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

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Abstract (249)

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

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% (95% CI: 78.13 - 92.01%) and 80.85% (95% CI: 73.38 - 86.99%), respectively, while those of the CarbaNP were lower 84.04% (95% CI: 75.05 - 90.78%) and 91.37% (95% CI: 85.41 - 95.46%), respectively, due to indeterminate results. The rCIM-A was capable to discriminate 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 NDM-1, 1 LMB-1, 1 TMB-1 and 1 IMP-13) producers. One chromosomally-encoded AmpC variant, MIR-10, gave repeatedly positive results using the three tests and was thus considered a false positive.

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

Introduction

Strains belonging to the *Enterobacter cloacae* complex, *Citrobacter* spp., *Serratia* spp., *Morganella morganii* or *Providencia* spp. are Gram-negative bacilli that naturally-produce an inducible chromosomally-encoded AmpC β -lactamase (cAmpC).¹ At the basal level of production, AmpC confers resistance to penicillins, first- and second-generation cephalosporins whereas when overproduced it also hydrolyses oxyimino-cephalosporins (such as ceftriaxone, cefotaxime, and ceftazidime) and monobactams but spares cefepime and 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 Although not as widely distributed as other *Enterobacterales* species such as *Escherichia coli* or *Klebsiella pneumoniae*, AmpC-producers are responsible for a wide range of nosocomial 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 of Infection in Intensive Care) study, an international 1-day, prospective, point prevalence study conducted in 2007 by JL Vincent *et al.*, revealed that *Enterobacter* spp. infections 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 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 AmpC-producing *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).

Materials and Methods

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

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

Biochemical test for the presence of a carbapenemase

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

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

The rCIM was used as previously described.⁷ The rCIM-A differs from rCIM by the addition 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 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 sterile distilled water (stock concentrations of 400 µg/µL). Stock solution was aliquoted and kept in the freezer (-20 °C) until use. One to 10 µL were added to the Eppendorf tube (concentrations of 400 - 4,000 µg/mL) before the addition of the bacteria. The rest of the protocol remained unchanged.⁷ Briefly, after incubation and centrifugation, the resulting 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)). 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, respectively, were used for each batch of tests.

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

Whole Genome Sequencing

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

Nucleotide accession numbers

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

Results and discussion

Evaluation of effect of Cloxacillin

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

Reference strains of the *Enterobacter cloacae* complex.

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

obtained in a cluster I *E. asburiae* strain harbouring only its AmpC and in the AmpC-derepressed reference ATCC 13047 *E. cloacae* subsp. *cloacae* (Δ ampD).

The standard rCIM protocol had a specificity of 91.49% (95% CI: 79.62 - 97.63%). rCIM was falsely positive for 4 strains not producing carbapenemases. This included the two strains with indeterminate CarbaNP results as well as two other strains from clusters IV and V (Table S1). With the addition of 400 μ g/mL of cloxacillin, the specificity of the rCIM-A rose to 100 % (95% CI: 92.45 – 100%). Both rCIM protocols correctly identified the presence of the OXA-204 carbapenemase (Table S1).

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

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

CarbaNP had a sensitivity and specificity of 93.75% (95% CI: 79.19 - 99.23%) and 76% (95% CI: 61.83 – 86.94%), respectively. The CarbaNP test failed to identify a GES-5 producing 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 IMI-17). This result is not surprising, as IMI-enzymes are known to preferentially hydrolyse imipenem, and to a weaker extent meropenem.¹⁸ When these tests were performed with

bacteria grown next to the imipenem discs from a routine disk diffusion test, they were repeatedly positive. These results are consistent with our previous findings, with IMI-17-producing isolates that gave false-negative results in the absence of induction.^{7,18} Using cloxacillin, one IMI-13 isolate gave an additional false-negative result. The initial positive result is likely due to the overproduced AmpC and not to IMI-13. Lastly, one TMB-1 producing *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 and are known to give false-positive Carba NP and rCIM results.¹⁹ Indeed, false positive results 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 acquired carbapenemase activity. Alongside these, one strain of *E. cloacae*, characterized as a cAmpC hyperproducer on the basis of its antibiogram, gave consistent positive results, even after addition of cloxacillin (400 as well as 4,000 µg/mL). This strain was whole-genome sequenced and was found to harbour a novel variant of MIR-10 cephalosporinase. Interestingly, this showed a dose-dependent response inhibition of cAmpC activity with cloxacillin (2.39 baseline-subtracted growth with 400 µg/mL and 0.71 baseline-subtracted growth with 4,000 µg/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

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

specificity were 92.59% (95% CI: 75.71 - 99.09%) and 84% (95% CI: 63.92 - 95.46%), respectively. Using the rCIM-A, the sensitivity slightly decreased to reach 88.89% (95% CI: 70.84 - 97.65%) but the specificity reached 100% (95% CI: 86.28 - 100%) (Tables 1 and 3). One NDM-1-producing *M. morganii* isolate tested positive with the rCIM but turned negative with the addition of cloxacillin (400 µg/mL), suggesting that the initial positivity was likely due to the over-produced AmpC and not to the low-level production of NDM-1-carbapenemase. This isolate tested positive in Carba NP test. Similarly, another NDM-producing *M. morganii* isolate tested negative in both rCIM and was indeterminate in Carba NP. In both cases, colonies taken from carbapenem-containing plates (bioMérieux chromID® CarbaSmart), UriSelect™ 4 and an antibiogram (close to the meropenem disc) gave positive rCIM-A results, likely due to induction.¹⁸ Finally, *C. freundii* producing LMB-1, was negative in both rCIM tests but became 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.

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}

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 literature, which are often linked to the appearance of slight colour changes.²⁴ A total of 16 isolates gave an indeterminate result in Carba NP. Proceeding as per CLSI guidelines, these 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 the specificity was 91.37% (95% CI: 85.41 - 95.46%). Taking a different, pragmatic approach, 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: 78.13 - 92.01%) and a specificity between 90.07% (95% CI: 83.9 - 94.46%) and 91.49% (95% CI: 85.61 - 95.52%) (Table 3).

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 MIR-10 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).

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* 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 incubation time to 45 minutes and by exchanging sterile purified water with TSB for incubation, 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 one an ESBL-AmpC co-producer. Of note, these results were obtained after cut-offs were changed to 2.0 baseline-subtracted *E. coli* ATCC 25922 growth for positives and 1.0 for negatives.²⁷

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.

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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291 **Transparency declaration**

292 None to declare

293

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297 **References**

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28. Bonnin RA, Jousset AB, Emeraud C, *et al.* Genetic Diversity, Biochemical Properties, and Detection Methods of Minor Carbapenemases in Enterobacterales. *Front Med* 2021; **7**:616490.

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	β -lactamase	Count	Carba NP	rCIM/rCIM-A	
					Cloxacillin	
					0 μ g/mL	400 μ 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. morganii</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	+	+	+

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

No carbapenemase detected	OXA-48	<i>E. coli</i>	1	+	+	+
		<i>C. koseri</i>	1	+	+	+
		<i>K. pneumoniae</i>	1	+	+	+
	OXA-58	<i>P. mirabilis</i>	1	+/-	+	+
	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 ^a	95.74% (85.46 - 99.48%)	1/1 ^a	91.49% (79.62 - 97.63%).	1/1 ^a	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% ^b (63.76 - 81.22%) - 86.11% ^c (78.13 - 92.01%)	90.07% ^a (83.9 - 94.46%) - 91.49% ^b (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 ^a Sensitivity could not be calculated as only one CPE was present

383 ^b when the 16 indeterminate results considered negative;

384 ^c when the 16 indeterminate results considered positive

385

Figure legends:

Figure 1. Boxplot showing the baseline subtracted McFarland Index for classical rCIM (no cloxacillin) and AmpC optimized rCIM-A (400 µg/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 false-positive results in classical rCIM protocol, are marked with boxed filled circles.

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.

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

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Supplemental Tables S1 and S2

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

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 ^a	n	Carba NP	rCIM/rCIM-A	
					Cloxacillin	
					0 μ g/mL	400 μ g/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	1-	1-	1-
	IMI-17	<i>E. cloacae</i>	2	+	2-	2-
	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 ^b	<i>E. kobei</i>	10	10+	10+	10-
	ACT-64	<i>E. kobei</i>	1	-	-	-
	ACT-60/7-like ^c	<i>E. cloacae</i>	1	-	-	-
	MIR-6	<i>E. cloacae</i>	1	-	-	-
	MIR-10-like ^c	<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	1+	2^d	-
		<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 ^a Species were determined through MALDI-ToF-MS analysis, using Biotyper (Bruker Daltonic)

22 ^b ACT with weak carbapenem-hydrolyzing activity ¹⁹

23 ^c single point mutant derivatives of the indicated enzymes

24 ^dTwo isolates (143A8 and 143A9) were previously tested and gave false-positive results.

25