



Use of animal models to support revising Meningococcal breakpoints of β -Lactams.

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

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18 Keywords: *Neisseria meningitidis*, breakpoints, beta-lactam, animal models, dynamic
19 imaging, inflammation

26 **Abstract**

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

34 We used experimental intraperitoneal infection in 8-week-old transgenic female mice
35 expressing human transferrin and human factor H. Dynamic bioluminescence imaging was
36 performed to follow the infection by bioluminescent meningococci with different MIC. Three
37 hours later, infected mice were treated intramuscularly using several doses of amoxicillin or
38 penicillin G. Signal decreased during infection with meningococci with the strain showing
39 MIC of 0.064 mg/L of penicillin G with all doses. Signals only decreased for the strain with
40 MIC of 0.5 mg/L of penicillin G after treatment with the highest doses corresponding to
41 250,000 units/kg of penicillin G or 200 mg/kg of amoxicillin although to a slower rate than
42 the strain with MIC of 0.064 mg/L. The decrease of bioluminescent signals was associated
43 with a decrease in the levels of the pro-inflammatory cytokine, IL-6. Our data suggest that
44 high dose of amoxicillin or penicillin G can reduce growth during infection by isolates
45 showing MIC of penicillin G of > 0.25 mg/L and ≤ 1mg/L.

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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 $\geq$ 0.125 mg/L and 0.250 mg/L for penicillin G and

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

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

Materials and Methods

Ethics Statement

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

Meningococcal isolates: Phenotypic and Genotypic characterization

Two clinical isolates of *N. meningitidis* were used (LNP24198 and LNP27704). Both isolates were of serogroup C and belonged to the clonal complex ST-11 (cc11). They harbored respectively the *penA* alleles *penA3* and *penA9* corresponding to a wild-type and a modified alleles respectively. MIC of penicillin G was determined as previously recommended using Etest with Mueller-Hinton agar supplemented with sheep blood (11) and were 0.064 mg/L and 0.5 mg/L respectively. MICs of amoxicillin were 0.125 and 1.5 mg/L respectively. Bioluminescent variants of both isolates were constructed by transformation with the recombinant plasmid pDG34, which carries the bioluminescent luxCDABE operon under the control of the *porB* promoter (12) and named LNP24198lux and 27704lux. Both strains were checked for their MICs of penicillin G, and for amoxicillin and their *penA* alleles verified by sequencing, showing that they were identical to the parent isolates. Both strains grew similarly on meningococcal growth medium.

Mice infection and dynamic live imaging studies

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

a transgenic mice expressing the human factor H (fH) that allowed binding of this negative regulator of complement pathway on bacterial surface and hence allowing meningococci to escape complement-mediated lysis (14). The two types of mice were crossed to generate transgenic mice expressing both human transferrin and human fH that we used in infection experiments using bioluminescent meningococcal strains with different MIC to penicillin G and amoxicillin. Mice were in-house bred and were kept in a biosafety containment facility, in filter-topped cages with sterile litter, water and food, according to institutional guidelines.

Mice were infected by intraperitoneal route (i.p.) with standardized inoculate of 5×10^6 bioluminescent colony forming units (CFU) per mouse in 0.5 ml of bacterial suspension. At the time point of 3 h the mice were divided into three groups that were treated by either penicillin G or amoxicillin only once by intramuscular injection in the interior face of the left thigh. The following increasing unique doses (per mouse) of penicillin G (60,000 units/kg, 120,000 units/kg or 250,000 units/kg; corresponding to 37mg/kg, 75 mg/kg and 150 mg/ml) or of amoxicillin (50 mg/kg, 100 mg/kg and 200 mg/kg). The highest doses of both antibiotics corresponded to a daily dose used in treatment of IMD in humans. A group of two mice were only injected with saline as control. Interleukin-6 (IL-6) levels were also assayed in blood at the end point (8 h) as previously described (15). As similar results were obtained from both experiments with penicillin G and amoxicillin, IL-6 was tested for mice experiment treated with amoxicillin.

Bacterial infection images were acquired after 0.5 h, 3 h, 6 h and 8 h of infection using an IVIS 100 system (Xenogen Corp., Alameda, CA) as previously described (13). Analysis and acquisition were performed using Living Image 4.3.1 software (Xenogen Corp.). Data were analyzed by linear regression using GraphPad InStat version 3.06 (GraphPad Software, San Diego, CA, USA).

Results

Impact of MIC on amoxicillin treatment during meningococcal infection in mice

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

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

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

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

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

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

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

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

For the mice infected by the strain LNP24198lux (MIC 0.064mg/L) and treated with penicillin G, the data were similar to those obtained with amoxicillin treatment (data not shown).

All these data taken together suggest the highest dose, similar to the daily dose used in humans, leads to decrease of bacterial counts in mice even if the strain is considered Pen^R.

Decreased inflammatory response in mice treated with optimal amoxicillin dose

We next assayed the levels of IL-6, a proinflammatory cytokine at 8 h post-infection. The data are showed in Table 1 and clearly suggest a dose dependent decrease of IL-6 levels in amoxicillin-treated mice that were infected by the strain LNP24198lux (MIC penicillin 0.064 mg/L and amoxicillin 0.125 mg/L). These levels were not detectable in mice treated with 200 mg/kg of amoxicillin. On the other hand, mice infected with the strain LNP27704lux (MIC penicillin 0.5 mg/L and amoxicillin 1.5 mg/L), and treated with 50 mg/kg and 100 mg/kg of amoxicillin showed similar levels of IL-6 as obtained in untreated mice (Table). IL-6 level was not detectable in mice treated with the highest dose (200 mg/kg of amoxicillin).

Discussion

We have previously emphasized the advantages of *in vivo* bioluminescent imaging for real-time monitoring of meningococcal infections and their treatment (13, 16). This technology is sensitive, rapid and non-invasive. It limits animal-to-animal variation and reduces the number of animal used. In the present study, we adapted this system to evaluate the correlation of beta-lactams breakpoints during experimental meningococcal infection in transgenic mice. *N. meningitidis* is intrinsically susceptible to many antibiotic classes and MIC₅₀ and MIC₉₀ values (antibiotic concentrations respectively inhibiting 50% and 90% of a sample of strains) are very low(17). Meningococcal resistance to beta-lactams remains rare and meningococcal strains remained susceptible to penicillin G with an MIC₅₀ of penicillin G of 0.06 mg/L. Few old reports described rare beta-lactamase producing strains with MICs that may reach or exceed 256 µg/ml (18, 19). These beta-lactamases are plasmid-borne TEM-1 type enzymes similar to those described in *Neisseria gonorrhoeae* that inactivate penicillin G and amoxicillin but does not hydrolyze third generation cephalosporins (18). However, isolates with reduced susceptibility to penicillin G and amoxicillin are quite frequent worldwide (7). The MICs of amoxicillin and penicillin G for these intermediate isolates remain low and defining the upper limit of the critical values remains problematic. Although treatment failures have been described for strains with the highest MICs (20), the severe infections caused by these strains generally resolve favorably using high doses of penicillin G or amoxicillin, which allow bactericidal concentration to be reached in cerebrospinal fluid. The effect of beta-lactams is dependant on the time the antibiotic concentration exceeds its MIC for the microorganism (T > MIC) (9). Our data with the highest dose of antibiotics are in agreement with this consideration. This dose corresponds to the recommended total daily dose for the treatment of IMD (21). Our data suggest that the 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 be 1 mg/L and thus
 254 intermediate isolates may be defined as those with $MIC \geq 0.125 \text{ mg/L}$ and $\leq 1 \text{ mg/L}$. This range
 255 also contains the isolates with altered *penA* alleles that all shared the same altered residues (7).
 256 Isolates with MICs $>0.25 \text{ mg/ml}$ and $\leq 1 \text{ mg/ml}$ 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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Legend to Figure

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

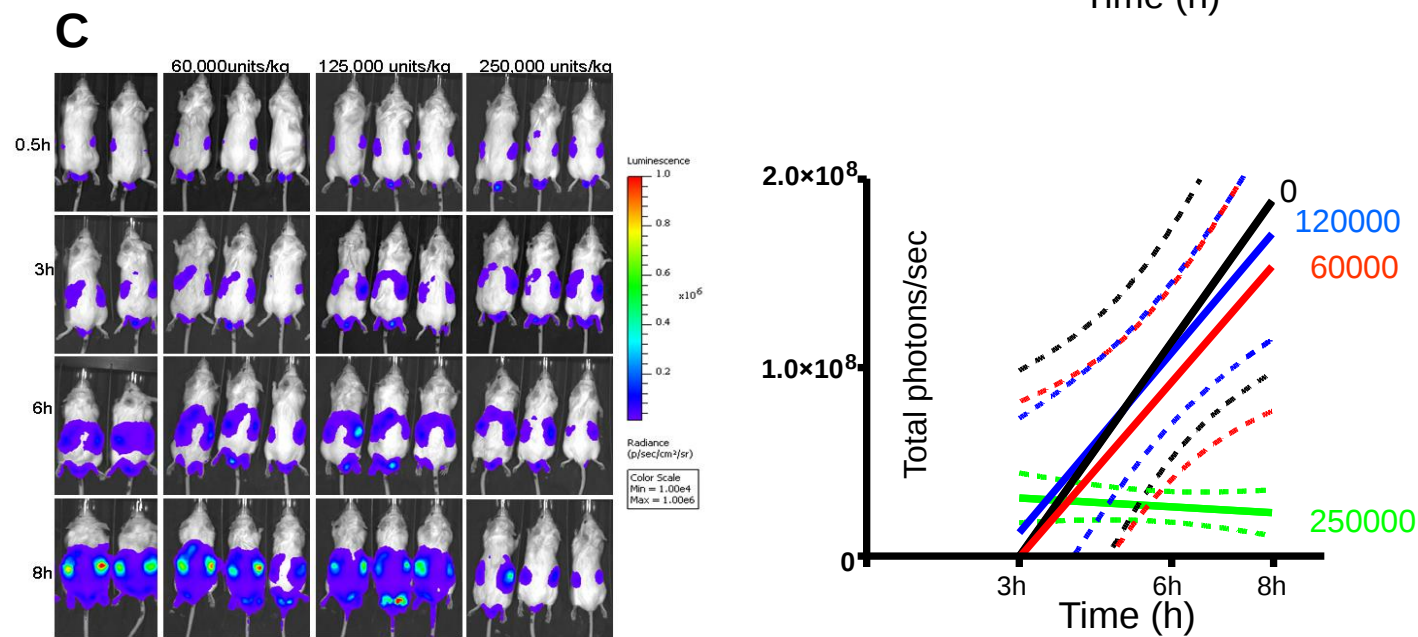
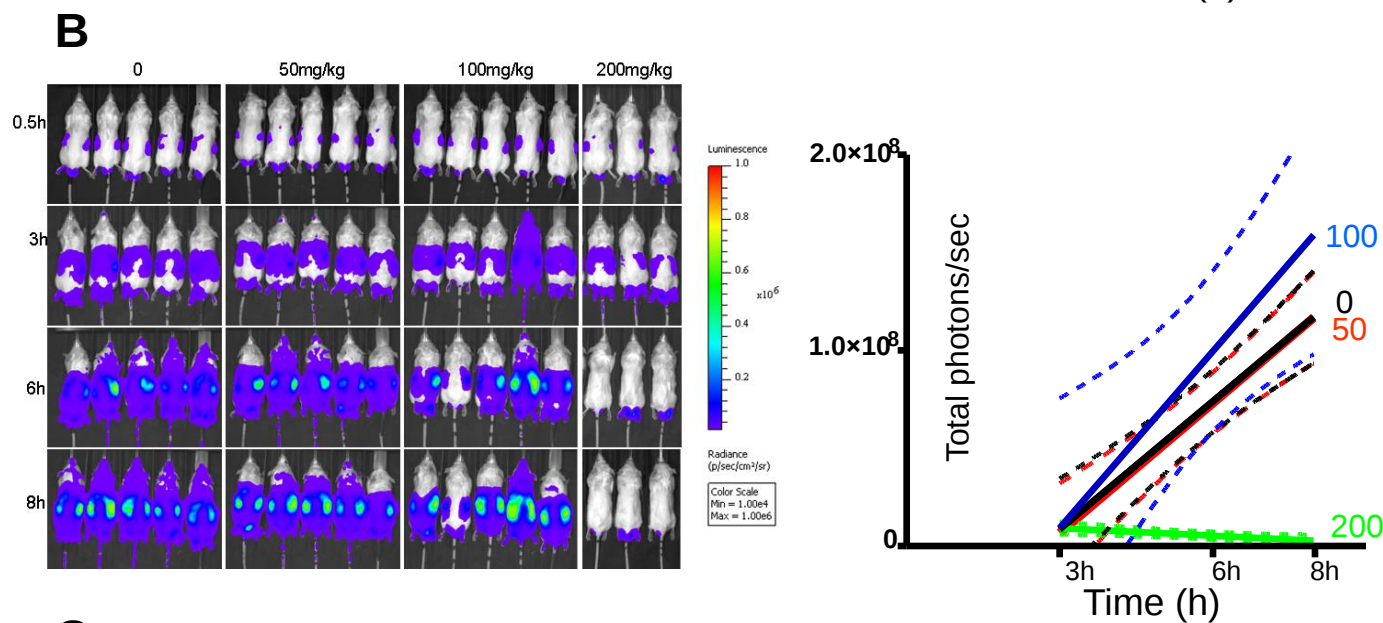
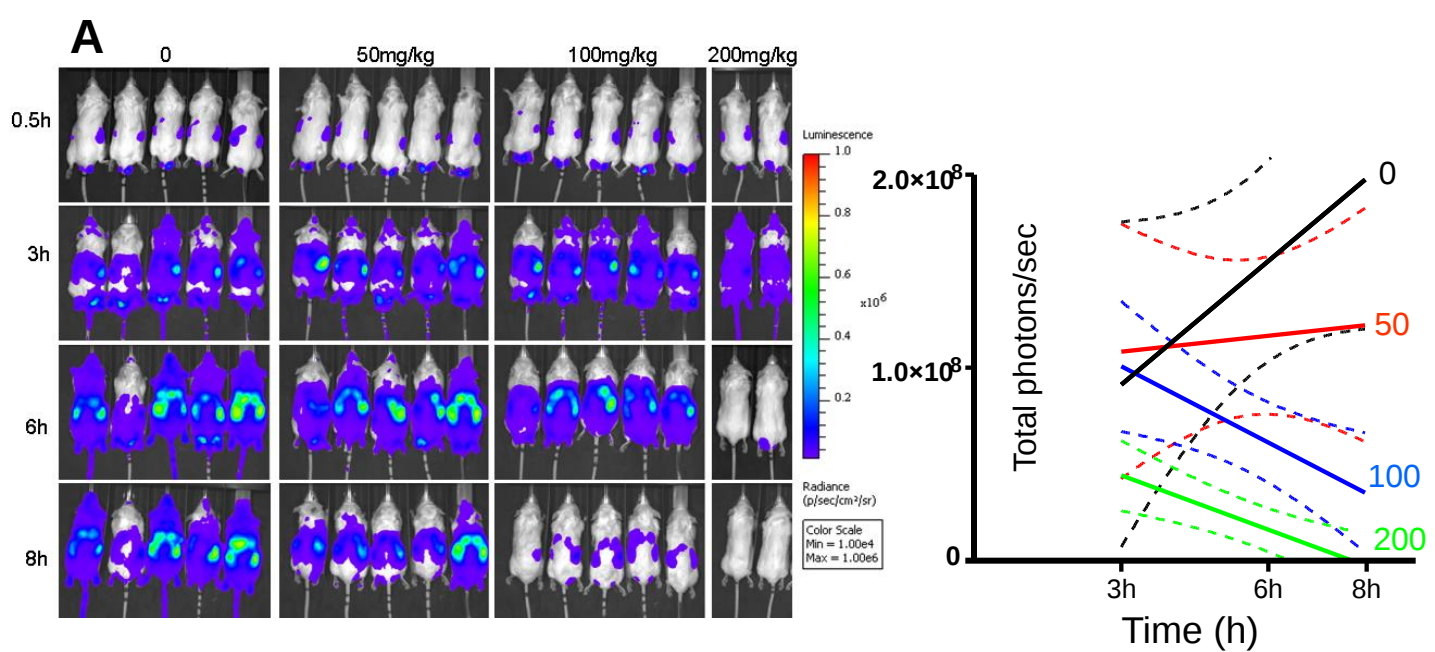


Table 1. IL-6 levels in mice presented in figure 1 after 8 hours of infection.

Strain tested	Antibiotic dose	IL-6 Mean ($\mu\text{g/ml}$)	95% CI of the mean	<i>P</i> 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).