



# Analysis of Paradoxical Efficacy of Carbapenems against carbapenemase-producing *Escherichia coli* in a Murine Model of Lethal Peritonitis

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1    **Analysis of Paradoxical Efficacy of Carbapenems against carbapenemase-producing**  
2    ***Escherichia coli* in a Murine Model of Lethal Peritonitis.**

3

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21

22    Running Title: Carbapenems to treat carbapenemase-producing *Escherichia coli* infections

23 **Abstract**

24 Clinical benefit of carbapenems against carbapenemase-producing Enterobacteriaceae (CPE)  
25 remains questioned. MICs of imipenem (IMP) and ertapenem (ERT) against isogenic  
26 derivatives of the wild-type strain *Escherichia coli* CFT073 producing KPC-3, OXA-48 or NDM-1  
27 were 0.25, 2, 16 and 64 mg/L for IMP and 0.008, 0.5, 8 and 64 mg/L, for ERT, respectively.  
28 Swiss ICR-strain mice with peritonitis were treated for 24h with IMP or ERT. Despite very  
29 limited duration of time during which free antibiotic concentrations were above the MIC  
30 (down to 0% for the NDM-1-producing strain), IMP and ERT significantly reduced bacterial  
31 counts in spleen and peritoneal fluid at 24h ( $P < 0.005$ ) and prevented mortality. Several  
32 possible explanations were investigated. Addition of 4% albumin or 50% normal human  
33 serum did not modify IMP activity. Bacterial fitness of resistant strains was not altered and  
34 virulence did not decrease with resistance. In the presence of sub-inhibitory concentrations  
35 of ERT, growth rates of OXA-48, KPC-3 and NDM-1 were significantly decreased and  
36 filamentation of NDM-1 strain was evidenced. *bla*<sub>NDM-1</sub> expression was not decreased *in vivo*  
37 as compared to *in vitro*. No zinc depletion was observed in infected mice as compared with  
38 MH broth. In conclusion, a paradoxical *in vivo* efficacy of IMP and ERT against highly resistant  
39 carbapenemase-producing *E. coli* was confirmed. Alternative mechanisms of antibacterial  
40 effects of sub-inhibitory concentrations of carbapenems may be involved to explain *in-vivo*  
41 activity. These results are in agreement with a potential clinical benefit of carbapenems to  
42 treat CPE infections, despite high carbapenem MICs.

43

## 44 Introduction

45 Antibiotic resistance, in particular in Gram negative bacilli, is a major threat facing modern  
46 medicine (1). The emergence and subsequent global spread of extended-spectrum  $\beta$ -  
47 lactamase-producing Enterobacteriaceae (ESBL-E) has led to a dramatic increase in  
48 carbapenem consumption. In this context, carbapenemase-producing Enterobacteriaceae  
49 (CPE) have appeared and spread worldwide. The emergence of CPE is of particular concern  
50 because such strains, as well as jeopardizing the use of carbapenems, are resistant to most  
51  $\beta$ -lactams as well as other classes of antibiotics such as fluoroquinolones, aminoglycosides  
52 and cotrimoxazole. Then, very few therapeutic options remain (2). The best known  
53 antibiotics available today are either toxic or poorly effective, explaining high mortality rates  
54 in CPE bacteremia, reaching up to 40% (3). Alternative therapeutic regimens are urgently  
55 needed; however, few novel antimicrobials are being developed, especially against metallo-  
56  $\beta$ -lactamase (MBL) producing CPE. Optimizing currently available antibiotics is therefore  
57 essential.

58 Interestingly, although CPE have different levels of resistance to carbapenems, antibiotic  
59 therapies including at least one carbapenem have been associated with a better outcome in  
60 severe infections due to CPE *Klebsiella pneumoniae*, especially in critically-ill patients (4-7).  
61 Concerning MBL specifically, although little clinical data is available, several reports of  
62 satisfactory outcomes with carbapenems, despite highly resistant strains *in vitro*, have been  
63 published (8,9). This unexpected *in vivo* activity of carbapenems alone or in combination  
64 against MBL producing strains has also been reported by others in mice models of either  
65 pneumonia or thigh infections (9, 11). Fitness cost associated with the CPE plasmids, poor *in*  
66 *vivo* expression of resistance or zinc depletion have been, among other explanations,  
67 suggested to support this phenomenon (12).

68 To gain insight into the discrepancy between *in vitro* resistance and the reports of *in vivo*  
69 efficacy of carbapenems in patients and animal models, we used a severe *Escherichia coli*  
70 peritonitis murine model complicated by severe sepsis and bacteremia to evaluate the  
71 activity of carbapenems against CPE with different levels of carbapenem resistance and  
72 explore several biological hypotheses which could explain how carbapenems maintain some  
73 efficacy *in vivo* against *in vitro* resistant isolates.

74

## 75 Results

76 **Antibiotic activities *in vitro*.** MICs of imipenem (IMP) and ertapenem (ERT) for each strain  
77 are shown in Table 1. *E. coli* strains CFT073 and OXA-48 remained in the susceptible range  
78 while KPC-3 and NDM-1 strains were highly resistant according to EUCAST breakpoints.

79 **Antibiotic concentrations and PK/PD parameters.** Antibiotic concentrations over time and  
80 PK/PD indexes for each antibiotic and strain are shown in Table 1. Regimens of one injection  
81 of IMP every 2 hours, and one injection every 6 hours of ERT reproduced the  $fT>MIC$   
82 obtained in humans for the susceptible strain (13-15). Values of  $fT>MIC$  were 0% for ERT  
83 against KPC3- and for ERT and IMP against NDM1-producing strains.

84 **Antibiotic activities in murine peritonitis.** Mean bacterial load two hours after inoculation  
85 was  $6.61 \log_{10}$  CFU/mL (range 2.53-8.17) in peritoneal fluid, with no differences between the  
86 four study strains except NDM-1 which tended to have higher bacterial titers ( $7.17 \log_{10}$   
87 CFU/mL,  $P<0.005$ ). All infected and untreated mice were bacteremic and spontaneous  
88 mortality at 24h was 100%. When treated, survival rates at 24h were 100% in all treatment  
89 groups for all strains except for ERT-treated mice infected with NDM-1, with a 90% (9/10)  
90 survival rate. Figure 1 represents bacterial counts in peritoneal fluid before and after  
91 treatment, for each strain. The two therapeutic regimens (IMP, ERT) produced a significant

decrease in viable bacterial counts compared to baseline, against TOPO, OXA-48, and NDM-1 strains ( $P < 0.05$ ). Against KPC-3 strains, decrease was significant only with ERT. For all strains, there was no significant difference in bacterial counts after 24h treatment between the therapeutic groups. For the OXA-48 and KPC-3 strains, as well as for the susceptible control, all mice survived when treated by ERT or IMP. No IMP- or ERT-resistant mutant was detected at the end of treatment for any of the tested strains.

**Analysis of discrepancy between *in vitro* and *in vivo* efficacy (Table 2).**

As PK/PD parameters could not explain the activity observed *in vivo* with both carbapenems, particularly against the carbapenem-resistant NDM-1 strain (Table 1), analysis of the possible causes of discrepancy between *in vitro* and *in vivo* efficacy was performed (Table 2).

**Technical issues.** The first explanations to be ruled out were technical issues. No plasmid loss was evidenced in bacterial strains at the end of treatment, indicating that the resistant gene was still present at the end of the experiment. Antibiotic concentrations in peritoneal fluid were below the detection limit for IMP and ERT at the time of sacrifice (0.5 mg/L). Therefore, there was no potential carry-over effect.

**Resistance gene expression *in vivo*.** In order to verify that the resistant gene was not only present but fully expressed *in vivo*, expression of *bla*<sub>NDM-1</sub> (as compared with *rrsA*) was measured in *E. coli* CFT073-NDM-1, in cells grown *in vitro* or *in vivo*, but no difference was found (Figure 2).

**Influence of albumin and human serum.** The presence of 4% albumin did not modify MICs of IMP and ERT (Table 1). The activity of IMP against CFT073 (Figure 3A) and NDM-1 (Figure 3B) in time-kill studies was not modified by the presence of 50% normal human serum. Zinc depletion did not modify IMP MICs against CFT073, OXA-48 and KPC-3 but decreased IMI MICs from 64 to  $< 0.06$  mg/L against NDM-1, as previously described (12).

116 **Bacterial growth and morphology.** No difference was found in terms of maximal growth  
117 rate (MGR) or time necessary to achieve maximal growth rate (T<sub>max</sub>) between the four  
118 study strains growing without antibiotics (data not shown), suggesting there was no fitness  
119 cost of resistance. Similarly, no difference was found in terms of MGR or T<sub>max</sub> for the  
120 strains growing with sub-inhibitory concentrations of IMP against pTOPO, OXA-48, KPC-3 or  
121 NDM-1 (data not shown). However, in the presence of sub-inhibitory concentrations of ERT,  
122 T<sub>max</sub> of OXA-48, KPC-3 and NDM-1 was significantly decreased (Figure 4). MGR was also  
123 significantly decreased against NDM-1 only (data not shown). Microscopic examination of  
124 NDM-1 strains growing under sub-inhibitory concentrations (1/8 x MIC) of ERT found  
125 filamentation of *E. coli*, which was not the case for the isolates exposed to IMP or to no  
126 antibiotics (Figure 5).

127 **Zinc concentrations.** Median zinc concentration in MH broth was 1.1 mg/L (range: 1.086 to  
128 1.177 mg/L). The median concentration of zinc in the sera from healthy mice was 2.02 mg/L  
129 [1.59-3.36] and 2.72 mg/L [1.94-3.87] in the sera from infected mice treated during 24h with  
130 IMP (*P* = 0.41).

131

## 132 Discussion

133 Thanks to the use of isogenic *E. coli* strains carrying different types of carbapenemases with  
134 varying IMP and ERT MICs *in vitro* and in a murine peritonitis model, we attempted to  
135 decipher here the fact that IMP and ERT alone were unexpectedly effective *in vivo* against  
136 isogenic CPE according to usual PK/PD indexes associated with *in vivo*  $\beta$ -lactam activity,  
137 despite *in vitro* resistance (Table 2). Indeed, both IMP and ERT were effective in decreasing  
138 bacterial counts in spleen and peritoneal fluid and allowing all mice to survive in this  
139 otherwise lethal model. However, considering free time over MIC (*f*<sub>T>MIC</sub>) as the relevant

140 PK/PD index associated with carbapenem activity, values of 0% were obtained for ERT  
141 against KPC3- as well as ERT and IMP against NDM1-producing strains (Table 1). The results  
142 for NDM-1 are especially striking, given that the levels of resistance were the highest  
143 (MIC=64mg/L) and the  $fT>MIC$  for both ERT and IMP was 0%. For OXA-48 and KPC-3 isolates,  
144 because the levels of resistance were lower, some  $fT>MIC$  was achieved, which may explain  
145 at least partially the efficacy of the drugs.

146 This unexpected *in vivo* activity of carbapenems against CPE, which cannot be explained by  
147 current PK/PD indexes (16), has been reported by others mainly for NDM-1 producers and in  
148 one report for KPC (10-12, 17). Here, we purposefully tested isogenic strains to assure that  
149 all things were otherwise equal, apart from resistance mechanisms and levels. This allowed  
150 us to investigate several hypothesis that may be involved in this phenomenon (Table 2).  
151 First, technical issues were eliminated, such as loss of resistant plasmid or a carry-over  
152 effect. A fitness cost of resistance (as measured by *in vitro* growth) was not involved either.  
153 Decreased virulence of carbapenemase-producing strains was not an issue here as mortality  
154 rates of untreated mice reached 100% for all strains.

155 A lower than expected impact of protein binding may explain part of what we found here.  
156 Indeed, we found no difference in the experiments evaluating *in vitro* bacteriostatic or  
157 bactericidal activity of ERT and IMP whether they were performed with or without 4%  
158 albumin, even though ERT protein binding is theoretically 95%. Taking into account total  
159 concentrations of the antibiotics, time above the MIC would be in a range of values  
160 associated with *in vivo* antibacterial effect against KPC3 producing strain (44-45% for ERT  
161 and IMP, respectively) and 11 and 17% for ERT and IMP against NDM-1 strain (Table 1).  
162 Although the percentage of protein binding is considered as “gold standard” for the  
163 evaluation of protein binding, the impact of protein binding on the activity of antibiotics is



164 multifaceted and more complex than indicated by the numerical value of protein binding  
165 alone (18). In particular, intrinsic properties of serum proteins may mediate serum-antibiotic  
166 synergism. However, standardization of the experiments to substantiate that phenomenon  
167 remains to be done (18).

168 Because NDM-1 has the highest resistance levels, we focused further investigations on this  
169 strain. One hypothesis was that a decrease in the expression of the NDM-1 could occur *in*  
170 *vivo* explaining the discrepancy between *in vitro* and *in vivo* resistance. However, normalized  
171 expression of bla<sub>NDM-1</sub> did not differ between cells grown *in vitro* and *in vivo* (Figure 2). The  
172 hypothesis that zinc concentrations in the infectious focus, may play a role in the expression  
173 of the MBL enzymes has recently been suggested by Asempa *et al.* (12). Indeed, authors  
174 found concentrations of zinc in the epithelial lining fluid of mice with lung infections to be  
175 much lower than zinc concentrations in the broth used to test the strains' susceptibility,  
176 suggesting they may not have been resistant to carbapenems in the focus of infection,  
177 despite *in vitro* resistance. However, we assayed zinc in MH broth and in the plasma of  
178 control and infected mice and found concentrations in the same range (#1-2mg/L),  
179 comparable to those found by Asempa *et al* in MHB broth and in mice plasma.

180 Another explanation could be related to an antibacterial effect resulting from a direct  
181 antibiotic/bacteria interaction but at sub-inhibitory concentrations. Our results suggest an  
182 impact of ERT on bacterial growth associated with filamentation at sub-inhibitory  
183 concentrations of 1/8 X MIC against NDM-1 producing strain; however, this was not  
184 observed with IMP. ERT inhibits predominantly penicillin-binding protein (PBP)-3 although it  
185 also has an impact on PBP-2. The former is responsible for cell septation during division. Sub-  
186 MIC concentrations of other antibiotics that target the PBP-3 such as piperacillin-tazobactam

187 alter septation of *E. coli* due to PBP-3 inhibition without inhibiting division, leading to the  
188 generation of filamentous cells (19, 20). Several reports have found that these filamentous *E.*  
189 *coli* are accompanied by the inhibition of production of virulence factors, biofilm formation  
190 and motility leading to a reduced pathogenicity of these strains in mice (20). IMP, on the  
191 other hand, targets only PBP-2 and at sub-MIC concentrations does not induce filaments but  
192 round forms (21, 22).

193 Other mechanisms may involve the interaction between antibiotic and host immune  
194 responses leading to an enhanced antimicrobial effect targeted by the antibiotic. Bacteria  
195 exposed even to sub-inhibitory concentrations of antibiotics may change their adherence  
196 properties, cell surface antigen, excretion of enzymes and toxins, and cell wall thickness (23).

197 Reports have found that *E. coli* grown in the presence of sub-MIC of IMP were phagocytized  
198 and killed in numbers significantly higher than untreated bacteria. Prior treatment with sub-  
199 MIC of IMP also resulted in an increased susceptibility of *E. coli* to the bactericidal activity of  
200 immune serum (21). IMP demonstrated a stimulating effect of innate immunity in mice that  
201 was greater with the lowest IMP doses, and also modified some acquired immune functions  
202 that would be useful to investigate in human (20, 24). More recently, Ulloa *et al.* found that  
203 avibactam had immune sensitizing activities against NDM producing *K. pneumonia* that was  
204 not appreciated by standard antibiotic testing and led to reduction in bacterial counts in lung  
205 from infected mice (25). To begin to explore the innate immune hypothesis, we tested the  
206 bactericidal activity of IMP in the presence and absence of normal human serum, but  
207 observed no difference, suggesting the intrinsic bactericidal activity of serum may not play a  
208 pivotal role here. An addition of these different phenomena, and maybe of others, is not  
209 excluded.

210 In conclusion, we found an unexpected *in vivo* efficacy of both ERT and IMP against  
211 carbapenemase-producing *E. coli* with high levels of resistance to both drugs. This was  
212 especially surprising for NDM-1 producers, where therapeutic options are particularly  
213 limited, as carbapenems, despite reaching no time above the MIC levels, allowed for mice  
214 survival and significant decrease in bacterial counts.

215 This may be the result of the impact of sub-inhibitory concentrations of antibiotics both on  
216 bacterial fitness and the host's immune response and/or other unknown factors. These data  
217 may explain clinical reports indicating therapeutic benefit from combinations including at  
218 least one carbapenem against CPE, even against highly resistant strains (4, 7).

219

## 220 **Material and methods**

221 **Bacterial strains, conjugation assays.** Three clinical isolates were used as sources of  
222 carbapenemase genes: KPC-3-producing *E. coli* 13832, OXA-48-producing *E. coli* 13828, and  
223 NDM-1-producing *E. coli* UR20. *E. coli* DH5a was used for cloning experiments, and  
224 uropathogenic *E. coli* CFT073 (O6:K2:H1), previously used to produce pyelonephritis and  
225 peritonitis in mice, was used as the final recipient strain (26, 27). The plasmid pCR-Blunt II-  
226 TOPO (Life Technologies, Saint-Aubin, France), which carried a kanamycin resistance gene,  
227 was used for cloning experiments. In order to avoid any problem of expression of resistance  
228 in vitro, carbapenemase genes were cloned into p-TOPO but with their own promotor. In  
229 order to avoid plasmid loss, all subcultures were done in the presence of kanamycin (400  
230 mg/L). PCR, cloning and DNA sequencing have been described previously (27). For NDM-1,  
231 cloning into pTOPO was performed using the NDM-1-F (5'- AGAGAAATTTGCTCAGCTTGTGA  
232 -3') and NDM1-R (5'- GATGGCAGATTGGGGGTGAC -3') primers. The four isogenic strains

233 carrying the empty vector (pTOPO) and recombinant plasmids with the *bla*<sub>KPC-3</sub>, *bla*<sub>OXA-48</sub> and  
234 *bla*<sub>NDM-1</sub> genes are referred to as TOPO, KPC3, OXA48 and NDM1, respectively.

235 ***In vitro* antibiotic activities.** MIC values of imipenem (IMP) and ertapenem (ERT) were  
236 determined by the broth microdilution method in accordance with EUCAST guidelines  
237 (www.eucast.org). Time-kill curves were performed to test for the bactericidal effect of each  
238 drug. To assess the impact of protein-binding on antibiotic activity, tests were also  
239 performed in the presence of 4% albumin (to approach plasma albumin concentrations in  
240 human around 40 g/L). To assess the impact of human serum on IMP activity, time-kill  
241 curves were also performed in the presence of 50% normal human serum (S4190-100  
242 EUROBIO). To assess the influence of zinc depletion, MICs were also performed in zinc  
243 depleted Mueller-Hinton (MH) broth by addition with 300 mg/L of EDTA, as described (12).

244 For time-kill curves, exponentially-growing *E. coli* cells were diluted in glass tubes containing  
245 10 ml MH broth to obtain an inoculum of ca. 10<sup>6</sup> CFU/mL and incubated with IMP or ERT in  
246 concentrations equal to 0,25-2 times the MIC value for the tested strain, or with no  
247 antibiotic. Viable counts were enumerated by plating 100-μL serial dilutions of cultures onto  
248 MH agar plates after 0, 1, 3, 6, and 24 h of incubation at 37°C, and expressed in log<sub>10</sub> CFU per  
249 milliliter. The lower limit of detection was 1 log<sub>10</sub> CFU/ml. A bactericidal effect was defined  
250 as a decrease of at least 3 log<sub>10</sub> in CFU counts compared to the initial inoculum.

251 **Growth experiments.** To measure growth rates of the different isolates with or without  
252 subinhibitory concentrations of ERT and IMP, bacterial cells grew overnight in Luria-Bertani  
253 medium at 37°C with constant agitation at 250 rpm, and were transferred into a fresh  
254 medium at a dilution of 1:10000. On the same plate, wells contained no antibiotic, ERT or  
255 IMP at concentrations of 1/4, 1/8 and 1/16 of the MIC for each strain (from 0.0625 to 0.0156  
256 mg/L of IMP for TOPO, to 4 to 16mg/L IMP for the NDM-1 strain). Growth was recorded by

an Infinite 200 Tecan® spectrophotometer, which measured OD<sub>600nm</sub> in each well every 5 minutes at 37°C for 24h. OD<sub>600nm</sub> were collected and log-transformed. The time in minutes to reach maximum growth rate was measured and the maximum growth rate was calculated and expressed in h<sup>-1</sup> (27). All *in vitro* experiments were repeated at least three times. Gram staining was performed on the bacteria growing at 24h.

**Experimental murine model.** Swiss ICR-strain immunocompetent female mice aged 5-6 weeks and weighing 25-30 g were used in the experimental model of intra-abdominal infection (27).

**Antibiotic concentrations in mice.** To determine the therapeutic regimen in mice that best reproduces the percentage of time during which free-drug concentrations of antibiotic exceeded the MIC ( $fT > MIC$ ) obtained in humans with standard regimens (1 g every 8 hours for IMP and 1 g every 24 hours for ERT), pharmacokinetic studies were performed on mice. A dose of 100 mg/kg was chosen for both IMP and ERT as they had previously been shown to reproduce the plasma peak concentrations that corresponded to human concentrations (28, 29). Free-drug concentrations of IMP and ERT had previously been determined in mice in our laboratory, and were found to be 37% for IMP and 1% for ERT (28).

Blood samples of a least 500 µL were obtained by intracardiac puncture from 4 anesthetized mice 30, 60, 90, and 120 minutes after a single subcutaneous injection of IMP (100 mg/kg) or 30, 60, 120, 240, 360 and 480 minutes after a single subcutaneous injection of ERT (100 mg/kg) [16]. Concentrations of IMP and ERT were determined by high-performance liquid chromatography (HPLC) with ultraviolet detection at 237 nm, and the limit of detection was 0.5 mg/L for both compounds (29).

**Treatment experiments.** Pellets of overnight *E. coli* cultures were mixed with porcine mucin 10% (Sigma-Aldrich, Saint-Quentin Fallavier, France). Mice, kept in single units during the

281 experimental time, were inoculated with a 250- $\mu$ L intra-peritoneal injection of  
282 bacteria/mucin mix, corresponding to a final inoculum of approximately  $10^8$  CFU/mL. Two  
283 hours after inoculation, 15 mice for each strain were sacrificed to determine start-of-  
284 treatment bacterial load. We had previously shown that the death rate of this model with  
285 the same bacterial strains was almost 100% in the absence of treatment and all mice were  
286 bacteremic; thus, for ethical reasons no untreated control group was used (27).

287 In the therapeutic groups, treatment was started 2h post infection and lasted 24h. Mice  
288 received either IMP 100 mg/kg subcutaneously every 2 hours or ERT 100 mg/kg  
289 subcutaneously every 6 hours. Mice were sacrificed by intraperitoneal injection of 200  $\mu$ L of  
290 sodium thiopental 4h after the last antibiotic injection. Immediately after sacrifice, a  
291 peritoneal wash was performed by intraperitoneal injection of 2 mL of sterile saline solution  
292 followed by gentle massage of the abdomen and opening of the peritoneum to collect 1 mL  
293 of fluid. Spleen was extracted and homogenized in 1 mL of sterile saline solution. In mice  
294 that did not survive the infection, only spleen was extracted within 2h after death to avoid  
295 sample contamination. Samples diluted by a factor of 10 were plated onto MH agar for  
296 quantitative culture, containing or not IMP and ERT at a concentration of 4 $\times$ MIC to detect  
297 the selection of resistant mutants *in vivo*. Results were expressed as log<sub>10</sub> CFU/g for spleen  
298 and log<sub>10</sub> CFU/mL for peritoneal fluid. The lower limit of detection was 1.3 log<sub>10</sub> CFU/g or  
299 mL. Lack of plasmid loss was verified on surviving micro-organisms by determining ERT and  
300 IMP MICs and by enumerating CFUs on kanamycin containing plates (400 mg/L).

301 **RNA extraction and qRT-PCR.** To measure the expression of the *bla*<sub>NDM-1</sub> gene, total RNAs  
302 were extracted from bacterial cells grown to the late-exponential phase in vitro and from  
303 bacterial cells extracted from the peritoneal cavity 2 hours after inoculation in vivo. Total  
304 RNAs were extracted the RNeasy PowerMicrobiome kit (Qiagen Hilden, Germany) and  
305 residual chromosomal DNA was removed by treating samples with the TURBO DNA-free kit  
306 (Life Technologies, Saint-Aubin, France). Samples were quantified using the NanoDrop One  
307 spectrophotometer (Thermo Fisher Scientific, Courtaboeuf, France). cDNA was synthesized  
308 from total RNAs (approximately 1 µg) using the QuantiTect reverse transcription kit (Qiagen)  
309 according to the manufacturer's instructions.

310 Since it is quite impossible to know if cells are in the same growth phase in vitro and in vivo,  
311 we performed an absolute quantification of the expression of *bla*<sub>NDM-1</sub> gene. The amounts of  
312 transcript levels were normalized using the *rrsA* gene as a housekeeping control gene. The  
313 amounts of transcript copies were determined by extrapolation from the linear regression of  
314 the standard curve obtained for each set of primers amplifying *bla*<sub>NDM-1</sub> and *rrsA* genes (Table  
315 3). Ratio *bla*<sub>NDM-1</sub> copy number / *rrsA* copy number was calculated to compare the *bla*<sub>NDM-1</sub>  
316 expression in *in vitro* and *in vivo* samples.

317 **Zinc concentrations.** Levels of Zn<sup>2+</sup> were measured in MH broth and in mouse serum by  
318 inductively coupled serum mass spectrometry (ICP-MS) on an X-Series II from Thermo  
319 Scientific® (Platform AEM2, University of Rennes 1/Biochemistry Laboratory, University  
320 Rennes Hospital) (30).

321

## 322 **Statistical analysis**

323 Continuous variables are expressed as the median followed by the range (minimum to  
324 maximum) in brackets, and were compared using a nonparametric test (Kruskall-Wallis).

325 Maximal growth rates and time to achieve maximal growth rate values were compared using  
326 the Kruskal-Wallis test. A P value of less than 0.05 was considered significant. All statistical  
327 analyses were conducted using the Prism software (version 7.0a).

328

329



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335 **Conflict of interest**

336 All authors declare they have no conflict of interest with this publication.

337

338 **Ethical issues**

339 Animal experiments were performed in our laboratory in accordance with prevailing

340 regulations regarding the care and use of laboratory animals and approved by the

341 Departmental Direction of Veterinary Services (Paris, France, agreement no. 75-861). The

342 peritonitis protocol (n° APAFIS#4949-2016021215347422 v5) was approved by the French

343 Ministry of Research and the ethics committee for animal experiment.

344

345

346

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453 **Table 1.** MICs and pharmacokinetics/pharmacodynamics in mice treated with ERT and IMP for each study strain, as compared with Human. For  
454 ERT:  $S \leq 0.5$  ;  $R > 1$  and for IMP:  $S \leq 2$ ;  $R > 8$ .

Antibiotics	Species	Regimens	Strains	MIC (mg/L)	MIC + 4%	T>MIC (%)	fT>MIC (%)
albumin (mg/L)							
ERT	Human	1g q 24h	TOPO	0.008	0.008	100	100
	Mouse	100mg/kg q 6h	TOPO	0.008	0.008	100	100
			OXA-48	0.5	0.5	100	14
			KPC-3	8	8	44	0
			NDM-1	64	64	11	0
IMP	Human	1g q 8h	TOPO	0.25	0.25	100	100
	Mouse	100mg/kg q 2h	TOPO	0.25	0.25	100	100
			OXA-48	2	2	75	65
			KPC-3	16	16	45	40
			NDM-1	64	64	17	0

455 -

456 **Table 2.** List of possible mechanisms to explain the paradoxal activity of carbapenems *in vivo* against carbapenemase-producing strains.

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Hypothesis	Test /Results	Comments
Loss of plasmid	All subcultures were performed in the presence of kanamycin (400 mg/L)s (a kanamycin resistance gene was present within the pTOPO plasmid)	No loss of plasmid
Carry-over effect	Antibiotic concentrations in peritoneal fluid were below the detection limit (0.5 mg/L) for IMP and ERT at the time of sacrifice	No carry over effect
<i>In vitro</i> fitness cost of resistance	Comparison of <i>in vitro</i> growth between susceptible and resistant strains: no difference found	No <i>in vitro</i> fitness cost of resistance
Decrease <i>in vivo</i> virulence	Mortality of untreated mice was 100% for all strains	No decrease in <i>in vivo</i> virulence
Impact of antibiotic protein binding	<i>In vitro</i> bacteriostatic and bactericidal activity of ERT and IMP tested with or without 4% albumin: no difference found	If total antibiotic concentrations taken into account (instead of free concentrations only), PK/PD parameters allow for some T>MIC of



		ERT ad IMP against all strains (see table 1).  The impact of protein binding on the activity of antibiotics may be more complex than indicated by the numerical value of protein binding
Decrease in expression of carbapenemase <i>in vivo</i> vs <i>in vitro</i>	No difference was found in terms of expression of <i>bla</i> <sub>NDM-1</sub> (compared to <i>rrsA</i> ) in <i>E. coli</i> CFT073-NDM-1 between cells grown <i>in vitro</i> or <i>in vivo</i>	No decrease in <i>bla</i> <sub>NDM-1</sub> expression <i>in vivo</i>
Zinc concentrations <i>in vivo</i> may differ and explain discrepancy between resistance levels measure <i>in vitro</i> and <i>in vivo</i> for NDM-1 strains	Measured zinc concentrations in MH broth, serum of control and infected mice  Median zinc concentrations in MH broth, healthy mice and infected mice were 1.1 mg/L [1.086 to 1.177], 2.02 mg/L [1.59-3.36] and 2.72 mg/L [1.94-3.87] respectively ( $P = 0.41$ )	No difference was found in zinc concentrations between Mueller-Hinton broth and mice serum
Antibacterial effect	<i>In vitro</i> growth curves in the presence of sub-inhibitory	Sub-MIC concentrations of ERT (which targets

of sub-inhibitory concentrations of antibiotics	concentrations of IMP and ERT. <ul style="list-style-type: none"><li>- For IMP: no effect</li><li>- For ERT: prolonged Tmax for study strains and decreased maximum growth rate for NDM-1. Filamentation of <i>E. coli</i> at 1/8 x MIC was evidenced.</li></ul>	PBP-3) may inhibit septation without inhibiting division (as other beta-lactams), leading to the generation of filamentous cells and growth impairment as seen here. IMP targets only PBP-2 and does not induce filamentation.
Interaction between antibiotic and host immune response leading to an enhanced antimicrobial effect	Bactericidal activity of IMP in the presence and absence of pooled human serum was not different	No intrinsic bactericidal activity of serum was evidenced.  The interaction between antibiotic and host immune response should be further explored.

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459 **Table 3.** Deoxynucleotide primers used in this study

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Primer	Nucleotide sequence (5' to 3')	Gene	Purpose
RT-Q-PCR-NDM1_F	TGGCCCGCTCAAGGTATTTT	Quantification of <i>bla</i> <sub>NDM-1</sub>	
RT-Q-PCR-NDM1_R	TGCTGTCCTTGATCAGGCAG		
RT-qPCR_rrsA_F	CTCTTGCCATCGGATGTGCCCA	Quantification of <i>rrsA</i> (Housekeeping gene)	
RT-qPCR_rrsA_R	CCAGTGTGGCTGGTCATCCTCTCA		

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465 **Figure Legends.**

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467 **Figure 1.** Bacterial counts in peritoneal fluid before treatment and after 24 h treatment with IMP, and ERT for each strain. Results are  
468 expressed in log<sub>10</sub> CFU/mL.

469 **Figure 2.** Normalized expression of bla<sub>NDM-1</sub> (compared to *rrsA*) in *E. coli* CFT073-NDM1 cells grown in vitro or in vivo conditions. ns, denotes  
470 not significant.

471 **Figure 3.** Time-kill curves of IMP at MIC concentration alone or in combination with or without 50% pooled human serum against susceptible *E.*  
472 *coli* CFT073 (A. top) and *E. coli* CFT073-NDM-1 (B. bottom).

473 **Figure 4.** Time to achieve maximal growth rate (T<sub>max</sub>, x 10<sup>4</sup> sec) of the study strains in the presence of sub-inhibitory concentrations of ERT  
474 (left panel) and IMP (right panel).

475 **Figure 5.** Photographs of rod shape NDM-1 producing *E. coli* after 24h growth in MH medium with no antibiotic (A), ERT 8 mg/L (0,125xMIC) (B)  
476 and IMP 8mg/L (0,125xMIC) (C). Photographs taken using a light microscope at magnification of x1000 before - upper panels - and after Gram  
477 staining – lower panels).

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Figure 1

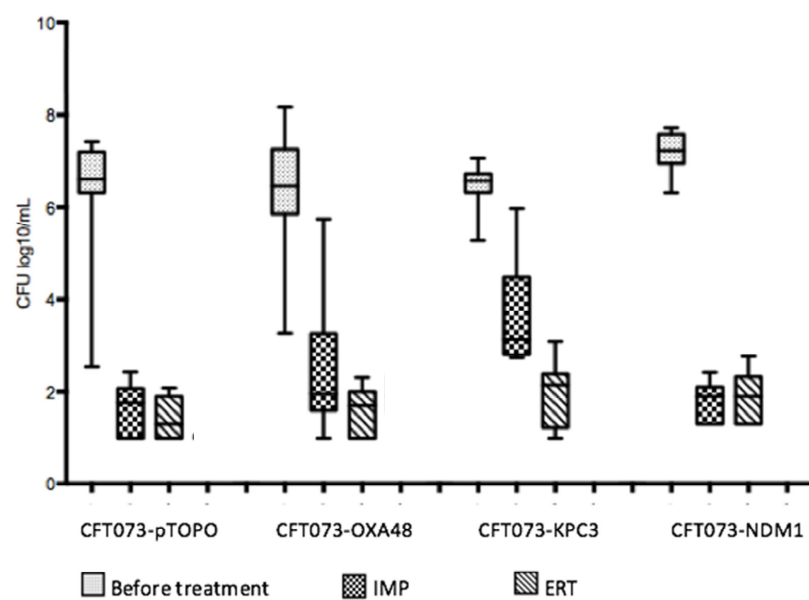
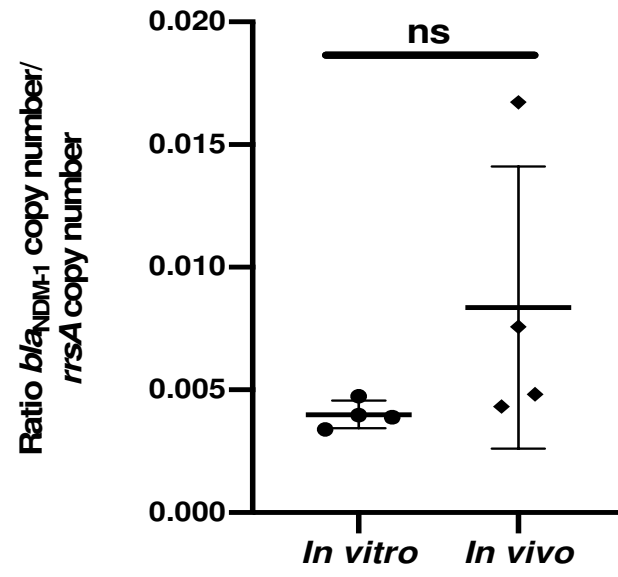


Figure 2



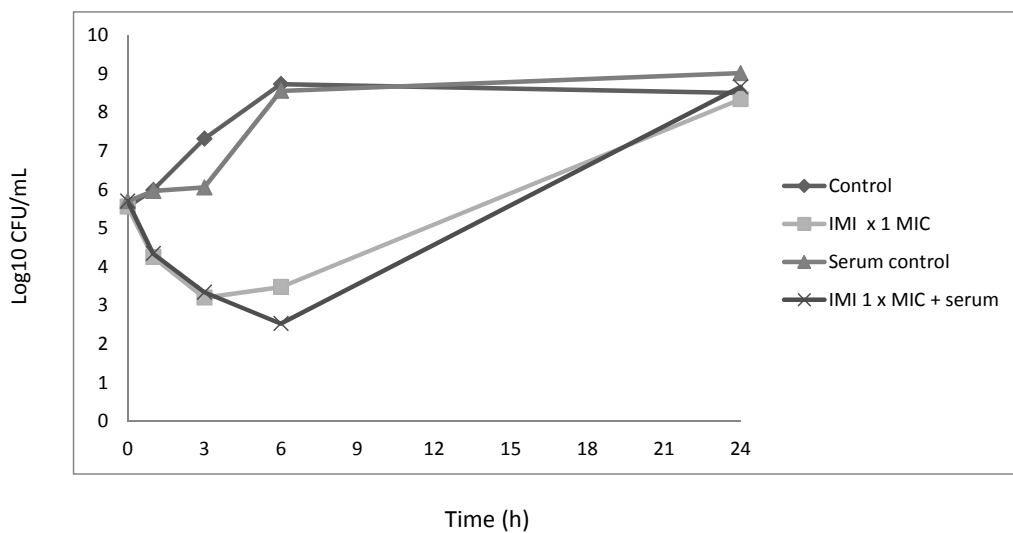
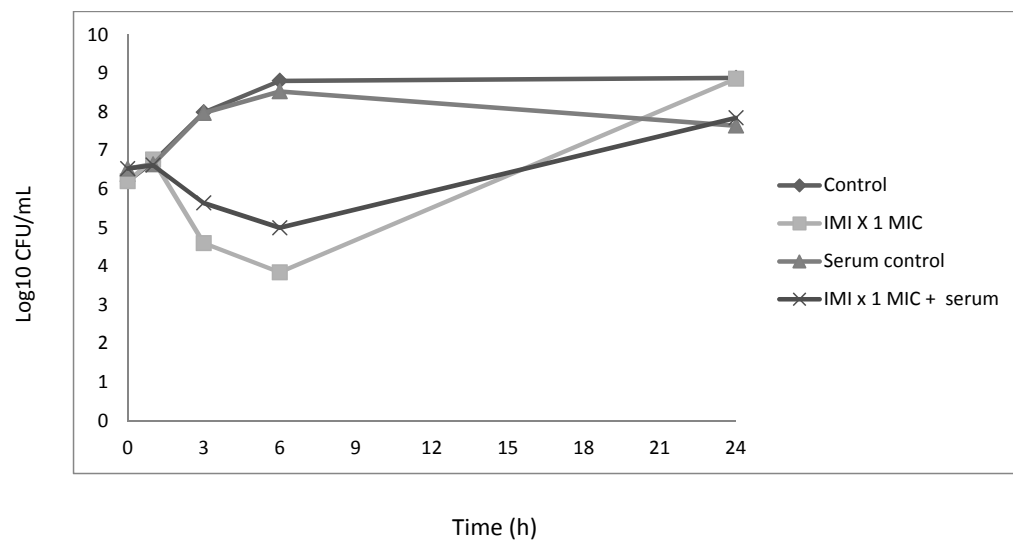
**Figure 3.****A. Stain *E. coli* CFT073****B. Stain *E. coli* CFT073-NDM-1**

Figure 4

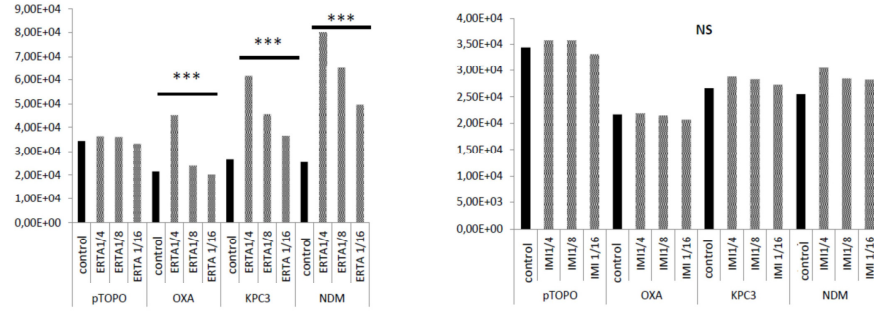




Figure 5.

