusters I (*E. asburiae*) and II (*E. kobei*) each, 8 strains from Cluster III, 4 strains  
97 from Cluster IV and V (*E. ludwigii*), 5 strains from Cluster VI (*E. hormaechei* subsp. *oharae*)  
98 and VIII (*E. hormaechei* subsp. *steigerwallti*), 6 strains from Cluster XI (*E. cloacae* subsp.  
99 *cloacae*) and 2 strains from Cluster VII (*E. hormaechei* subsp. *hormaechei*), IX, X (*E.*  
100 *nimipressuralis*), XII (*E. cloacae* subsp. *dissolvens*) and XIII, each (Table S1).<sup>9</sup>

101           Hundred and thirty-four other AmpC-producing *Enterobacterales* including *E. cloacae*  
102 complex (ECC, n = 82), *Morganella* spp., *Serratia* spp., *Hafnia alvei*, and *Citrobacter* spp. (n=  
103 52) came from F-NRC-CREs (Tables S2 and 1). Finally, 67 control *Enterobacterales* (*E. coli*,  
104 *K. pneumoniae*, *K. oxytoca*, *Proteus mirabilis*) that included 48 true CPEs were evaluated  
105 whether optimization has no incidence on the analytical performances previously obtained with  
106 the rCIM (Table 2).<sup>7</sup>

107           All isolates were sub-cultured from frozen stocks (stored at -80 °C) on UriSelect™ 4  
108 (BioRad, Marne-la-Coquette, France) for 24 h at 37 °C, and on Mueller-Hinton agar for an  
109 additional 24 h at 37 °C.

110 **Biochemical test for the presence of a carbapenemase**

111 The in-house Carba NP test was performed as previously described.<sup>7,10</sup>

112

113 **Rapid Carbapenem Inactivation Method (rCIM) and Rapid Carbapenem Inactivation**  
114 **Method for AmpC producers (rCIM-A)**

115 The rCIM was used as previously described.<sup>7</sup> The rCIM-A differs from rCIM by the addition  
116 of cloxacillin to the initial antibiotic incubation step. Injectable cloxacillin powder (Orbenin®  
117 1g injectable powder, Astellas Pharma, Levallois Perret, France) was used instead of the  
118 purified active principle, as no difference in potency was observed in preliminary experiments  
119 (data not shown). Cloxacillin was prepared by suspending 1g of active substance in 2.5 mL of  
120 sterile distilled water (stock concentrations of 400 µg/µL). Stock solution was aliquoted and  
121 kept in the freezer (-20 °C) until use. One to 10 µL were added to the Eppendorf tube  
122 (concentrations of 400 - 4,000 µg/mL) before the addition of the bacteria. The rest of the  
123 protocol remained unchanged.<sup>7</sup> Briefly, after incubation and centrifugation, the resulting  
124 supernatant is used to challenge growth of the susceptible *Escherichia coli* ATCC 25922 strain,  
125 as monitored by nephelometry (Densitometer DEN-1B, Grant Bio, Cambridgeshire, UK)).  
126 Growth was expressed as baseline subtracted growth in McFarland units, as previously  
127 described.<sup>7</sup> A positive and a negative control of a *K. pneumoniae* ATCC BAA 1705 and 1706,  
128 respectively, were used for each batch of tests.

129

130 **Statistical analysis**

131 The overall characteristics of the test (sensitivity, specificity, positive and negative predictive  
132 values) were calculated, with their respective 95% CI, using the R v. 4.0.2 software suite and  
133 epiR v. 2.0-19.<sup>11-13</sup>

134

135 **Whole Genome Sequencing**

136 Discrepant results were further characterized using WGS using a DNA library prepared using  
137 the Nextera XT-v2 kit (Illumina, Paris, France) and then run on NextSeq 500 automated system  
138 (Illumina), using a 2 × 100-bp paired-end approach, as previously described.<sup>14</sup>

139

140 **Nucleotide accession numbers**

141 The genome determined in this study is available at NCBI under the accession number of  
142 JAFEWA000000000.

143

144 **Results and discussion**

145 **Evaluation of effect of Cloxacillin**

146 The effect of cloxacillin was firstly evaluated on *E. coli* ATCC 25922 indicator strain. No  
147 inhibitory effect was observed on the baseline-subtracted growth of the *E. coli* indicator strain  
148 neither with 400 µg/mL, which is the concentration at which cloxacillin will be used for the  
149 rCIM-A nor with 4,000 µg/mL, a much higher concentration to clearly demonstrate the non-  
150 susceptibility of *E. coli* ATCC 25922 to cloxacillin (data not shown).

151

152 **Reference strains of the *Enterobacter cloacae* complex.**

153 Forty-eight reference strains of the *Enterobacter cloacae* complex were studied, among which  
154 15 were AmpC overproducers, as revealed by phenotypical methods (31,25%) and one was an  
155 OXA-204 carbapenemase-producer.<sup>9</sup> Results are presented in Table S1, Table 3 and Figure 1.  
156 Inconclusive (+/-) CarbaNP results were considered as false positive results, thus resulting in  
157 a specificity of 95.74% (95% CI: 85.46 - 99.48%). CarbaNP was not able to detect the OXA-  
158 204 producing *E. hormaechei* subsp. *oharae* (cluster VI; Table S1). Indeterminate results were



159 obtained in a cluster I *E. asburiae* strain harbouring only its AmpC and in the AmpC-  
160 derepressed reference ATCC 13047 *E. cloacae* subsp. *cloacae* ( $\Delta$ ampD).  
161 The standard rCIM protocol had a specificity of 91.49% (95% CI: 79.62 - 97.63%). rCIM was  
162 falsely positive for 4 strains not producing carbapenemases. This included the two strains with  
163 indeterminate CarbaNP results as well as two other strains from clusters IV and V (Table S1).  
164 With the addition of 400  $\mu$ g/mL of cloxacillin, the specificity of the rCIM-A rose to 100 % (95%  
165 CI: 92.45 – 100%). Both rCIM protocols correctly identified the presence of the OXA-204  
166 carbapenemase (Table S1).

167

#### 168 ***Enterobacter* spp. isolates from the French National Reference Center for CREs**

169 *Enterobacter* spp. strains (n = 82) with reduced susceptibility to at least one carbapenem  
170 corresponded to isolates from the F-NRC-CREs reference strain collection and to those  
171 prospectively received during September 2020 were tested. This collection included both CPEs  
172 (n = 32) as well as isolates producing or overproducing their natural AmpCs, associated with  
173 ESBL production and/or impaired outer membrane permeability. Results are presented in Table  
174 S2, and Table 3.

175 CarbaNP had a sensitivity and specificity of 93.75% (95% CI: 79.19 - 99.23%) and 76% (95%  
176 CI: 61.83 – 86.94%), respectively. The CarbaNP test failed to identify a GES-5 producing  
177 isolate, and one IMI-13-producer, as shown by others.<sup>15-17</sup>

178 The sensitivity and specificity of the rCIM were 84.38% (95% CI: 67.21 - 94.72%) and 62%  
179 (95% CI: 47.17 – 75.35%), respectively. The addition of cloxacillin resulted in a sensitivity of  
180 81.25% (95% CI: 63.56 - 92.79%) and a specificity of 98% (95% CI: 89.35 – 99.95%). Both,  
181 rCIM and rCIM-A, failed to identify several IMI-producing isolates (IMI-6, IMI-13 and 2 IMI-  
182 17). This result is not surprising, as IMI-enzymes are known to preferentially hydrolyse  
183 imipenem, and to a weaker extend meropenem.<sup>18</sup> When these tests were performed with

184 bacteria grown next to the imipenem discs from a routine disk diffusion test, they were  
185 repeatedly positive. These results are consistent with our previous findings, with IMI-17-  
186 producing isolates that gave false-negative results in the absence of induction.<sup>7,18</sup> Using  
187 cloxacillin, one IMI-13 isolate gave an additional false-negative result. The initial positive  
188 result is likely due to the overproduced AmpC and not to IMI-13. Lastly, one TMB-1 producing  
189 *E. cloacae* isolate gave a negative rCIM-A result, even though the CarbaNP test was positive.<sup>14</sup>  
190 Of note, we included 10 *E. kobei* ST125 isolates that harbour a chromosome-encoded ACT-28  
191 and are known to give false-positive Carba NP and rCIM results.<sup>19</sup> Indeed, false positive results  
192 were obtained in all ACT-28-producing *E. kobei* strains with the Carba NP test and the rCIM  
193 protocol. However, using rCIM-A, all ACT-28 isolates were correctly categorized as lacking  
194 acquired carbapenemase activity. Alongside these, one strain of *E. cloacae*, characterized as a  
195 cAmpC hyperproducer on the basis of its antibiogram, gave consistent positive results, even  
196 after addition of cloxacillin (400 as well as 4,000 µg/mL). This strain was whole-genome  
197 sequenced and was found to harbour a novel variant of MIR-10 cephalosporinase. Interestingly,  
198 this showed a dose-dependent response inhibition of cAmpC activity with cloxacillin (2.39  
199 baseline-subtracted growth with 400 µg/mL and 0.71 baseline-subtracted growth with 4,000  
200 µg/mL).

201 The two AmpC-hyperproducing *E. cloacae* isolates (Table S2, 143A8 and 143A9) that gave  
202 false-positive results in the course of the initial evaluation of the rCIM, gave consistently  
203 negative results with the rCIM-A.<sup>7</sup>

204

### 205 **Impact of chromosomally-encoded AmpCs in various other *Enterobacterales***

206 A collection of 52 Group 3 *Enterobacterales* were evaluated; of these 27 were carbapenemase  
207 producers. CarbaNP had a sensitivity of 92.59% (95% CI: 75.71 - 99.09%) and a specificity  
208 100% (95% CI: 86.28 - 100%). Using the standard rCIM protocol, the sensitivity and the

209 specificity were 92.59% (95% CI: 75.71 - 99.09%) and 84% (95% CI: 63.92 - 95.46%),  
210 respectively. Using the rCIM-A, the sensitivity slightly decreased to reach 88.89% (95% CI:  
211 70.84 - 97.65%) but the specificity reached 100% (95% CI: 86.28 - 100%) (Tables 1 and 3).  
212 One NDM-1-producing *M. morgani* isolate tested positive with the rCIM but turned negative  
213 with the addition of cloxacillin (400 µg/mL), suggesting that the initial positivity was likely  
214 due to the over-produced AmpC and not to the low-level production of NDM-1-carbapenemase.  
215 This isolate tested positive in Carba NP test. Similarly, another NDM-producing *M. morgani*  
216 isolate tested negative in both rCIM and was indeterminate in Carba NP. In both cases, colonies  
217 taken from carbapenem-containing plates (bioMérieux chromID® CarbaSmart), UriSelect™ 4  
218 and an antibiogram (close to the meropenem disc) gave positive rCIM-A results, likely due to  
219 induction.<sup>18</sup> Finally, *C. freundii* producing LMB-1, was negative in both rCIM tests but became  
220 positive upon induction (growth next to imipenem disc) and failed to grow on chromID®  
221 CarbaSmart media (Table 1).<sup>20</sup> This strain was positive using the Carba NP test.

222

### 223 **Other *Enterobacterales* and plasmid encoded AmpCs**

224 To validate that the addition of cloxacillin did not influence results on other *Enterobacterales*,  
225 67 additional isolates were tested including 48 CPEs, especially difficult to detect enzymes,  
226 such as the slow-hydrolysing OXA-48-variants. In addition, 7 isolates presented acquired  
227 cephalosporinases and 1 isolate a derepressed chromosomally-encoded cephalosporinase.  
228 Results are presented in Tables 2 and 3.

229 Carba NP had a sensitivity and specificity of 50% (95% CI: 35.23 - 64.77%) and 100% (95%  
230 CI: 82.35 - 100%), respectively, while the values for rCIM and rCIM-A were 83.33% (95%  
231 CI: 69.78 - 92.52%) and 100% (95% CI: 82.35 - 100%), respectively.

232 As already published, Carba NP has a low sensitivity for certain OXA-48-variants.<sup>21,22</sup> The  
233 rCIM-A clearly identified OXA-162, OXA-181, OXA-204 and OXA-232 harbouring isolates.  
234 Only OXA-23 producing *P. mirabilis* (n = 10) were not reliably identified by either rCIM.<sup>22,23</sup>

235

### 236 **Overall sensitivity and specificity.**

237 Overall, 249 *Enterobacterales* isolates were tested, 48 ECC reference isolates and 201 from  
238 the French National Reference Center for CPEs, of which 108 were CPEs.

239 The difficulties in interpreting the Carba NP test result have already been described in the  
240 literature, which are often linked to the appearance of slight colour changes.<sup>24</sup> A total of 16  
241 isolates gave an indeterminate result in Carba NP. Proceeding as per CLSI guidelines, these  
242 tests would be considered invalid and would be eliminated from statistical analysis. Thus, the  
243 overall Carba NP sensitivity for n=233 was 84.04% (95% CI: 75.05 - 90.78%) and  
244 the specificity was 91.37% (95% CI: 85.41 - 95.46%). Taking a different, pragmatic approach,  
245 if we were to consider the Carba NP indeterminate as either a positive or a negative, then this  
246 would result in a sensitivity between 73.15% (95% CI: 63.76 - 81.22%) and 86.11% (95% CI:  
247 78.13 - 92.01%) and a specificity between 90.07% (95% CI: 83.9 - 94.46%) and 91.49% (95%  
248 CI: 85.61 - 95.52%) (Table 3).

249 The sensitivity and specificity of the rCIM were 86.11% (95% CI: 78.13 - 92.01%) and 80.85%  
250 (95% CI: 73.38 - 86.99%), respectively. The rCIM gave false positive results in isolates  
251 harbouring a derepressed chromosomal cephalosporinase, such as ACT-28, or the novel MIR-  
252 10 variant.

253 The addition of cloxacillin to the rCIM test, led to a sensitivity and specificity for AmpC  
254 producers of 84.26% (95% CI: 76 - 90.55%) and 99.29% (95% CI: 96.11 - 99.98%),  
255 respectively (Table 3, and Figure 2).

256 The strength of this study is that we have evaluated a very broad range of carbapenem-resistant  
257 *Enterobacterales*, which included true CPEs, as well as AmpC hyperproducers (both natural  
258 and acquired).

259 Potential other false-positive results of CIM-derived tests have been reported both with the  
260 CIM and the modified CIM (mCIM).<sup>25</sup> In one recent study two false positive *E. cloacae*  
261 complex isolates, were reported with positive colorimetric tests, of which one also produced  
262 the ESBL SHV-12.<sup>26</sup> Similarly, Chinese researchers, using a modified rCIM, by extending the  
263 incubation time to 45 minutes and by exchanging sterile purified water with TSB for incubation,  
264 have reported 2 false-positive results, but without specifying the responsible species. It is  
265 however indicated, that phenotypically one was considered an ESBL producer, and the other  
266 one an ESBL-AmpC co-producer. Of note, these results were obtained after cut-offs were  
267 changed to 2.0 baseline-subtracted *E. coli* ATCC 25922 growth for positives and 1.0 for  
268 negatives.<sup>27</sup>

269 The limitation of the rCIM-A is that it requires cloxacillin. Commercially-available injectable  
270 powder may be used, by resuspending one gram into water, and by freezing aliquots of this  
271 suspension. Thus, there is no need for a precision scale.

272

## 273 **Conclusions**

274 The use of cloxacillin allows an efficient inhibition of AmpC enzymes, thus improving the  
275 specificity of the assay. The rCIM-A is a rapid, cheap and simple test that can easily be  
276 implemented in low resource laboratories for the detection of carbapenemases in all  
277 *Enterobacterales* isolates, including those overproducing AmpC enzymes, while keeping the  
278 total operating time to less than 3 hours. The assay is still lacking sensitivity with IMI-type  
279 carbapenemases and OXA-23, which is likely due to their weak meropenem hydrolysis, unlike  
280 imipenem that is strongly hydrolysed.<sup>18,23,28</sup>

281

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290

## 291 **Transparency declaration**

292 None to declare

293

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365 Method (reCIM) to Detect Carbapenemase and Distinguish Metallo-Carbapenemase in  
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368 and Detection Methods of Minor Carbapenemases in Enterobacterales. *Front Med* 2021;  
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- 370

371 Table 1. Carba NP, rCIM and rCIM-A results with 52 Enterobacterales naturally-producing a chromosomally-encoded cephalosporinase  
 372 (*Morganella* spp., *Serratia* spp., *Hafnia alvei*, *Citrobacter* spp.) from the French National Reference Center for CREs.

Species	Class	$\beta$ -lactamase	Count	Carba NP	rCIM/rCIM-A	
					Cloxacillin	
					0 $\mu$ g/mL	400 $\mu$ g/mL
<i>C. freundii</i>	A	KPC-2	1	+	+	+
	B	LMB-1	1	+	-	-
		NDM-1	3	+	+	+
	B + D	VIM-1 + OXA-48	1	+	+	+
	D	OXA-181	2	+	+	+
		OXA-204	1	+/-	+	+
		OXA-204	4	+	+	+
		OXA-372	1	+	+	+
		OXA-48	1	+	+	+
		D + D	OXA-48 + OXA-198	1	+	+
<i>M. morgani</i>	B	NDM	1	+/-	-	-
		NDM-1	1	+	+	-
<i>S. fonticola</i>		SFH-1	1	+	+	+
<i>S. marcescens</i>	A	KPC-2	1	+	+	+
		Sme-1	1	+	+	+
		Sme-2	1	+	+	+
	B	IMP-10	1	+	+	+

		IMP-10 like	1	+	+	+
		IMP-11	1	+	+	+
	D	OXA-181	1	+	+	+
		OXA-48	1	+	+	+
		ESBL + Overproduced cAmpC	1	-	-	-
<i>C. freundii</i>		cAmpC (CMY-135)	1	-	-	-
		Overproduced cAmpC	9	-	<b>2+</b>	-
	No carbapenemase detected	TEM-1 + OXA-101 + cAmpC	1	-	-	-
<i>H. alvei</i>		ACC-1	5	-	-	-
<i>M. morgani</i>		Overproduced cAmpC	3	-	-	-
		Overproduced cAmpC	4	-	<b>1+</b>	-
<i>S. marcescens</i>		OXA-405	1	-	+	-

373 Inconclusive colorimetric results from the CarbaNP results are marked with „+/-“. Discordant results are **bolded**. In case not all the tested isolates  
374 tested gave the same results, the number of erroneous results is indicated.

375

376 Table 2. Carba NP, rCIM and rCIM-A results with 67 Enterobacterales isolates from the French National Reference Center for CREs.

Class	Enzyme	Species	Count	Carba NP	rCIM/rCIM-A	
					Cloxacillin	
					0 µg/mL	400 µg/mL
A	KPC-2	<i>E. coli</i>	1	+	+	+
		<i>K. pneumoniae</i>	1	+	+	+
	GES-5	<i>K. pneumoniae</i>	1	+	+	+
B	IMP-1	<i>E. coli</i>	1	+	+	+
		<i>K. pneumoniae</i>	1	+	+	+
	IMP-4	<i>K. pneumoniae</i>	1	+	+	+
	NDM-1	<i>E. coli</i>	1	+	+	+
		<i>K. pneumoniae</i>	1	+	+	+
	VIM-1	<i>E. coli</i>	1	+	+	+
		<i>K. pneumoniae</i>	2	+	+	+
	D	OXA-162	<i>K. pneumoniae</i>	1	+/-	+
OXA-181		<i>E. coli</i>	5	2+;1+/-;2-	5+	5+
		<i>K. pneumoniae</i>	1	+	+	+
		<i>C. koseri</i>	1	+	+	+
OXA-204		<i>E. coli</i>	1	+	+	+
		<i>K. pneumoniae</i>	2	+	+	+
OXA-23		<i>P. mirabilis</i>	10	2+/-;8-	2+/-;8-	2+/-;8-
OXA-232		<i>K. pneumoniae</i>	5	1+;3+/- ;1-	5+	5+
OXA-244	<i>E. coli</i>	7	2+; 2+/- ;3-	7+	7+	

OXA-48	<i>E. coli</i>	1	+	+	+	
	<i>C. koseri</i>	1	+	+	+	
	<i>K. pneumoniae</i>	1	+	+	+	
OXA-58	<i>P. mirabilis</i>	1	+/-	+	+	
No carbapenemase detected	Plasmid encoded ACC-1	<i>E. coli</i>	1	-	-	-
		<i>P. mirabilis</i>	1	-	-	-
	Plasmid encoded CMY-16	<i>K. pneumoniae</i>	1	-	-	-
	Plasmid encoded CMY-2	<i>E. coli</i>	1	-	-	-
	Plasmid encoded CTX-M-1	<i>E. coli</i>	1	-	-	-
	Plasmid encoded CTX-M-14	<i>E. coli</i>	2	-	-	-
	Plasmid encoded CTX-M-15	<i>E. coli</i>	1	-	-	-
		<i>K. pneumoniae</i>	1	-	-	-
	Plasmid encoded CTX-M-3	<i>E. coli</i>	1	-	-	-
		<i>K. pneumoniae</i>	1	-	-	-
	Plasmid encoded DHA-1	<i>E. coli</i>	1	-	-	-
		<i>K. pneumoniae</i>	1	-	-	-
	Plasmid encoded DHA-2	<i>K. pneumoniae</i>	1	-	-	-
	Overproduced cAmpC	<i>E. coli</i>	1	-	-	-
	Plasmid encoded TEM-1	<i>K. pneumoniae</i>	1	-	-	-
	<i>Wild Type</i>	<i>E. coli</i>	1	-	-	-
		<i>K. oxytoca</i>	1	-	-	-

377 Inconclusive colorimetric results from the Carba NP results are marked with „+/-“. Discordant results are **bolded**. In case not all the tested isolates  
378 tested gave the same results, the number of erroneous results is indicated.  
379

380 Table 3: Summary of Carba NP, rCIM and rCIM-A tests on the different panels of isolates tested  
 381

Isolates	#	Carba NP		rCIM		rCIM-A	
		Sensitivity (95% CI:)	Specificity (95% CI:)	Sensitivity (95% CI:)	Specificity (95% CI:)	Sensitivity (95% CI:)	Specificity (95% CI:)
<i>E. cloacae</i> complex isolates (from Table S1)	48	0/1 <sup>a</sup>	95.74% (85.46 - 99.48%)	1/1 <sup>a</sup>	91.49% (79.62 - 97.63%)	1/1 <sup>a</sup>	100 % (92.45 – 100%)
<i>Enterobacter cloacae</i> complex isolates from the F-NRC for CREs (from Table S2)	82	93.75% (79.19 - 99.23%)	76% (61.83 – 86.94%)	84.38% (67.21 - 94.72%)	62% (47.17 – 75.35%)	81.25% (63.56 - 92.79%)	98% (89.35 – 99.95%)
<i>Enterobacterales</i> isolates naturally-producing a chromosomally-encoded cephalosporinase from the F NRC for CREs (from Table 2)	52	92.59% (75.71 - 99.09%)	100% (86.28 - 100%)	92.59% (75.71 - 99.09%)	84% (63.92 - 95.46%)	88.89% (70.84 - 97.65%)	100% (86.28 - 100%)
<i>Enterobacterales</i> isolates from the French National	67	50%	100%	83.33%	100%	83.33%	100%

Reference Center for CREs. (Table3)		(35.23 - 64.77%)	(82.35 - 100%),	(69.78 - 92.52%)	(82.35 - 100%),	(69.78 - 92.52%)	(82.35 - 100%),
Overall	249	73.15% <sup>b</sup> (63.76 - 81.22%) - 86.11% <sup>c</sup> (78.13 - 92.01%)	90.07% <sup>a</sup> (83.9 - 94.46%) - 91.49% <sup>b</sup> (85.61 - 95.52%).	86.11% (78.13 - 92.01%)	80.85% (73.38 - 86.99%),	84.26% (76 - 90.55%)	99.29% (96.11 - 99.98%),

382 <sup>a</sup> Sensitivity could not be calculated as only one CPE was present

383 <sup>b</sup> when the 16 indeterminate results considered negative;

384 <sup>c</sup> when the 16 indeterminate results considered positive

385

386 Figure legends:

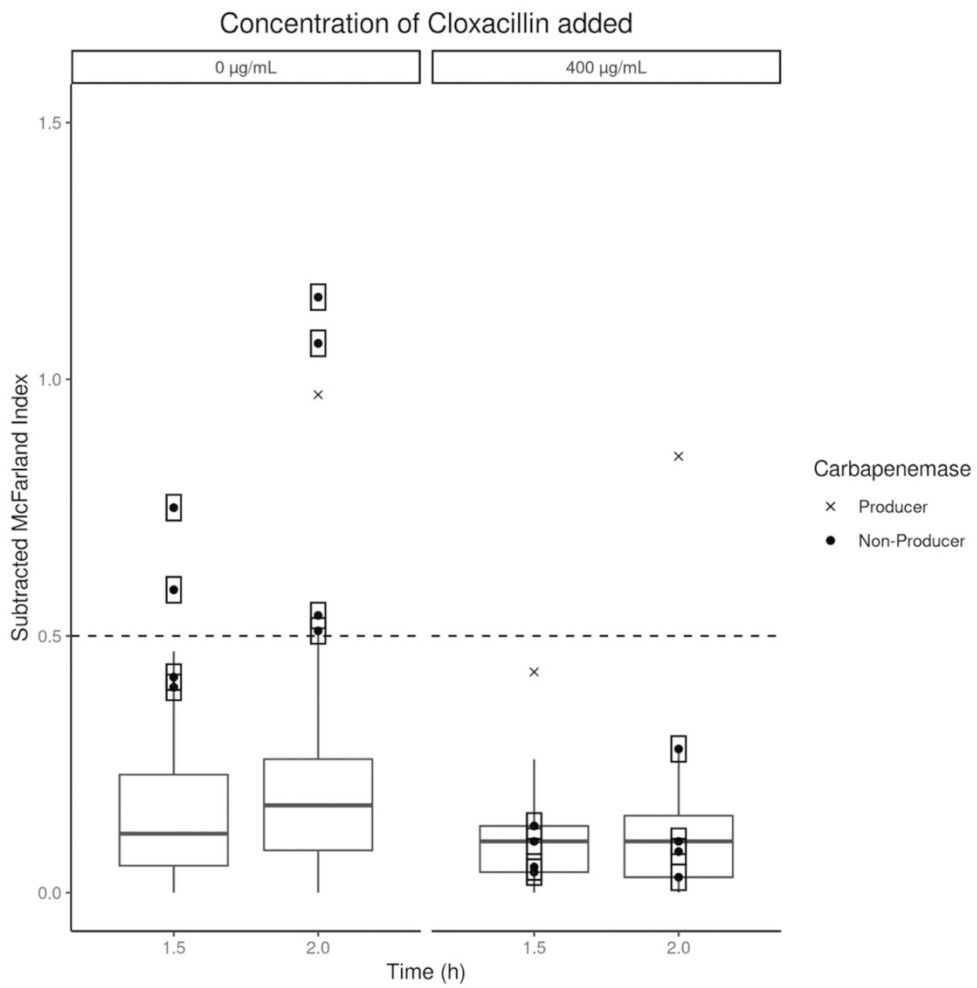
387 **Figure 1.** Boxplot showing the baseline subtracted McFarland Index for classical rCIM (no  
388 cloxacillin) and AmpC optimized rCIM-A (400 µg/mL) with the 48 *E. cloacae* complex  
389 reference isolates belonging to the different subclasses.<sup>9</sup> Individual results are expressed as  
390 nephelometric readings (baseline subtracted McFarland Index) of the *E. coli* ATCC 25922  
391 growth indicator strain at 1.5 and 2 hours of incubation. One strain harbouring OXA-204 is  
392 marked with a cross. Outliers and carbapenem-resistant hyper-AmpC producers that gave false-  
393 positive results in classical rCIM protocol, are marked with boxed filled circles.

394

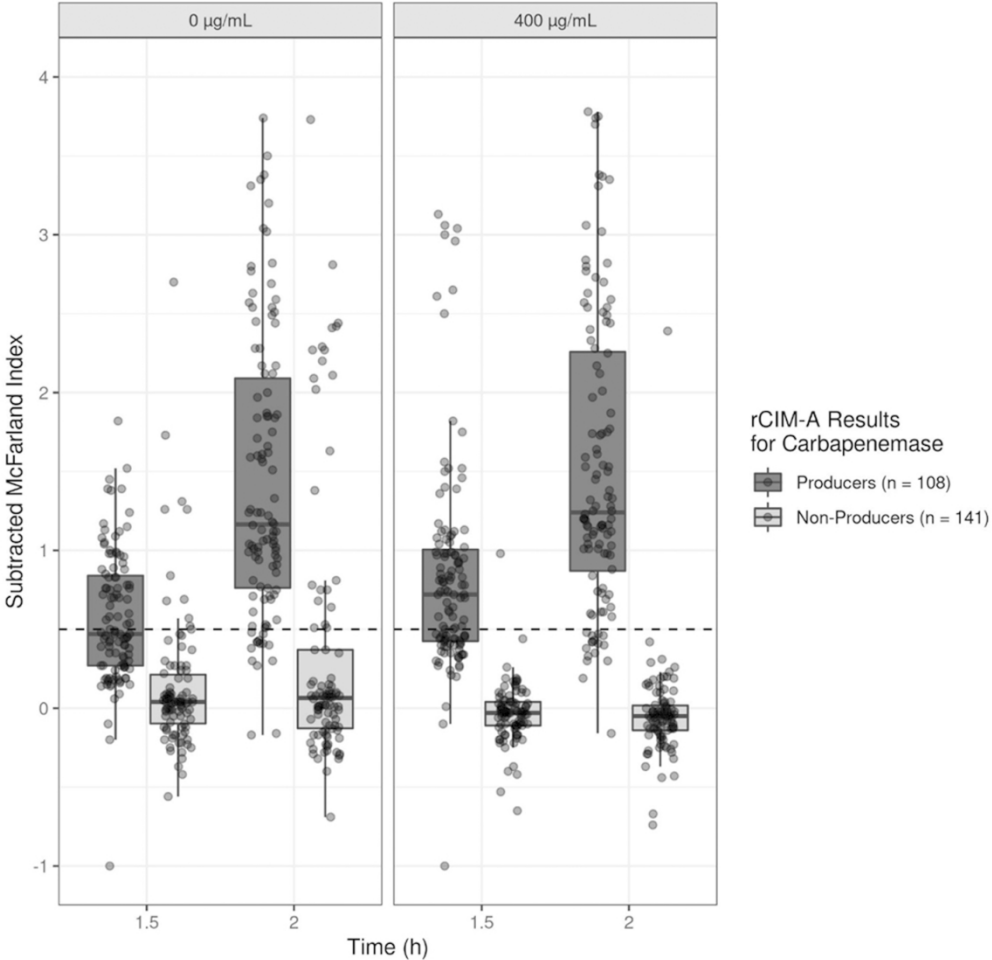
395 **Figure 2.** Graph showing overall results of classical (0 µg/mL cloxacillin) and modified rCIM  
396 (400 µg/mL cloxacillin) protocols. Results are presented as nephelometric readings, using  
397 baseline subtracted McFarland Index. Scatterplot with overlaid boxplots present Median and  
398 InterQuartile Ranges (IQR) 25-75, while the whiskers extend to the data point which is no more  
399 than 1.5 times the IQR. Outliers are presented as filled circles outside the whiskers of the plot.  
400 Carbapenemase producers and non-producers are presented side-by-side for each measurement  
401 time point (1.5 and 2 hours). A dashed horizontal line marks the 0.5 McFarland index cut-off.

402





Concentration of Cloxacillin added



1 **OPTIMISATION OF THE RAPID CARBAPENEM INACTIVATION METHOD FOR**  
2 **USE WITH AMPC HYPERPRODUCERS**

3

4 Mădălina-Maria MUNTEAN,<sup>1,2,3§</sup> Andrei-Alexandru MUNTEAN,<sup>1,2,3§</sup> François GUERIN,<sup>4,5</sup>

5 Vincent CATTOIR,<sup>4,5,6</sup> Elodie CRETON,<sup>1,7,8,9</sup> Garance COTELLON,<sup>1,7,8,9</sup> Saoussen

6 OUESLATI,<sup>1,7,8,9</sup> Mircea Ioan POPA,<sup>2,3</sup> Delphine GIRLICH,<sup>1,8,9</sup> Bogdan I. IORGA,<sup>10</sup> Rémy

7 A. BONNIN,<sup>1,8,9</sup> and Thierry NAAS<sup>1,7,8,9 \*</sup>

8

9

Supplemental Tables S1 and S2

10

11 **Table S1.** Carba NP, rCIM and rCIM-A results with 48 *E. cloacae complex* strains<sup>9</sup>.

Cluster	Species (Where available)	ID	Carbapenemase	CarbaNP	rCIM/rCIM-A		Phenotype
					cloxacillin		
					0 µg/mL	400 µg/mL	
I	<i>E. asburiae</i>	ECL 28	-	+/-	+	-	CASE
I	<i>E. asburiae</i>	CHV23	-	-	-	-	WT
I	<i>E. asburiae</i>	CCH43	-	-	-	-	WT
II	<i>E. kobei</i>	ECL 16	-	-	-	-	CASE
II	<i>E. kobei</i>	ECL 123	-	-	-	-	WT
II	<i>E. kobei</i>	ECL 140	-	-	-	-	WT
III		ECL 26	-	-	-	-	CASE
III		ECL 31	-	-	-	-	CASE
III		ECL 34	-	-	-	-	WT
III		ECL 35	-	-	-	-	WT
III		ECL 41	-	-	-	-	ESBL
III		ECL Ep03	-	-	-	-	ESBL

III		ECL Ep08	-	-	-	-	ESBL
III		ECL Ep16	-	-	-	-	ESBL
IV		ECL 18	-	-	+	-	CASE
IV		ECL 166	-	-	-	-	CASE
IV		ECL 101	-	-	-	-	WT
IV		NAN31	-	-	-	-	WT
V	<i>E. ludwigii</i>	ECL 32	-	-	+	-	CASE
V	<i>E. ludwigii</i>	ECL 11	-	-	-	-	CASE
V	<i>E. ludwigii</i>	ECL 33	-	-	-	-	WT
V	<i>E. ludwigii</i>	ECL 130	-	-	-	-	WT
VI	<i>E. hormaechei subsp. oharae</i>	ECL 25	-	-	-	-	CASE
VI	<i>E. hormaechei subsp. oharae</i>	ECL 30	-	-	-	-	CASE
VI	<i>E. hormaechei subsp. oharae</i>	ECL 38	-	-	-	-	WT
VI	<i>E. hormaechei subsp. oharae</i> (OXA-204)	ECL Ep013	+	+/-	+	+	ESBL
VI	<i>E. hormaechei subsp. oharae</i>	ECL Ep025	-	-	-	-	ESBL
VII	<i>E. hormaechei subsp. hormaechei</i>	ECL 121	-	-	-	-	WT

VII	<i>E. hormaechei subsp. hormaechei</i>	ECL 122	-	-	-	-	WT
VIII	<i>E. hormaechei subsp. steigerwallti</i>	ECL 01	-	-	-	-	CASE
VIII	<i>E. hormaechei subsp. steigerwallti</i>	ECL 29	-	-	-	-	CASE
VIII	<i>E. hormaechei subsp. steigerwallti</i>	ECL 40	-	-	-	-	ESBL
VIII	<i>E. hormaechei subsp. steigerwallti</i>	ECL Ep02	-	-	-	-	ESBL
VIII	<i>E. hormaechei subsp. steigerwallti</i>	ECL Ep022	-	-	-	-	ESBL
IX		ECL 146	-	-	-	-	WT
IX		ECL JO36	-	-	-	-	WT
X	<i>E. nimipressuralis</i>	CHV27	-	-	-	-	WT
X	<i>E. nimipressuralis</i>	CAE15	-	-	-	-	WT
XI	<i>E. cloacae subsp. cloacae</i>	ECL JO18	-	-	-	-	WT
XI	<i>E. cloacae subsp. cloacae</i>	ECL JO24	-	-	-	-	WT
XI	<i>E. cloacae subsp. cloacae</i>	ATCC 13047	-	-	-	-	WT
XI	<i>E. cloacae subsp. cloacae</i>	ATCC 13047 <i>ΔampD</i>	-	+/-	+	-	CASE
XI	<i>E. cloacae subsp. cloacae</i>	ATCC 13047 <i>ΔdacA</i>	-	-	-	-	CASE

XI	<i>E. cloacae subsp. cloacae</i>	ATCC 13047 <i>ΔdacB</i>	-	-	-	-	CASE
XII	<i>E. cloacae subsp. dissolvens</i>	ECL JO20	-	-	-	-	WT
XII	<i>E. cloacae subsp. dissolvens</i>	RMS39	-	-	-	-	WT
XIII		GIP-ECC99	-	-	-	-	WT
XIII		GIP-ECC8	-	-	-	-	WT

12 “Δ” denotes deletion of genes. Positive results are marked with a plus; negative results are marked with a minus. Inconclusive colorimetric results from the  
13 CarbaNP results are marked with „+/-”. Discordant results are **bolded**. WT – Wild type; ESBL – Extended Spectrum Beta-Lactamase; CASE –  
14 Hyperproduced chromosomal cephalosporinase.

15

16 Table S2. Carba NP, rCIM and rCIM-A results with 82 *Enterobacter cloacae* complex isolates from the French National Reference Center for  
 17 CREs.

Class	β-lactamase	Species <sup>a</sup>	n	Carba NP	rCIM/rCIM-A	
					Cloxacillin	
					0 µg/mL	400 µg/mL
A	TEM-1B	<i>E. aerogenes</i>	1	-	-	-
	VEB-1	<i>E. cloacae</i>	1	-	-	-
	GES-5	<i>E. cloacae</i>	1	+/-	+	+
	IMI-1	<i>E. cloacae</i>	2	2+	2+	2+
	IMI-13	<i>E. cloacae</i>	2	<b>1-</b>	<b>1-</b>	<b>1-</b>
	IMI-17	<i>E. cloacae</i>	2	+	<b>2-</b>	<b>2-</b>
	IMI-2	<i>E. asburiae, E. cloacae</i>	2	+	+	+
	IMI-6	<i>E. ludwigii</i>	1	+	-	-
	NmcA	<i>E. cloacae</i>	1	+	+	+
	FRI-1	<i>E. cloacae</i>	1	+	+	+
	KPC-2	<i>E. cloacae</i>	1	+	+	+
A + B	KPC-4 + NDM-7	<i>E. cloacae</i>	1	+	+	+
B	GIM-1	<i>E. cloacae</i>	1	+	+	+
	IMP-8	<i>E. cloacae</i>	1	+	+	+



	NDM-1	<i>E. cloacae</i>	2	2+	2+	2+
	NDM-5	<i>E. xiangfang</i>	1	+	+	+
	TMB-1	<i>E. cloacae</i>	1	+	-	-
	VIM-1	<i>E. cloacae</i>	1	+	+	+
	VIM-2	<i>E. cloacae</i>	1	+	+	+
	VIM-4	<i>E. cloacae</i>	1	+	+	+
	VIM-1 + VIM-4	<i>E. cloacae</i>	2	2+	2+	2+
B + D	NDM-5 + OXA-181	<i>E. cloacae</i>	1	+	+	+
	VIM-4 + OXA-48	<i>E. cloacae</i>	1	+	+	+
D	OXA-181	<i>E. aerogenes</i>	1	+	+	+
	OXA-204	<i>E. cloacae</i>	2	2+	2+	2+
	OXA-48	<i>E. aerogenes, E. cloacae</i>	2	2+	2+	2+
C	ACT-16	<i>E. cloacae</i>	1	-	-	-
	ACT-28 <sup>b</sup>	<i>E. kobei</i>	10	<b>10+</b>	<b>10+</b>	10-
	ACT-64	<i>E. kobei</i>	1	-	-	-
	ACT-60/7-like <sup>c</sup>	<i>E. cloacae</i>	1	-	-	-
	MIR-6	<i>E. cloacae</i>	1	-	-	-
	MIR-10-like <sup>c</sup>	<i>E. cloacae</i>	1	+	+	+
	Basal AmpC	<i>K. aerogenes</i>	4	4-	4-	4-
	Basal ACT-like	<i>E. cloacae</i>	1	-	-	-

	Basal ACT-like	<i>E. hormaechei</i>	1	-	-	-
	Hyperexpressed chromosomal cephalosporinases	<i>K. aerogenes</i>	2	2-	2-	2-
		<i>E. asburiae</i>	1	-	+	-
		<i>E. cloacae</i>	20	<b>1+</b>	<b>2+<sup>d</sup></b>	-
		<i>E. hormaechei</i>	1	-	+	-
		<i>E. hormaechei</i>	1	-	-	-
		<i>E. kobei</i>	2	2-	2-	2-

18

19 Discordant results are **bolded**. Inconclusive CarbaNP results are marked as “+/-”. In case not all the tested isolates gave the same results, the  
20 number of erroneous results is indicated.

21 <sup>a</sup> Species were determined through MALDI-ToF-MS analysis, using Biotyper (Bruker Daltonic)

22 <sup>b</sup> ACT with weak carbapenem-hydrolyzing activity <sup>19</sup>

23 <sup>c</sup> single point mutant derivatives of the indicated enzymes

24 <sup>d</sup>Two isolates (143A8 and 143A9) were previously tested and gave false-positive results.

25