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1 **Use of Animal models to support revising meningococcal breakpoints of beta-lactams**

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19 imaging, inflammation

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Abstract

Antibiotic susceptibility testing (AST) in *Neisseria meningitidis* is an important part of the management of invasive meningococcal disease. It defines minimal inhibitory concentrations (MICs) of antibiotics that are used in treatment and/or prophylaxis and that mainly belong to beta-lactams. The interpretation of the AST requires breakpoints to classify the isolates into susceptible, intermediate or resistant. The resistance to penicillin G is defined by MIC>0.25 mg/L and that of amoxicillin is defined by MIC>1 mg/L. We provide data that may support revision of resistance breakpoints for beta-lactams in meningococci.

We used experimental intraperitoneal infection in 8-week-old transgenic female mice expressing human transferrin and human factor H. Dynamic bioluminescence imaging was performed to follow the infection by bioluminescent meningococci with different MIC. Three hours later, infected mice were treated intramuscularly using several doses of amoxicillin or penicillin G. Signal decreased during infection with meningococci with the strain showing MIC of 0.064 mg/L of penicillin G with all doses. Signals only decreased for the strain with MIC of 0.5 mg/L of penicillin G after treatment with the highest doses corresponding to 250,000 units/kg of penicillin G or 200 mg/kg of amoxicillin although to a slower rate than the strain with MIC of 0.064 mg/L. The decrease of bioluminescent signals was associated with a decrease in the levels of the pro-inflammatory cytokine, IL-6. Our data suggest that high dose of amoxicillin or penicillin G can reduce growth during infection by isolates showing MIC of penicillin G of > 0.25 mg/L and ≤ 1mg/L.

51 **Introduction**

52 *Neisseria meningitidis* is a Gram negative bacterium frequently encountered in human
53 nasopharynx but it is also the causative agent of invasive meningococcal disease (IMD) that
54 provokes mainly septicemia and meningitis. *Neisseria meningitidis* remains susceptible to
55 beta-lactams, the antibiotics of choice in the treatment of IMD (1). Resistance to beta-lactams
56 in meningococci is extremely rare, but reduced susceptibility has been described to penicillin
57 G and to amoxicillin (intermediate isolates, Pen^I). However, neither resistance nor reduced
58 susceptibility to cephalosporin of third generation has been detected so far (2). The
59 proportions of Pen^I isolates differ worldwide and are increasing in several countries and can
60 reach >30% of total meningococcal isolates (3-7).

61 We have previously shown direct correlation between the polymorphism of *penA* gene
62 encoding the penicillin binding protein 2 (PBP2) and the Pen^I phenotype. This phenotype
63 seems to result from the reduced affinity of penicillin G and amoxicillin to PBP2 as well as to
64 modification of peptidoglycan structure in Pen^I isolates with increased pentapeptide-
65 containing muropeptides (8). Horizontal interspecies DNA exchanges in the genus *Neisseria*
66 are suggested to drive the polymorphism of *penA* (7). Antibiotic susceptibility testing (AST)
67 is mandatory for beta-lactam antibiotics and requires reliable breakpoints to inform decision
68 making in patient treatment.

69 In order to consistently define breakpoints, sequencing of *penA* from a large collection of
70 isolates allowed linking wild-type alleles of *penA* to low minimal inhibitory concentration
71 (MIC) for penicillin G (<0.125 mg/L)(7). This defined the epidemiological cut off values for
72 susceptibility to penicillin G of MIC to be lower than 0.125 mg/L and <0.250 mg/L for
73 amoxicillin (7). It divided the meningococcal population into one part containing isolates
74 harboring wild-type alleles of *penA* and another part comprising isolates showing highly
75 diverse *penA* alleles and MICs of ≥0.125 mg/L and 0.250 mg/L for penicillin G and

amoxicillin, respectively (7). The value of MIC<0.125 mg/L was preferred to define the susceptibility to penicillin G as it allows to fill the important rule to not split wild-type MIC distribution (9) as isolates with wild type *penA* showed MIC of 0.094 mg/L(7). These values fitted with those used by the European Committee for Antimicrobial Susceptibility Testing (EUCAST; http://www.eucast.org) and the Clinical and Laboratory Standards Institute (CLSI)(10).

Intermediate isolates are expected to be treatable by beta-lactams, i.e. bacteria growth is reduced and/or bacteria are cleared from biological fluids. However, the higher limit of Pen^{I} isolates is still to be determined. EUCAST and CLSI indicate that isolates with MIC of penicillin G and amoxicillin >0.250 mg/L and >1mg/L respectively are resistant (Pen^{R}) to these beta-lactams (i.e. non treatable/treatment failure). However, isolates with MIC of penicillin G >0.250 mg/L harbor similar modified *penA* alleles as Pen^{I} isolates (7). The definition of resistant breakpoints is mainly driven by pharmacokinetic (PK) and pharmacodynamic (PD) indices that reflect antibiotic concentration and its effect respectively. However, experimental data are needed to correlate breakpoints to treatment. The use of animal models may help testing whether these breakpoints correspond to resistance and treatment failure.

101 **Materials and Methods**

102 **Ethics Statement**

103 This study was carried out in strict accordance with the European Union Directive
104 2010/63/EU (and its revision 86/609/EEC) on the protection of animals used for scientific
105 purposes. Our laboratory has the administrative authorization for animal experimentation
106 (Permit Number 75-1554) and the protocol was approved by the Institut Pasteur Review
107 Board that is part of the Regional Committee of Ethics of Animal Experiments of Paris region
108 (Permit Number: 99-174).

109 **Meningococcal isolates: Phenotypic and Genotypic characterization**

110 Two clinical isolates of *N. meningitidis* were used (LNP24198 and LNP27704). Both isolates
111 were of serogroup C and belonged to the clonal complex ST-11 (cc11). They harbored
112 respectively the *penA* alleles *penA*3 and *penA9* corresponding to a wild-type and a modified
113 alleles respectively. MIC of penicillin G was determined as previously recommended using
114 Etest with Mueller-Hinton agar supplemented with sheep blood (11) and were 0.064 mg/L
115 and 0.5 mg/L respectively. MICs of amoxicillin were 0.125 and 1.5 mg/L respectively.
116 Bioluminescent variants of both isolates were constructed by transformation with the
117 recombinant plasmid pDG34, which carries the bioluminescent luxCDABE operon under the
118 control of the *porB* promoter (12) and named LNP24198lux and 27704lux. Both strains were
119 checked for their MICs of penicillin G, and for amoxicillin and their *penA* alleles verified by
120 sequencing, showing that they were identical to the parent isolates. Both strains grew
121 similarly on meningococcal growth medium.

122 **Mice infection and dynamic live imaging studies**

123 We took advantage from the availability of an animal model to study *N. meningitidis*
124 infection, the transgenic mice expressing the human transferrin, since an iron source is
125 required for meningococcal growth (13). We have recently developed another animal model,

126 a transgenic mice expressing the human factor H (fH) that allowed binding of this negative
127 regulator of complement pathway on bacterial surface and hence allowing meningococci to
128 escape complement-mediated lysis (14). The two types of mice were crossed to generate
129 transgenic mice expressing both human transferrin and human fH that we used in infection
130 experiments using bioluminescent meningococcal strains with different MIC to penicillin G
131 and amoxicillin. Mice were in-house bred and were kept in a biosafety containment facility, in
132 filter-topped cages with sterile litter, water and food, according to institutional guidelines.
133 Mice were infected by intraperitoneal route (i.p.) with standardized inoculate of 5×10^6
134 bioluminescent colony forming units (CFU) per mouse in 0.5 ml of bacterial suspension. At
135 the time point of 3 h the mice were divided into three groups that were treated by either
136 penicillin G or amoxicillin only once by intramuscular injection in the interior face of the left
137 thigh. The following increasing unique doses (per mouse) of penicillin G (60,000 units/kg,
138 120,000 units/kg or 250,000 units/kg; corresponding to 37mg/kg, 75 mg/kg and 150 mg/ml)
139 or of amoxicillin (50 mg/kg, 100 mg/kg and 200 mg/kg). The highest doses of both antibiotics
140 corresponded to a daily dose used in treatment of IMD in humans. A group of two mice were
141 only injected with saline as control. Interleukin-6 (IL-6) levels were also assayed in blood at
142 the end point (8 h) as previously described (15). As similar results were obtained from both
143 experiments with penicillin G and amoxicillin, IL-6 was tested for mice experiment treated
144 with amoxicillin.
145 Bacterial infection images were acquired after 0.5 h, 3 h, 6 h and 8 h of infection using an
146 IVIS 100 system (Xenogen Corp., Alameda, CA) as previously described (13). Analysis and
147 acquisition were performed using Living Image 4.3.1 software (Xenogen Corp.). Data were
148 analyzed by linear regression using GraphPad InStat version 3.06 (GraphPad Software, San
149 Diego, CA, USA).
150

151 **Results**

152

153 **Impact of MIC on amoxicillin treatment during meningococcal infection in mice**

154 In order to test the evolution of the experimental infection *in vivo*, transgenic mice were
155 infected i.p. by the LNP24198lux (MIC penicillin 0.064 mg/L and amoxicillin 0.125 mg/L) or
156 the strain LNP27704 (MIC penicillin 0.5mg/L and amoxicillin 1.5 mg/L). After 3 h of
157 infection, infected mice were divided into groups that were treated by one of the three
158 antibiotic doses (Amoxicillin or penicillin G). A group of mice was left untreated as a control
159 group.

160 After bacterial intraperitoneal challenge, dynamic bioluminescence imaging showed that
161 30 min after the bacterial suspension injection, bacteria were mainly present in the peritoneal
162 cavity in both types of strains LNP24198lux (MIC penicillin 0.064 mg/L and amoxicillin
163 0.125 mg/L) and LNP27704lux (MIC penicillin 0.5 mg/L and amoxicillin 1.5 mg/L). Signal
164 increased in all mice after 3 h of infection. For mice infected with the strain LNP24198lux
165 and 6 h after infection (3 h after treatment with amoxicillin), the signal decreased in mice
166 treated with 200 mg/kg dose and were cleared after 8 h of infection (5 h after treatment). For
167 the two other doses, the signal only decreased after 8 h of infection (5 h after treatment). In
168 the untreated mice, signal continued to increase in all time points (Fig.1A). For mice infected
169 with the strain LNP27704lux (MIC penicillin 0.5 mg/L and amoxicillin 1.5 mg/L), the
170 bioluminescent signal decreased only in mice treated with the highest dose (200 mg/kg) while
171 signal continued to increase in mice treated with 50 mg/kg and 100 mg/kg and did not differ
172 from the signal observed in untreated mice (Fig.1B).

173 Linear regression analysis confirmed the significant decrease of bacterial viability for the
174 strain LNP24198lux in amoxicillin-treated mice in a dose-dependent manner. Negative slopes
175 were also significantly different from the slope of untreated mice (P<0.01 and r^2 of 0.38 and

176 0.85 respectively for 100 mg/kg and 200 mg/kg doses) (Fig. 1A; 1B). However, for the dose
177 of 50 mg/kg, the slope was not significantly different from zero (P=0.77 and r^2=0.007)
178 suggesting bacteriostasic effect (Fig.1A; 1B).

179 For the strain LNP27704lux, only the 200 mg/kg dose resulted in a negative slope that
180 differed significantly from that observed with untreated mice and from the other doses of
181 amoxicillin-treated mice ($P<0.001$ and r^2=0.59). For the strain LNP27704lux treated with the
182 two other doses (50 mg/kg and 100 mg/kg) the slopes were positive and similar to those of
183 untreated mice suggesting bacterial growth (Fig. 1A; 1B). For the 200 mg/kg-treated mice,
184 the r^2 values for the strain LNP27704lux was lower than that for strain LNP24198lux (0.59
185 versus 0.85 respectively) suggesting slower clearance of the former strain.

186 We also performed similar experiments using penicillin G with (doses of 60,000 units/kg;
187 125,000 units/kg and 250,000 units/kg). Mice were infected i.p. by the strain LNP27704
188 (MIC penicillin 0.5mg/L and amoxicillin 1.5 mg/L) and after 3 h of infection, three groups of
189 infected mice were treated by one of the three penicillin G doses. A group of mice was left
190 untreated as a control.

191 Bioluminescent signal increased and spread after 3 h of infection. After treatment, only the
192 mice group treated with the highest dose (250,000 units/kg) showed a decrease of
193 bioluminescent signal indicating loss of bacterial growth. The signal continued to increase in
194 mice that were infected by LNP27704lux and treated with either 60,000 units/kg or 125,000
195 units/kg of penicillin G (Fig. 1C).

196 Linear regression analysis confirmed the impact of the highest dose in mice infected with
197 the strain LNP27704lux. The highest dose resulted in a negative slope that differed
198 significantly from that obtained with the other doses and untreated mice ($P<0.001$) with an r^2
199 value of 0.12.

200 For the mice infected by the strain LNP24198lux (MIC 0.064mg/L) and treated with
201 penicillin G, the data were similar to those obtained with amoxicillin treatment (data not
202 shown).
203 All these data taken together suggest the highest dose, similar to the daily dose used in
204 humans, leads to decrease of bacterial counts in mice even if the strain is considered Pen^R.
205
206 **Decreased inflammatory response in mice treated with optimal amoxicillin dose**
207 We next assayed the levels of IL-6, a proinflammatory cytokine at 8 h post-infection. The
208 data are showed in Table 1 and clearly suggest a dose dependent decrease of IL-6 levels in
209 amoxicillin-treated mice that were infected by the strain LNP24198lux (MIC penicillin 0.064
210 mg/L and amoxicillin 0.125 mg/L). These levels were not detectable in mice treated with 200
211 mg/kg of amoxicillin. On the other hand, mice infected with the strain LNP27704lux (MIC
212 penicillin 0.5 mg/L and amoxicillin 1.5 mg/L), and treated with 50 mg/kg and 100 mg/kg of
213 amoxicillin showed similar levels of IL-6 as obtained in untreated mice (Table). IL-6 level
214 was not detectable in mice treated with the highest dose (200 mg/kg of amoxicillin).
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225 **Discussion**

226 We have previously emphasized the advantages of *in vivo* bioluminescent imaging for real-
227 time monitoring of meningococcal infections and their treatment (13, 16). This technology is
228 sensitive, rapid and non-invasive. It limits animal-to-animal variation and reduces the number
229 of animal used. In the present study, we adapted this system to evaluate the correlation of
230 beta-lactams breakpoints during experimental meningococcal infection in transgenic mice.
231 *N. meningitidis* is intrinsically susceptible to many antibiotic classes and MIC_{50} and MIC_{90}
232 values (antibiotic concentrations respectively inhibiting 50% and 90% of a sample of strains)
233 are very low(17). Meningococcal resistance to beta-lactams remains rare and meningococcal
234 strains remained susceptible to penicillin G with an MIC_{50} of penicillin G of 0.06 mg/L. Few
235 old reports described rare beta-lactamase producing strains with MICs that may reach or
236 exceed 256 µg/ml (18, 19). These beta-lactamases are plasmid-borne TEM-1 type enzymes
237 similar to those described in *Neisseria gonorrhoeae* that inactivate penicillin G and
238 amoxicillin but does not hydrolyze third generation cephalosporins (18). However, isolates
239 with reduced susceptibility to penicillin G and amoxicillin are quite frequent worldwide (7).
240 The MICs of amoxicillin and penicillin G for these intermediate isolates remain low and
241 defining the upper limit of the critical values remains problematic.

242 Although treatment failures have been described for strains with the highest MICs (20), the
243 severe infections caused by these strains generally resolve favorably using high doses of
244 penicillin G or amoxicillin, which allow bactericidal concentration to be reached in
245 cerebrospinal fluid. The effect of beta-lactams is dependant on the time the antibiotic
246 concentration exceeds its MIC for the microorganism (T > MIC) (9). Our data with the
247 highest dose of antibiotics are in agreement with this consideration. This dose corresponds to
248 the recommended total daily dose for the treatment of IMD (21). Our data suggest that the
249 strain with MIC of 0.5 mg/L may not be classified as resistant to penicillin G as it was

250 treatable. However, lower doses of penicillin G and amoxicillin failed to control the bacterial
251 growth and may lead to treatment failure. As the threshold of 1 mg/L corresponds to the
252 effective therapeutic concentration in the CSF obtained during treatment with penicillin G
253 (22), we may suggest that the upper breakpoint for penicillin G to by 1 mg/L and thus
254 intermediate isolates may be defined as those with MIC ≥0.125mg/L and ≤1mg/L. This range
255 also contains the isolates with altered *penA* alleles that all shared the same altered residues (7).
256 Isolates with MICs >0.25 mg/ml and ≤ 1mg/ml may not be considered as Pen^R isolates.
257 Indeed, the drug effect, *in vivo*, is subjective to bacterial growth rate but is also dependant on
258 host defense mechanisms. These latter aspects are not directly considered by the conventional
259 PK/PD model but can be addressed using relevant animal models as described in this work.
260 IL-6 is considered to be an important mediator of acute inflammatory responses to bacterial
261 infection (23). After 8 h of infection, the levels of IL-6 increased in mice infected by both
262 isolates and were higher in mice infected by the susceptible strain (LNP24198lux) than in
263 mice infected by the resistant isolate (LNP27704lux). This is in agreement with our previous
264 study that meningococcal isolates with modified PBP2 showed significant lower induction of
265 the inflammatory response (15). The mice treated with the higher dose did not show any
266 detectable IL-6. The trends of IL-6 levels in amoxicillin treated groups followed the
267 microbiological results. Our data are consistent with previous reports suggesting that IL-6 was
268 a possible indicator of bacterial killing (24).
269 Finally, our data suggest that if amoxicillin and penicillin G are to be used in treatment of
270 IMD prior determination of MIC, the first dose should be 200 mg/Kg and 250,000 units/kg
271 respectively.
272 Our findings clearly highlight a powerful approach in defining breakpoints through the
273 analysis the polymorphism of the gene encoding the targets of the antibiotics to characterize
274 the alterations of the targets and their correlation with MIC. The use of animal models in

275 association with PK/PD analysis can then allow defining the breakpoints. Finally, the increase
276 of MIC in meningococci may also correspond to other undefined mechanisms that require
277 additional studies. However, our proposal for a breakpoint for resistance to penicillin G
278 higher than 1 mg/L fits with other breakpoints for pathogens showing meningeal tropism (25).

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402 **Legend to Figure**

403 **Figure 1**. Transgenic mice were infected i.p. with standardized inocula of 5×10^6
404 bioluminescent colony forming units (CFU) per mouse in 0.5 ml of bacterial suspension.
405 Quantification was performed after 30 min, 3 h, 6 h and 8 h of infection by defining regions
406 of interest (the whole mouse). After 3 h of infection, mice were treated with the unique
407 indicated doses of amoxicillin or penicillin G by intramuscular injection in the interior face of
408 the left thigh. The untreated mice received an injection with the same volume of physiological
409 serum. Left side is the bioluminescent image and the right side is the linear regression
410 analysis of the evolution of bioluminescent signals after treatment. Linear regression data are
411 shown as solid lines with colors corresponding to the indicated tested condition with dashed
412 color-matched lines corresponding to the 95% confidence band (A). Amoxicillin treatment
413 data for the strain LNP24198lux (MIC of 0.064 mg/L and 0.125 mg/L of penicillin G and
414 amoxicillin respectively). (B) and (C) Amoxicillin treatment and penicillin G treatment
415 respectively for the strain LNP27704lux (MIC of 0.5 mg/L and 1.5 mg/L of penicillin G and
416 amoxicillin respectively). The doses of amoxicilline (in mg/kg A and B) and those of
417 penicillin G (in units/kg in C) are shown to the left.

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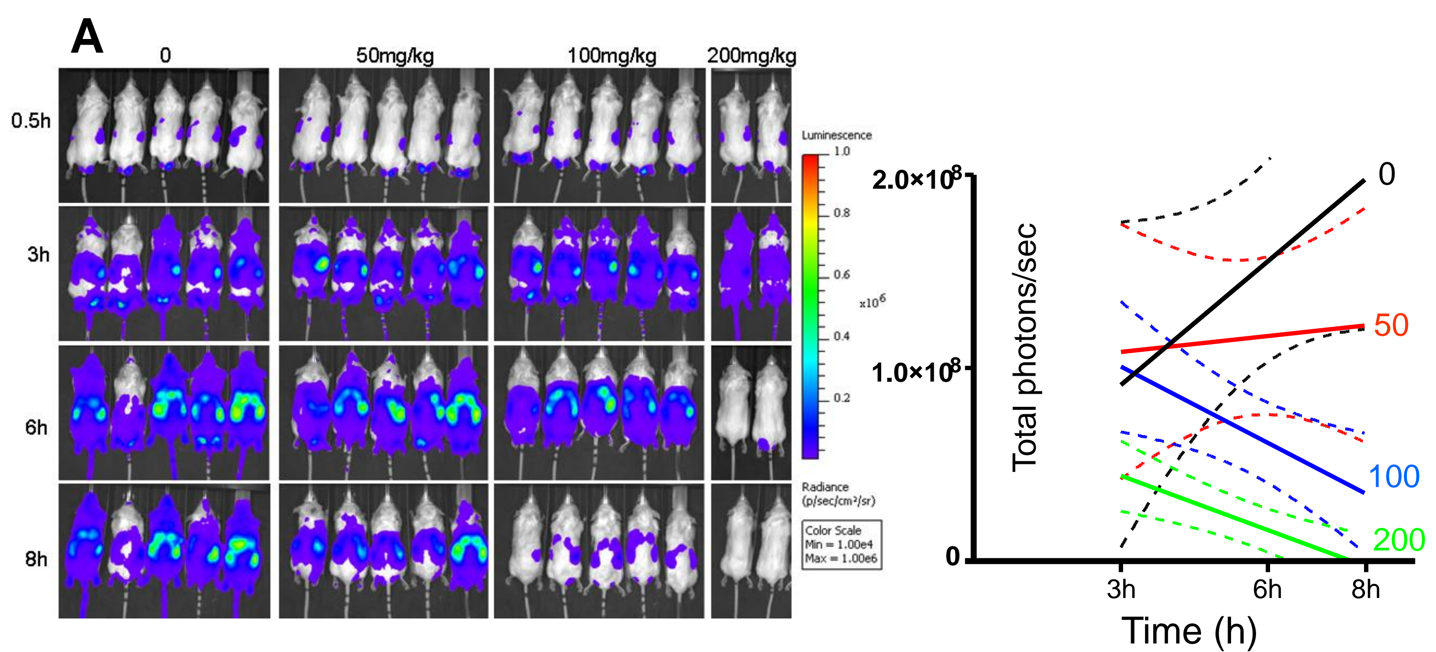
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0
50mg/kg
100mg/kg
200mg/kg
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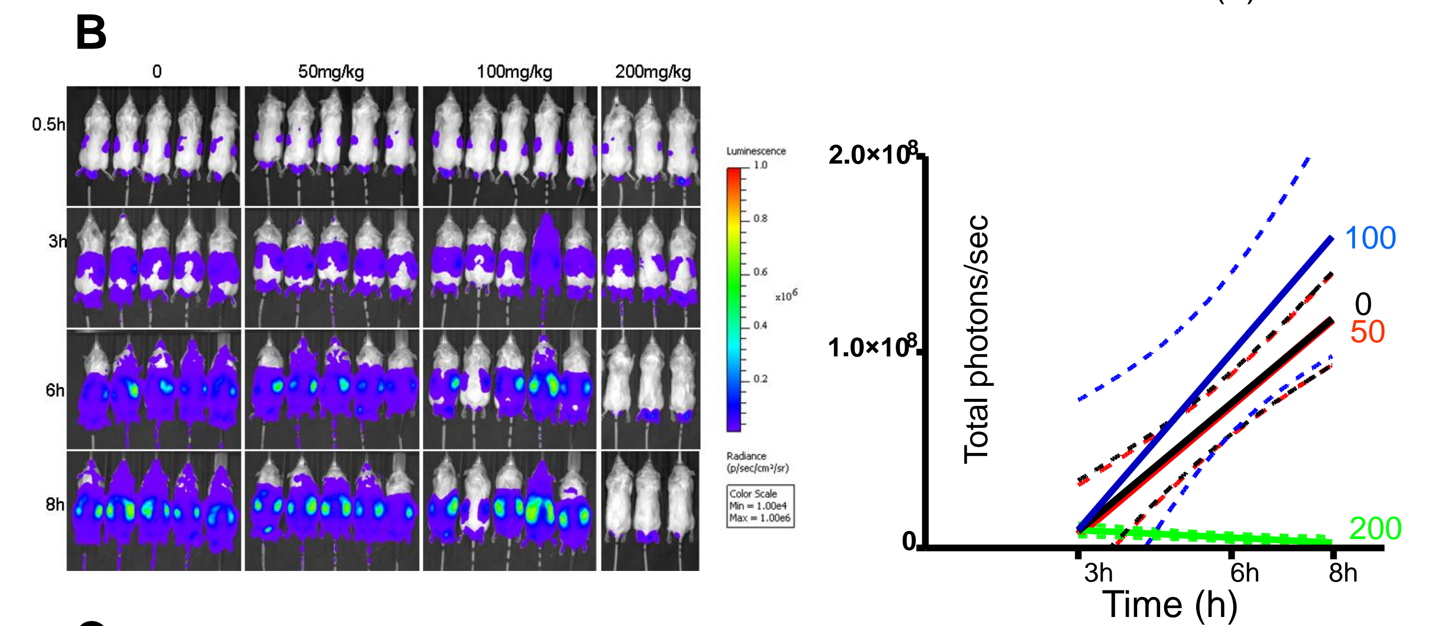
3h
6h
8h
Luminescence
Radiance (p/sec/cm²/sr)
Color Scale
Min = 1.00e4
Max = 1.00e6
2.0×10^8
1.0×10^8
Total photons/sec
Time (h)
3h
6h
8h
0
50
100
200


B
0
50mg/kg
100mg/kg
200mg/kg
0.5h
3h
6h
8h
Luminescence
Radiance (p/sec/cm²/sr)
Color Scale
Min = 1.00e4
Max = 1.00e6
2.0×10^8
1.0×10^8
Total photons/sec
Time (h)
3h
6h
8h
100
0
50
200

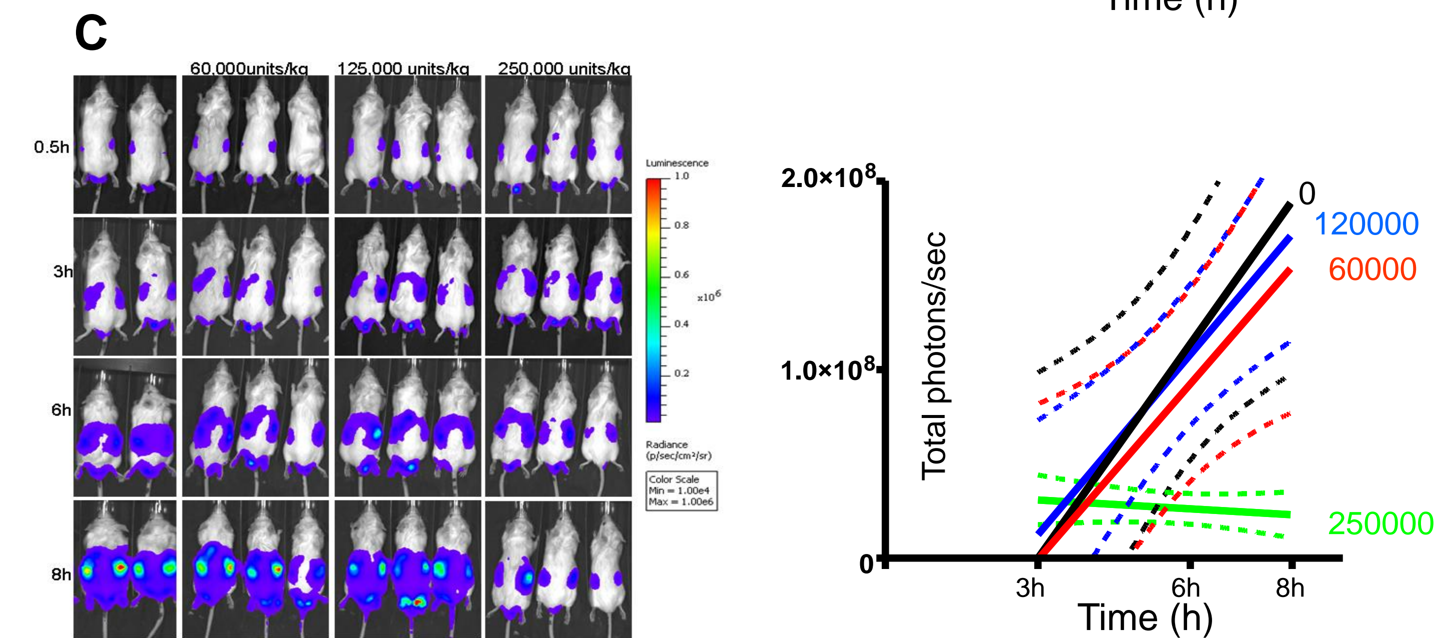

C
60,000units/kg
125,000 units/kg
250,000 units/kg
0.5h
3h
6h
8h
Luminescence
Radiance (p/sec/cm²/sr)
Color Scale
Min = 1.00e4
Max = 1.00e6
2.0×10^8
1.0×10^8
Total photons/sec
Time (h)
3h
6h
8h
0
120000
60000
250000

Table 1. Il-6 levels in mice presented in figure 1 after 8 hours of infection.

Strain tested	Antibiotic dose	IL-6 Mean (µg/ml)	95% CI of the mean	*P* value
LNP24198lux (0.125 mg/L)				
	untreated	10.4	3.8 - 16.9	Ref
	50 mg/kg	14.4	-8.1 - 36.9	Ref
	100 mg/kg	1.8	-0.7 - 4.2	Ref
	200 mg/kg	ND		
LNP27704lux (1.5 mg/L)				
	untreated	2.8	0.6 - 5	0.01
	50 mg/kg	4.2	0.6-7.7	0.25
	100 mg/kg	4.3	0.1 - 8.5	0.2
	200 mg/kg	ND		

ND: Non Detectable

CI: Confident interval

Ref: The Pen^S isolate (LNP24198lux) was used as a reference for the comparison with the Pen^R isolate (LNP27704).