

Optimization of the rapid carbapenem inactivation method for use with AmpC hyperproducers

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2	OPTIMISATION OF THE RAPID CARBAPENEM INACTIVATION METHOD FOR
3	USE WITH AMPC HYPERPRODUCERS
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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.

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

47 **Results**: The rCIM-A yielded a sensitivity and a specificity of 84.26% (95% CI: 76 - 90.55%) and 99.29% (95% CI: 96.11 - 99.98%), respectively, while those of the rCIM were 86.11% 48 (95% CI: 78.13 - 92.01%) and 80.85% (95% CI: 73.38 - 86.99%), respectively, while those of 49 the CarbaNP were lower 84.04% (95% CI: 75.05 - 90.78%) and 91.37% (95% CI: 85.41 -50 95.46%), respectively, due to indeterminate results. The rCIM-A was capable to discriminate 51 52 between AHEs and true CPEs, but still fails to identify OXA-23 producing *P. mirabilis* isolates and remains only partially reliable to identify IMI-like- and a few metallo-β-lactamase- (2 53 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 a false positive. 56

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.

61 Introduction

62 Strains belonging to the *Enterobacter cloacae* complex, *Citrobacter* spp., *Serratia* spp., Morganella morganii or Providencia spp. are Gram-negative bacilli that naturally-produce an 63 inducible chromosomally-encoded AmpC B-lactamase (cAmpC).¹ At the basal level of 64 production, AmpC confers resistance to penicillins, first- and second-generation 65 cephalosporins whereas when overproduced it also hydrolyses oxyimino-cephalosporins (such 66 67 as ceftriaxone, cefotaxime, and ceftazidime) and monobactams but spares cefepime and 68 carbapenems, and it is not inhibited by classical β -lactamase inhibitors such as clavulanic acid. Noteworthy, these enzymes are inhibited by cloxacillin and 3-aminophenylboronic acid.¹ 69 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 infections, infections in orthopaedic or transplant patients.²⁻⁴ The EPIC II (Extended Prevalence 73 of Infection in Intensive Care) study, an international 1-day, prospective, point prevalence 74 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.⁵ Carbapenem resistance mechanisms in AmpC-producers may be complicated to decipher, as it can be linked 77 78 to AmpC hyperproduction associated with a decrease in outer membrane permeability.⁶

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

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

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92 Materials and Methods

93 Bacterial isolates tested

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

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

107 All isolates were sub-cultured from frozen stocks (stored at -80 °C) on UriSelectTM 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.

Biochemical test for the presence of a carbapenemase

111 The in-house Carba NP test was performed as previously described.^{7,10}

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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.⁷ The rCIM-A differs from rCIM by the addition 116 of cloxacillin to the initial antibiotic incubation step. Injectable cloxacillin powder (Orbenin® 1g injectable powder, Astellas Pharma, Levallois Perret, France) was used instead of the 117 118 purified active principle, as no difference in potency was observed in preliminary experiments (data not shown). Cloxacillin was prepared by suspending 1g of active substance in 2.5 mL of 119 120 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 μ g/mL) before the addition of the bacteria. The rest of the protocol remained unchanged.⁷ Briefly, after incubation and centrifugation, the resulting 123 124 supernatant is used to challenge growth of the susceptible *Escherichia coli* ATCC 25922 strain, as monitored by nephelometry (Densitometer DEN-1B, Grant Bio, Cambridgeshire, UK)). 125 126 Growth was expressed as baseline subtracted growth in McFarland units, as previously described.⁷ A positive and a negative control of a *K. pneumoniae* ATCC BAA 1705 and 1706, 127 128 respectively, were used for each batch of tests.

129

130 Statistical analysis

The overall characteristics of the test (sensitivity, specificity, positive and negative predictive
values) were calculated, with their respective 95% CI, using the R v. 4.0.2 software suite and
epiR v. 2.0-19.¹¹⁻¹³

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. ¹⁴
139	
140	Nucleotide accession numbers
141	The genome determined in this study is available at NCBI under the accession number of
142	JAFEWA00000000.
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 μ g/mL, which is the concentration at which cloxacillin will be used for the
149	rCIM-A nor with 4,000 μ g/mL, a much higher concentration to clearly demonstrate the non-
150	susceptibility of <i>E. coli</i> 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. ⁹ 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-

Whole Genome Sequencing

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 (ΔampD).
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 μ 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).

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.¹⁵⁻¹⁷

The sensitivity and specificity of the rCIM were 84.38% (95% CI: 67.21 - 94.72%) and 62%
(95% CI: 47.17 - 75.35%), respectively. The addition of cloxacillin resulted in a sensitivity of
81.25% (95% CI: 63.56 - 92.79%) and a specificity of 98% (95% CI: 89.35 - 99.95%). Both,
rCIM and rCIM-A, failed to identify several IMI-producing isolates (IMI-6, IMI-13 and 2 IMI17). This result is not surprising, as IMI-enzymes are known to preferentially hydrolyse

183 imipenem, and to a weaker extend meropenem.¹⁸ 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-17producing isolates that gave false-negative results in the absence of induction.^{7,18} Using 186 187 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.¹⁴ Of note, we included 10 E. kobei ST125 isolates that harbour a chromosome-encoded ACT-28 190 and are known to give false-positive Carba NP and rCIM results.¹⁹ Indeed, false positive results 191 192 were obtained in all ACT-28-producing E. kobei strains with the Carba NP test and the rCIM protocol. However, using rCIM-A, all ACT-28 isolates were correctly categorized as lacking 193 194 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 μ g/mL and 0.71 baseline-subtracted growth with 4,000 200 $\mu g/mL$).

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

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

A collection of 52 Group 3 *Enterobacterales* were evaluated; of these 27 were carbapenemase
producers. CarbaNP had a sensitivity of 92.59% (95% CI: 75.71 - 99.09%) and a specificity
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. morganii* isolate tested positive with the rCIM but turned negative with the addition of cloxacillin (400 μ g/mL), suggesting that the initial positivity was likely 213 214 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. morganii* 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), UriSelectTM 4 and an antibiogram (close to the meropenem disc) gave positive rCIM-A results, likely due to 218 219 induction.¹⁸ 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® CarbaSmart media (Table 1).²⁰ This strain was positive using the Carba NP test. 221

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223 Other Enterobacterales and plasmid encoded AmpCs

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

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

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

236 Overall sensitivity and specificity.

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

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

The sensitivity and specificity of the rCIM were 86.11% (95% CI: 78.13 - 92.01%) and 80.85%
(95% CI: 73.38 - 86.99%), respectively. The rCIM gave false positive results in isolates
harbouring a derepressed chromosomal cephalosporinase, such as ACT-28, or the novel MIR10 variant.

The addition of cloxacillin to the rCIM test, led to a sensitivity and specificity for AmpC producers of 84.26% (95% CI: 76 - 90.55%) and 99.29% (95% CI: 96.11 – 99.98%), respectively (Table 3, and Figure 2). The strength of this study is that we have evaluated a very broad range of carbapenem-resistant *Enterobacterales*, which included true CPEs, as well as AmpC hyperproducers (both natural and acquired).

259 Potential other false-positive results of CIM-derived tests have been reported both with the CIM and the modified CIM (mCIM).²⁵ In one recent study two false positive E. cloacae 260 261 complex isolates, were reported with positive colorimetric tests, of which one also produced the ESBL SHV-12.²⁶ Similarly, Chinese researchers, using a modified rCIM, by extending the 262 incubation time to 45 minutes and by exchanging sterile purified water with TSB for incubation, 263 264 have reported 2 false-positive results, but without specifying the responsible species. It is however indicated, that phenotypically one was considered an ESBL producer, and the other 265 266 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 negatives.27 268

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

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273 Conclusions

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

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290	

- 291 Transparency declaration
- 292 None to declare
- 293

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Table 1. Carba NP, rCIM and rCIM-A results with 52 Enterobacterales naturally-producing a chromosomally-encoded cephalosporinase

					rCIM/rCIM-A	
Species	Class	ß-lactamase	Count	Carba NP	Cloxacillin	
					0 μg/mL	400 μg/mL
	А	KPC-2	1	+	+	+
-	D	LMB-1	1	+	-	-
	D	NDM-1	3	+	+	+
-	B + D	VIM-1 + OXA-48	1	+	+	+
-		OXA-181	2	+	+	+
C. jreunali		OXA-204	1	+/-	+	+
	D	OXA-204	4	+	+	+
		OXA-372	1	+	+	+
		OXA-48	1	+	+	+
-	D + D	OXA-48 + OXA-198	1	+	+	+
14		NDM	1	+/-	-	-
M. morganii	В	NDM-1	1	+	+	-
S. fonticola		SFH-1	1	+	+	+
		KPC-2	1	+	+	+
C	А	Sme-1	1	+	+	+
S. marcescens		Sme-2	1	+	+	+
-	В	IMP-10	1	+	+	+

372 (*Morganella* spp., *Serratia* spp., *Hafnia alvei*, *Citrobacter* spp.) from the French National Reference Center for CREs.

	IMP-10 like	1	+	+	+
	IMP-11	1	+	+	+
D	OXA-181	1	+	+	+
D	OXA-48	1	+	+	+
	ESBL + Overproduced cAmpC	1	-	-	-
No	cAmpC (CMY-135)	1	-	-	-
	Overproduced cAmpC	9	-	2+	-
	TEM-1 + OXA-101 + cAmpC	1	-	-	-
detected	ACC-1	5	-	-	-
-	Overproduced cAmpC	3	-	-	-
-	Overproduced cAmpC	4	-	1+	-
	OXA-405	1	-	+	-
-	D No carbapenemase detected	IMP-10 likeIMP-11DOXA-181OXA-48OXA-48ESBL + Overproduced cAmpCcAmpC (CMY-135)Overproduced cAmpCTEM-1 + OXA-101 + cAmpCACC-1Overproduced cAmpCOverproduced cAmpC	$\frac{IMP-10 \text{ like}}{IMP-11} \qquad 1$ $D \qquad \frac{OXA-181}{OXA-48} \qquad 1$ $ESBL + Overproduced cAmpC \qquad 1$ $cAmpC (CMY-135) \qquad 1$ $Overproduced cAmpC \qquad 9$ $TEM-1 + OXA-101 + cAmpC \qquad 1$ $ACC-1 \qquad 5$ $Overproduced cAmpC \qquad 3$ $Overproduced cAmpC \qquad 4$ $OXA-405 \qquad 1$	$\frac{IMP-10 \text{ like}}{IMP-10 \text{ like}} 1 + $ $\frac{IMP-11}{IMP-11} 1 + $ $D \frac{OXA-181}{OXA-48} 1 + $ $\frac{ESBL + Overproduced cAmpC}{I} - $ $\frac{CAmpC (CMY-135)}{Overproduced cAmpC} 9 - $ $\frac{TEM-1 + OXA-101 + cAmpC}{I} - $ $\frac{ACC-1}{5} - $ $\frac{Overproduced cAmpC}{I} - $ $\frac{Overproduced cAmpC}{I} - $	$\frac{IMP-10 \text{ like}}{IMP-11} \qquad 1 \qquad + \qquad + \qquad + \\ \overline{IMP-11} \qquad 1 \qquad 1 \qquad + \qquad + \\ D \qquad \frac{OXA-181}{OXA-48} \qquad 1 \qquad + \qquad + \\ \overline{OXA-48} \qquad 1 \qquad + \qquad + \\ \overline{OXA-48} \qquad 1 \qquad + \qquad + \\ \overline{CMpC (CMY-135)} \qquad 1 \qquad - \qquad - \\ \overline{CAmpC (CMY-135)} \qquad 1 \qquad - \qquad - \\ \overline{Overproduced cAmpC} \qquad 9 \qquad - \qquad 2+ \\ \overline{Overproduced cAmpC} \qquad 9 \qquad - \qquad 2+ \\ \overline{TEM-1+OXA-101+cAmpC} \qquad 1 \qquad - \qquad - \\ \overline{ACC-1} \qquad 5 \qquad - \qquad - \\ \overline{ACC-1} \qquad 5 \qquad - \qquad - \\ \overline{Overproduced cAmpC} \qquad 3 \qquad - \qquad - \\ \overline{Overproduced cAmpC} \qquad 4 \qquad - \qquad 1+ \\ \overline{OXA-405} \qquad 1 \qquad - \qquad + \\ \end{array}$

373 Inconclusive colorimetric results from the CarbaNP results are marked with "+/-". Discordant results are **bolded**. In case not all the tested isolates

tested gave the same results, the number of erroneous results is indicated.

					rCIM/rCIM-A		
Class	Enzyme	Species	Count	Carba NP	Cloxacillin		
					0 μg/mL	400 μg/mL	
	KDC 2	E. coli	1	+	+	+	
А	KPC-2	K. pneumoniae	1	+	+	+	
	GES-5	K. pneumoniae	1	+	+	+	
	IMD 1	E. coli	1	+	+	+	
		K. pneumoniae	1	+	+	+	
	IMP-4	K. pneumoniae	1	+	+	+	
В	NIDM 1	E. coli	1	+	+	+	
		K. pneumoniae	1	+	+	+	
	VIM 1	E. coli	1	+	+	+	
	V 11VI-1	K. pneumoniae	2	+	+	+	
	OXA-162	K. pneumoniae	1	+/-	+	+	
		E. coli	5	2+;1+/-;2-	5+	5+	
	OXA-181	K. pneumoniae	1	+	+	+	
		C. koseri	1	+	+	+	
D	OVA 204	E. coli	1	+	+	+	
	0XA-204	K. pneumoniae	2	+	+	+	
	OXA-23	P. mirabilis	10	2+/-;8-	2+/-;8-	2+/-;8-	
	OXA-232	K. pneumoniae	5	1+;3+/- ;1-	5+	5+	
	OXA-244	E. coli	7	2+; 2+/- ;3-	7+	7+	

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

		E. coli	1	+	+	+
	OXA-48	C. koseri	1	+	+	+
		K. pneumoniae	1	+	+	+
	OXA-58	P. mirabilis	1	+/-	+	+
	Diagmid analysis ACC 1	E. coli	1	-	-	-
	Plasmid encoded ACC-1	P. mirabilis	1	-	-	-
	Plasmid encoded CMY-16	K. pneumoniae	1	-	-	-
	Plasmid encoded CMY-2	E. coli	1	-	-	-
	Plasmid encoded CTX-M-1	E. coli	1	-	-	-
	Plasmid encoded CTX-M-14	E. coli	2	-	-	-
	Plasmid encoded CTX-M-15	E. coli	1	-	-	-
		K. pneumoniae	1	-	-	-
No carbapenemase	Diamidance ded OTY M 2	E. coli	1	-	-	-
detected	Plasmid encoded CTX-M-3	K. pneumoniae	1	-	-	-
		E. coli	1	-	-	-
	Plasmia encoded DHA-1	K. pneumoniae	1	-	-	-
	Plasmid encoded DHA-2	K. pneumoniae	1	-	-	-
	Overproduced cAmpC	E. coli	1	-	-	-
	Plasmid encoded TEM-1	K. pneumoniae	1			-
	W-11 T	E. coli	1	-	-	-
	<i>Wild Туре</i>	K. oxytoca	1	-	-	-

377 Inconclusive colorimetric results from the Carba NP results are marked with "+/-". Discordant results are **bolded**. In case not all the tested isolates

tested gave the same results, the number of erroneous results is indicated.

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

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

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

382 ^a Senstivity could not be calculated as only one CPE was present

383 ^b when the16 indeterminate results considered negative;

384 ^c when the 16 indeterminate results considered positive

386 Figure legends:

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

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





1	OPTIMISATION OF THE RAPID CARBAPENEM INACTIVATION METHOD FOR
2	USE WITH AMPC HYPERPRODUCERS
3	
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8	
9	Supplemental Tables S1 and S2
10	

Cluster	Species (Where available)	ID Carbaj	Carbapenemase	CarbaNP	rCIM	/rCIM-A	Phenotype	
					cloxacillin		_	
					0 μg/mL	400	-	
						μg/mL		
Ι	E. asburiae	ECL 28	-	+/-	+	-	CASE	
Ι	E. asburiae	CHV23	-	-	-	-	WT	
Ι	E. asburiae	CCH43	-	-	-	-	WT	
II	E. kobei	ECL 16	-	-	-	-	CASE	
II	E. kobei	ECL 123	-	-	-	-	WT	
II	E. kobei	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	

Table S1. Carba NP, rCIM and rCIM-A results with 48 *E. cloacae complex* strains⁹.

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

VII	E. hormaechei subsp. hormaechei	ECL 122	-	-	-	-	WT
VIII	E. hormaechei subsp. steigerwallti	ECL 01	-	-	-	-	CASE
VIII	E. hormaechei subsp. steigerwallti	ECL 29	-	-	-	-	CASE
VIII	E. hormaechei subsp. steigerwallti	ECL 40	-	-	-	-	ESBL
VIII	E. hormaechei subsp. steigerwallti	ECL Ep02	-	-	-	-	ESBL
VIII	E. hormaechei subsp. steigerwallti	ECL Ep022	-	-	-	-	ESBL
IX		ECL 146	-	-	-	-	WT
IX		ECL JO36	-	-	-	-	WT
Х	E. nimipressuralis	CHV27	-	-	-	-	WT
Х	E. nimipressuralis	CAE15	-	-	-	-	WT
XI	E. cloacae subsp. cloacae	ECL JO18	-	-	-	-	WT
XI	E. cloacae subsp. cloacae	ECL JO24	-	-	-	-	WT
XI	E. cloacae subsp. cloacae	ATCC 13047	-	-	-	-	WT
XI	E. cloacae subsp. cloacae	ATCC 13047	-	+/-	+	-	CASE
		ΔampD					
XI	E. cloacae subsp. cloacae	ATCC 13047	-	-	-	-	CASE
		$\Delta dac A$					

XI	E. cloacae subsp. cloacae	ATCC 13047	-	-	-	-	CASE
		$\Delta dac B$					
XII	E. cloacae subsp. dissolvens	ECL JO20	-	-	-	-	WT
XII	E. cloacae subsp. dissolvens	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.

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.

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

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

	Basal ACT-like	E. hormaechei	1	-	-	-
		K. aerogenes	2	2-	2-	2-
Hyperexpressed chromosomal cephalosporinases	E. asburiae	1	-	+	-	
	E. cloacae	20	1+	2+ ^d	-	
	E. hormaechei	1	-	+	-	
		E. hormaechei	1	-	-	-
		E. kobei	2	2-	2-	2-

- 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 ^a Species were determined through MALDI-ToF-MS analysis, using Biotyper (Bruker Daltonic)
- 22 ^bACT with weak carbapenem-hydrolyzing activity ¹⁹
- 23 ^c single point mutant derivatives of the indicated enzymes
- ^dTwo isolates (143A8 and 143A9) were previously tested and gave false-positive results.