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## **In Vivo Assessment of Drug Efficacy against Mycobacterium abscessus Using the Embryonic Zebrafish Test System**

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1 ***In vivo* assessment of drug efficacy against *Mycobacterium***  
2 ***abscessus* using the embryonic zebrafish test system**

3  
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6

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17

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19

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21  
22

23 **ABSTRACT**

24

25 *Mycobacterium abscessus* is responsible of a wide spectrum of clinical syndromes and is one of  
26 the most intrinsically drug-resistant mycobacterial species. Recent evaluation of the *in vivo*  
27 therapeutic efficacy of the few potentially active antibiotics against *M. abscessus* was  
28 essentially performed using immune-compromised mice. Herein, we assessed the feasibility  
29 and sensitivity of fluorescence imaging for monitoring the *in vivo* activity of drugs against acute  
30 *M. abscessus* infection using zebrafish embryos. A protocol was developed where  
31 clarithromycin and imipenem were directly added to water containing fluorescent *M.*  
32 *abscessus*-infected embryos in a 96-well plate format. The status of the infection with  
33 increasing drug concentrations was visualized on a spatiotemporal level. Drug efficacy was  
34 assessed quantitatively by determining the index of protection, the bacterial burden by CFU  
35 plating and by monitoring the number of abscesses through fluorescence measurements. Both  
36 drugs were active in infected embryos and were capable of significantly increasing embryo  
37 survival in a dose-dependent manner. Protection from bacterial killing correlated with  
38 restricted mycobacterial growth in the drug-treated larvae and with reduced pathophysiological  
39 symptoms, such as the number of abscesses within the brain. In conclusion, we present here a  
40 new and efficient method for testing and compare the *in vivo* activity of two clinically-relevant  
41 drugs based on a fluorescent reporter strain in zebrafish embryos. This approach could be  
42 applied to a rapid determination of *in vivo* drug susceptibility profile of clinical isolates and to  
43 assess the preclinical efficacy of new compounds against *M. abscessus*.

44

45 **INTRODUCTION**

46

47 *M. abscessus* (*Mabs*) is an emerging pathogen and the etiological agent of a wide spectrum  
48 of infections in humans. It is responsible for severe chronic pulmonary and disseminated  
49 infections, mostly in immunosuppressed and in cystic fibrosis (CF) patients (1), and cutaneous  
50 diseases, often post-traumatic and post-surgical. This neglected pathogen causes a higher  
51 fatality rate compared to other RGMs and the infection of CF patients is becoming a major  
52 health-related issue in most CF centers worldwide (2). *Mabs* infections occur in early childhood  
53 (3), are severe and sometimes fatal, especially following transplantation (4-6), and have the  
54 potential to cause outbreaks of infection (6). *Mabs* is also the main RGM responsible for  
55 nosocomial and iatrogenic infections in humans (post-injection abscesses, cardiac surgery, and  
56 plastic surgery) (7-9). It has also been reported to cross the blood-brain barrier and to cause  
57 important central nervous system (CNS) lesions. Although a rapid grower, *Mabs* possesses  
58 several important pathogenic traits such as the ability to i) persist silently for years and even  
59 decades (10) in the human host, and to ii) induce lung disease associated with caseous lesions  
60 and granuloma formation in lung parenchyma (11, 12).

61 The major issue with *Mabs* relies on its intrinsic resistance to the majority of available  
62 antibiotics. The American Thoracic Society has recommended different groups of agents,  
63 namely macrolides (clarithromycin), aminoglycosides (amikacin), cephamycins (cefoxitin) and  
64 carbapenems (imipenem) for treatment of *Mabs* infections (13). Patients with severe infections  
65 are generally treated with long courses of combinatorial antibiotic therapy, often accompanied  
66 by surgical resection. As antibiotic susceptibility testing is not fully standardized, the clinical  
67 response to drugs does not correlate well with *in vitro* susceptibility test results and failure  
68 occurs frequently despite administration of two or three antibiotics for several months (14).

69 This further emphasizes the need of suitable animal models (15, 16). In addition, different  
70 clinical isolates of this emerging pathogen are not uniformly susceptible to the currently used  
71 antibiotics (17). As a consequence, an optimal regimen to cure the *Mabs* infections has not  
72 been yet established.

73 Thanks to the recent availability of efficient genetic tools (18), *Mabs* has been proposed as  
74 an attractive experimental model to study non-tuberculous mycobacteria associated diseases  
75 (1). Our poor understanding of the pathogenesis of *Mabs*, essentially hampered by the  
76 restricted panel of cellular/animal models available, prompted us to develop the zebrafish  
77 model of infection to describe the chronology of *Mabs* infection (19). In particular, the  
78 *Mabs*/zebrafish couple already provided important insights regarding the pathogenesis of *Mabs*  
79 such as the unexpected tropism for the CNS, a finding relevant in the light of recent clinical  
80 studies reporting the presence of *Mabs* in the CNS of infected human patients (20, 21). Since  
81 infection foci/abscesses within the CNS, particularly the brain, appear very rapidly and are very  
82 easy to detect and visualize, we reasoned that this alternative model could represent a valuable  
83 and cheap system to evaluate and compare the *in vitro* and *in vivo* activity of drugs against  
84 *Mabs*. Such a simple and innovative system would be particularly suited for screening active  
85 molecules and/or antibacterial activity assessment, representing a critical step in the context of  
86 drug discovery, urgent in the case of *Mabs*.

87 Here, we report on the development of experimental conditions for *in vivo* imaging of  
88 *Mabs* and demonstrate that it is compatible with *in vivo* observations, at a spatiotemporal  
89 level, of the effects of drug treatment on the infection process. This represents a unique  
90 biological model that allows non-invasive observations to evaluate, in real time, the efficacy of  
91 antibiotics in living infected vertebrates, a system that could be applied to high-throughput *in*  
92 *vivo* testing of drug efficacy against the most drug-resistant mycobacterial species.

94 **MATERIALS AND METHODS**

95

96 ***M. abscessus* strains and growth conditions**

97 The rough variant of *M. abscessus sensu stricto* strain CIP104536<sup>T</sup> (ATCC19977T) was grown  
98 at 30°C in Middlebrook 7H9 broth supplemented with 10% Oleic  
99 acid/Albumin/Dextrose/Catalase (OADC) enrichment and 0.05% Tween 80 (7H9<sup>T</sup>) or on  
100 Middlebrook 7H10 agar containing 10% OADC. Recombinant *Mabs* carrying pTEC27 (Addgene,  
101 plasmid 30182) that allows to express tdTomato under the control of a strong mycobacterial  
102 promoter were grown in the presence of hygromycin 500 mg/L (19).

103

104 **Mice experiments and CFU counting**

105 6-8 weeks old BALB/c mice were divided in groups of 5-7 mice and used for either  
106 intravenous (*i.v.*) or aerosol challenges. Inocula were prepared from rapidly thawed frozen  
107 aliquots, and bacterial clumps were eliminated by iterative passages through a 29.5-gauge  
108 insulin needle (Becton Dickinson). Bacterial suspensions were then diluted in phosphate buffer  
109 saline (PBS). For *i.v.* inoculations, 10<sup>6</sup> CFU (in 200 µl) were injected into the lateral tail/caudal  
110 vein, as previously described (22, 23). Pulmonary infections were achieved with aerosolized  
111 *Mabs* using an aerosol generator, equipped with a Micro Mist<sup>®</sup> small volume nebulizer (Hudson  
112 RCI-Teleflex medical) containing 6 ml of bacterial solution at 4 × 10<sup>7</sup> CFU/ml. Pre-sleeping mice  
113 (isoflurane<sup>®</sup> Abbott) were anesthetized with 200 µl of Hypnomidate (Etomidate<sup>®</sup>, Janssen-Cilag)  
114 and placed into an opened 50 ml syringe fixed on the top of a closed compartment containing  
115 the nebulizer. In this device, nebulization lasted for 15 min to vaporize the entire bacterial  
116 suspension. Lungs, liver and spleen were collected in PBS, crushed and 10-folds serial dilutions  
117 were plated on Middlebrook 7H11 plates for CFU counting, as previously described (22, 23).

118 Plates were then incubated at 37°C for up to 7 days. The results were expressed as the mean  
119 Log<sub>10</sub> CFU per organ.

120

### 121 **Minimal inhibitory concentrations**

122 Antibiotics powder tested in drug susceptibility assays were pharmaceutical standards for  
123 imipenem/cilastatin (Mylan) or clarithromycin (Sigma-Aldrich). Stock solutions were dissolved  
124 in water (imipenem) or in DMSO (clarithromycin). Drug susceptibility testing was also  
125 determined using the microdilution method, in cation-adjusted Mueller-Hinton broth,  
126 according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (24). In addition,  
127 the susceptibility profile was also determined on LB agar supplemented with increasing  
128 concentrations of the compounds. Serial 10-fold dilutions of each actively growing culture were  
129 plated and incubated at 37°C for 3-4 days and the MIC was defined as the minimum  
130 concentration required to inhibiting 99% of the growth.

131

### 132 **Zebrafish care**

133 All zebrafish experiments were done at the University Montpellier 2, according to  
134 European Union guidelines for handling of laboratory animals  
135 ([http://ec.europa.eu/environment/chemicals/lab\\_animals/home\\_en.htm](http://ec.europa.eu/environment/chemicals/lab_animals/home_en.htm)) and approved by the  
136 Direction Sanitaire et Vétérinaire de l'Hérault and Comité d'Ethique pour l'Expérimentation  
137 Animale de la région Languedoc Roussillon (CEEA-LR) under the reference CEEA-LR-13007.  
138 Experiments were performed using the *golden* ZF mutant (25). Eggs were obtained by natural  
139 spawning and incubated at 28.5°C in water with 60 mg/L Ocean salts. Ages of the embryos are  
140 expressed as hours post fertilization (hpf).

141

142 **Microinjection of *M. abscessus* into embryos**

143 Mid-log phase cultures of *Mabs* expressing tdTomato were centrifuged, washed and  
144 resuspended in PBS supplemented with 0.05% Tween-80 (PBS<sup>T</sup>). Bacterial suspensions were  
145 then homogenized through a 26-gauge needle and sonicated three times for 10s and the  
146 remaining clumps were allowed to settle down to for 5-10 min. Bacteria were concentrated to  
147 an OD<sub>600</sub> of 1 in PBS<sup>T</sup> and *i.v.* injected ( $\approx$ 2nL containing 300 CFU) into the caudal vein in 30hpf  
148 embryos previously dechorionated and anesthetized. To follow infection kinetics and embryo  
149 survival, infected larvae were transferred into 96 well plates (2 embryos/plate) and incubated  
150 at 28.5°C. The inoculum size was checked by injection of 2nL in sterile PBS<sup>T</sup> and plated on  
151 Middlebrook 7H10 agar containing 10% OADC supplemented with hygromycin 500 mg/L.

152

153 **Drug efficacy assessment in *Mabs*-infected ZF**

154 Clarythromycin and imipenem/cilastatin were added at one day post-infection (dpi),  
155 directly into the water containing the embryos. Three doses were tested, corresponding to  
156 1.7X, 17X and 170X the MIC of clarithromycin and 0.5X, 5X or 28X the MIC of imipenem, based  
157 on the values determined using the microdilution method (Table S1). *In vivo* drug efficacy was  
158 determined for each concentration by following i) the bacterial burdens, ii) the kinetic of  
159 embryo survival, iii) the evolution of the infection foci/abscesses within the CNS and iv) the  
160 effect on bacterial cord formation/reduction. Survival curves were determined by recording  
161 dead embryos (no heartbeat) every day for up to 13 days. Regarding the kinetic of  
162 mycobacterial loads, groups of three infected embryos were collected, lysed individually in 2%  
163 Triton X100- PBS<sup>T</sup> with a 26-gauge needle (15 up-and-down sequences) and resuspended in  
164 PBS<sup>T</sup>. Several 10-fold dilutions of homogenates were plated on Middlebrook 7H10 agar  
165 supplemented with 10% OADC, the appropriate antibiotics and added of mix of “BBL<sup>TM</sup> MGIT<sup>TM</sup>



166 PANTA™ (Becton-Dickinson) using as recommended by the supplier. CFU were enumerated  
167 after 4 days of incubation at 30°C. This procedure was repeated at 0, 3, 5 dpi.

168

### 169 **Microscopy**

170 Widefield bright-field and fluorescence live microscopy of infected embryos were  
171 performed using an Olympus MVX10 epifluorescent microscope equipped with a X-Cite® 120Q  
172 (Lumen Dynamics) 120W mercury light source. Images are acquired with a digital color camera  
173 (Olympus XC50) and processed using CellSens software (Olympus). Fluorescence filter cube  
174 TRITC-MVX10 is used for detection of red light. For live imaging, anesthetized infected embryos  
175 were positioned in dishes and immobilized with 1% low-melting point agarose solution covering  
176 the entire larvae then immobilized embryos are immersed with fish water containing tricaine  
177 for direct visualization.

178

### 179 **Image Processing and Analysis**

180 Final images analysis and visualization are performed using GIMP 2.6 freeware to merge  
181 fluorescent and DIC images and to adjust levels and brightness and to remove out-of-focus  
182 background fluorescence.

183

### 184 **Statistical Analyses**

185 Statistical analyses of comparisons between Kaplan-Meier survival curves were performed  
186 using the log rank test with Prism 4.0 (Graphpad, Inc). CFU counts and quantifications  
187 experiments were analyzed using one-way ANOVA and Fisher's exact test, respectively.  
188 Statistical significance was assumed at  $p$  values  $<0.05$ .

189

## 190 RESULTS

### 191 ***M. abscessus* fails to establish a persistent infection in BALB/c mice**

192 Experiments were first aimed to determine the colonization rate of *Mabs* in a murine  
193 pulmonary infection model (Figure 1A). Aerosol infections of BALB/c mice was characterized by  
194 an initial and rapid increase of the bacterial burden from 1-3 days post-infection (dpi) in the  
195 lungs, followed by a phase of infection control that leads to a reduction (starting after 3dpi) and  
196 almost complete clearance of the bacilli at 27dpi. Very few bacteria were detected within the  
197 spleen or the liver of infected mice. The colonization profile after an *i.v.* challenge showed that  
198 bacilli were primarily found in the liver at 1dpi and to a lesser extent in the spleen and the lungs  
199 (Figure 1B). All heavily infected organs rapidly underwent a progressive reduction in the CFU  
200 levels with a 3-Log<sub>10</sub> CFU decrease in the liver and the lungs at 30dpi, highlighting a transient  
201 colonization process. These results indicate that the course of infection in immune-competent  
202 mice consists mostly as a progressive eradication of the pathogen. This model would, therefore,  
203 require to testing a very large number of animals to insure that the observed CFU decrease  
204 results to the antibiotic regimen rather than to the natural course of infection. Consequently,  
205 wild-type BALB/c mice are not well adapted to investigate the *in vivo* efficacy of therapeutic  
206 treatments. Therefore, the use of alternative animal models susceptible to *Mabs* infection,  
207 permissive to bacterial replication and leading to the development of infection foci/abscesses  
208 and death would be particularly advantageous. We hypothesized that ZF larvae may represent a  
209 valuable system for *in vivo* assessments of drugs against *Mabs*.

210

### 211 **Zebrafish larvae for *in vivo* assessment of drug activity in *M. abscessus***

212 To assess *in vivo* antimycobacterial drug activity against *Mabs* in ZF larvae, an experimental  
213 protocol has been established (Figure 2). tdTomato-expressing *Mabs*, exhibiting red

214 fluorescence, were *i.v.* injected in the caudal vein of embryos at 30 hours post-fertilization (hpf)  
215 and transferred into 96-well plates. Antibiotic were then directly added at 1dpi to the water  
216 containing the infected embryos and the drug-supplemented water was then changed on a  
217 daily basis for 5 days. Daily monitoring of mortality as well as determination of the bacterial  
218 burden at various time points were used as phenotypic read-outs. Thanks to the optical  
219 transparency of the embryos, the effect of the antibiotic treatment on evolution of the clinical  
220 signs of infection was also recorded by fluorescence microscopy. Since most infected *Mabs*  
221 infected-embryos developed infection abscesses within the CNS, especially the brain (Figure 2),  
222 the chemotherapeutic activity of the antibiotics was particularly easy to observe, on an  
223 individual basis, during the entire period of drug treatment. Drug-mediated toxicity was also  
224 investigated by determining the survival curves of non-infected embryos treated with  
225 increasing drug doses. We have previously shown that the rough *Mabs* exhibits a marked  
226 neurotropism with massive abscesses within the CNS (19), thus prompting us to assess the  
227 activity of drugs in *Mabs*-infected embryos with a special emphasis on infection within the CNS.

228

### 229 **Minimal inhibitory concentrations of antimycobacterial drugs against *M. abscessus***

230 We first determined the *in vitro* activity of various drugs, including antitubercular agents,  
231 against *Mabs* using the microdilution in cation-adjusted Mueller-Hinton broth, according to the  
232 Clinical and Laboratory Standards Institute guidelines (24). Table S1 shows that the activity  
233 varies considerably, in agreement with other studies. The first-line antitubercular drug isoniazid  
234 and second-line drug thiacetazone appeared inactive against *Mabs*. Among the few clinically  
235 used drugs for the treatment of *Mabs* infection, cefoxitin, amikacin, imipenem and  
236 erythromycin exhibit moderate activity *in vitro* on agar plates with MICs ranging from 60-125  
237  $\mu\text{M}$ , whereas clarithromycin demonstrated the highest activity with an MIC value of 4  $\mu\text{M}$ .

238 Because clarithromycin and imipenem exhibit different physicochemical properties (high  
239 molecular weight and hydrophobicity for clarithromycin versus low molecular weight and  
240 hydrophilicity for imipenem), they were further investigated for their *in vivo* therapeutic  
241 efficacy in *Mabs*-infected ZF.

242

### 243 ***In vivo* susceptibility of *M. abscessus* to clarithromycin**

244 Due to insufficient information concerning the mechanisms of drug uptake by ZF  
245 embryos/larvae, a wide range of concentrations, spanning from 6.6  $\mu$ M-668  $\mu$ M of  
246 clarithromycin (corresponding to 1.7X to 170X the *in vitro* MIC value obtained using the  
247 microdilution method) was tested. Supplementation of the embryo-containing water with low  
248 or intermediate doses (1.7X and 17X the MIC, respectively) displayed no toxicity as measured  
249 by larval survival, while used at the highest dose tested (170X MIC), a 10% reduction in larval  
250 survival was observed at 9dpi, with respect to the control group in which water was  
251 supplemented with 1% DMSO (26) (Figure 3A). Embryos that develop in the presence of high  
252 doses of clarithromycin had a curved body trunk with uninflated swim bladder as compared to  
253 the DMSO control embryos (Figure 3A, inset). These phenotypic alterations were hardly  
254 observed when exposed to intermediate or low doses of clarithromycin (data not shown).

255 Nevertheless, since these developmental abnormalities essentially occurred at the highest  
256 doses, we next assessed the *in vivo* efficacy of clarithromycin in *Mabs*-infected embryos. No  
257 significant increased survival was found when infected-embryos were exposed to low and  
258 intermediate drug concentrations (Figure 3B). In contrast, high doses extended the life span of  
259 infected embryos and fully protected the infected embryos up to 9dpi, when the first embryo  
260 started to dye, which coincidentally, corresponded to the toxicity-induced-killing effect (Figure  
261 3A). This shows that clarithromycin, using the highest regimen, is efficient in the ZF test system.

262 **Effects of clarithromycin on ZF survival, bacterial burden and abscesses**

263 Increased survival was associated with lower bacterial burdens after 3dpi in the presence  
264 of the highest dose (170X MIC), as determined quantitatively by CFU plating (Figure 3C),  
265 whereas treatment with the low or intermediate doses failed to restrict mycobacterial growth.  
266 *In vivo* drug efficacy was next monitored by time-lapse fluorescence microscopy (Figure 3D).  
267 Injection of *Mabs* led to the appearance of rapidly growing infection foci and abscesses in the  
268 larval brain at 3dpi, as reported previously (19). Imaging the same infected embryos at 3 and  
269 5dpi revealed that abscesses within the brain were already reduced at 3dpi when treated with  
270 high drug concentrations and this effect in reducing the clinical signs of the infection was even  
271 more accentuated at 5dpi. There was no visible reduction of the infection at 5dpi in ZF treated  
272 with low or intermediate drug concentrations, consistent with the survival curves and kinetic of  
273 bacterial growth. A quantitative analysis revealed that high doses of clarithromycin reduced by  
274 50% the number of embryos with abscesses (Figure 3E), and this drug effect was apparent in  
275 both the brain and the spinal cord (Figure 3F), albeit the impact of lower drug concentrations  
276 was not significant. This indicates that clarithromycin exerts a beneficial effect by inhibiting  
277 mycobacterial growth, preventing the development of abscesses within the CNS and protecting  
278 the embryos from bacterial killing.

279

280 **Effects of imipenem on ZF survival and reduction of the pathological signs**

281 To further confirm and extend the impact and usefulness of this biological system with  
282 respect to *in vivo* drug testing, embryos were exposed to water-soluble imipenem, a clinically  
283 relevant drug against *Mabs* known to act on L,D-transpeptidases (17, 27). A wide range of  
284 concentrations was tested, corresponding to 0.5X to 28X MIC of imipenem, and neither signs of  
285 toxicity-induced-killing nor developmental abnormalities were detected even at the highest

286 dose (data not shown). When assessing the effect of imipenem on infected ZF, no increased  
287 survival was found with low drug concentrations. However, treatment with intermediate doses  
288 led to a significant increase in survival and 100% of protection was observed in the presence of  
289 this highest drug concentration (Figure 4A). These survival rates correlated with the CFU loads  
290 as intermediate and high doses of imipenem started to restrict bacterial growth at 3dpi (after  
291 two days of drug treatment) (Figure 4B). With the highest dose, there was a 3 Log<sub>10</sub> decline in  
292 the CFU at 5dpi (four days of treatment) compared with the untreated control group. Time-  
293 lapse fluorescence microscopy further confirmed the *in vivo* efficacy of imipenem, illustrating  
294 the inhibition of bacterial growth and disappearance of abscesses in the larval brain at 3 and  
295 5dpi, respectively (Figure 4C). There was no visible reduction of the infection at 5dpi when  
296 treated with low imipenem doses, consistent with the survival curves and kinetic of bacterial  
297 growth. High doses significantly reduced the proportion of embryos with abscesses (Figure 4D),  
298 a phenotypic effect that was particularly apparent in the brain on infected embryos (Figure 4E),  
299 indicating that imipenem reduces the pathology signs of the infection.

300 These results also prompted us to examine whether imipenem can counteract/alter the  
301 progression of an already established infection, especially when given at 3dpi when brain  
302 abscesses are already apparent (Figure S1A). Death curves indicate that treatment with high  
303 doses of imipenem effectively extended the life span of embryos with pre-existing abscesses  
304 (Figure S1B). A large proportion (more than 60%) of the treated embryos survived to the  
305 infection compared to 10% for the non-treated individuals (P=0.008). The remaining 40%  
306 embryos that died despite the treatment showed increased bacterial loads in the CNS (data not  
307 shown). The increased index of protection rate was associated to a significant decrease in the  
308 number of embryos with abscesses (Figure S1C), particularly within the brain (Figure S1D). This

309 “curative” protocol shows that imipenem was able to cure embryos with pre-existing abscesses  
310 and to protect severely infected ZF.

311

### 312 ***In vivo* inhibition activity of imipenem on mycobacterial cording**

313 Rough *Mabs* displays a dry texture with organized serpentine cords on agar plates (19, 28,  
314 29) and large bacterial clumps consisting mainly of cords in liquid cultures (19). Our recent  
315 studies also unraveled the presence of serpentine cords within the brain or spinal cord of  
316 embryos infected with the rough morphotype and emphasized the role of cording in immune  
317 evasion by preventing phagocytosis of *Mabs* by macrophages and neutrophils (19). Since cords  
318 are easy to visualize and to numerate by fluorescence microscopy (Figure 5A), and because they  
319 promote extracellular replication, abscess formation and tissue damage, we investigated  
320 whether exposure of infected embryos to imipenem may also affect the development of  
321 mycobacterial cords. Figure 5B shows the presence of multiple cords within the brain at 5dpi  
322 (left panel) and the impact of imipenem treatment on the number of cords (right panel).

323 Quantitative analysis of the percentage of embryos with cords at 4dpi is shown in Figure  
324 5C. The presence of low doses of imipenem has little impact on mycobacterial cords, although a  
325 reduction of the number of embryos with cords was detected at 4dpi. However, this effect was  
326 more pronounced with higher drug concentrations with only 20% of cord-laden embryos at  
327 4dpi (compared to 60% for untreated embryos at 4dpi). This dose-dependent effect occurred  
328 essentially within the CNS whilst reduction of cord formation within the vasculature was not  
329 significant (Figure 5D).

330

331 **DISCUSSION**

332 At a basic research level, the appropriate use of animal models can help to improve our  
333 understanding of host-pathogen interactions. At a more applied level, preclinical evaluation of  
334 new drug compounds requires *in vivo* testing prior these can advance along the development  
335 pipeline. However, *in vivo* animal studies, when possible, are usually costly and time-consuming  
336 and present a major bottleneck in drug developments. Implementation of novel approaches,  
337 expected to accelerate the *in vivo* efficacy assessment of drugs, is particularly justified in two  
338 cases. First, such systems are useful for bacterial infections requiring extended periods of drug  
339 treatment such as mice infected with *M. tuberculosis*, for which rapid *in vivo* assessment of  
340 drug efficacy directly in infected mice using fluorescence imaging (30) or using improved firefly  
341 luciferase (31) were elegantly demonstrated. We similarly show in this study how the use of  
342 fluorescence imaging can be useful in evaluating antimicrobial activity against *Mabs*. Second,  
343 alternative biological systems are particularly relevant for infections lacking of a permissive  
344 animal model. In this context, we recently demonstrated the high susceptibility of ZF embryos  
345 to *Mabs* and how the number of CNS abscesses may represent a marker for establishing *in vivo*  
346 antibiotic activity against *Mabs*.

347 Due to its intrinsic and acquired resistance to commonly used antibiotics, treatment  
348 becomes more complicated, thereby leading to high failure rates, stressing the need for new  
349 drug discovery. One of the key steps of drug discovery process is to identify and evaluate the *in*  
350 *vitro* and *in vivo* potential of new hits against *Mabs*, which pre-requires adequate animal  
351 models. Assessing the murine model which, following *i.v.* or aerosol infection, led only to  
352 transient colonization. Therefore, the natural course of infection in immune-competent BALB/c  
353 mice impedes its use as a valuable animal model for drug susceptibility testing. Comparatively,  
354 SCID mouse model has been shown to produce a chronic infection of *Mabs*, but this model has



355 not been used for drug testing (29, 32). However, granulocyte-macrophage colony-stimulating  
356 (GM-CSF) knockout mice have recently been used to develop a new animal model of persistent  
357 pulmonary *Mabs* infection that can be used for preclinical efficacy testing of anti-microbial  
358 drugs (15). In particular, azithromycin treatment of *Mabs*-infected GM-CSF KO mice resulted in  
359 a lower bacterial burden in the lungs and spleen, weight gain and significant improvement in  
360 lung pathology (15). Another report proposed Nude mice as an adequate model for *in vivo*  
361 chemotherapy studies (16). However, both models raised the question of the adaptive  
362 response in addition to the antibiotic activity in eradicating the bacilli. It was previously shown  
363 that, albeit being a rapid-growing mycobacterium, *Mabs* infection was only controlled in mice  
364 with a functional adaptive immune response (22), as compared to *M. chelonae*, which was  
365 cleared even in T cell-deficient mice. In addition, and despite the fact that both immune-  
366 compromised mice present a significant advance as compared to wild-type mice in preclinical  
367 assessments, these models remain costly and time-consuming and remain most likely not  
368 suitable for a general use in drug screening campaigns.

369 New non-mammalian models of infection have been developed, including *Drosophila*  
370 *melanogaster* (33, 34), *Caenorhabditis elegans* (35) or *Danio rerio* (36, 37) which offer several  
371 advantages in terms of speed, cost, technical convenience and ethical acceptability over the  
372 mouse model. However, very few of these alternative models, except for the recent *Drosophila*  
373 model (34), have been reported for antibiotic assessments against *Mabs*. In this study, we  
374 propose the ZF model, to visualize by non-invasive imaging the progressive infection of *Mabs* in  
375 live animals, and to quantifying the effect of drug treatment. We successfully investigated the  
376 suitability and sensitivity of two clinically relevant drugs, clarithromycin and imipenem, to  
377 visualize in a dose- and time-dependent manner the dynamics of cord and abscess  
378 formation/resorption. One major advantage of this model compared to mice is the ease and

379 rapidity of experimentation within a restricted time scale and low cost. That both drugs had a  
380 positive impact in terms of embryo survival was correlated to a significant reduction in the  
381 number of CFU and abscesses, demonstrating a proof of concept that ZF embryos are suitable  
382 for drug efficacy testing. Since *in vitro* studies demonstrated decreased MICs in the presence of  
383 imipenem for clarithromycin, minocycline, levofloxacin and moxifloxacin (38), future work  
384 should also address the *in vivo* efficacy of these drug combinations using the *Mabs*/ZF couple.

385 It is, however, noteworthy that despite of their unique features for the *in vivo* drug testing,  
386 ZF embryos also present several disadvantages over mammalian models. In particular, there are  
387 some important anatomical differences between ZF embryos and mammals such as gills  
388 instead of lungs, hematopoiesis occurring in the anterior kidney instead of the bone marrow,  
389 lack on discernable lymph nodes as well as a very different reproductive system. In addition,  
390 the natural lack of an adaptive immunity early in the development is very likely to affect the  
391 outcome of the infection, thus making it difficult to directly correlating data obtained in ZF and  
392 in humans. In addition, as shown in this study, embryos allow to describing effects of antibiotics  
393 during acute *Mabs* infections but not during the chronic stages of the disease, which can be  
394 better modelled for instance using immuno-compromized mice (15). In a similar vein, since  
395 pharmacokinetics in are not known in ZF, it remains difficult at this stage to directly transpose  
396 the MIC data obtained in ZF to humans. As a consequence, this biological system should  
397 essentially be regarded as an early model for pre-clinical drug testing and/or to select for new  
398 active compounds which should then be evaluated in other models before clinical trials.

399 Nevertheless, the perspectives of application of these findings are multiple. First, this  
400 method could be implemented to address the *in vivo* drug susceptibility profiles of clinical  
401 isolates that include strains from CF and non-CF patients, as *Mabs* clinical strains are not  
402 uniformly susceptible to the currently used antibiotics. Due to these strain-to-strain variations

403 (17, 39), no optimal regimen has been established to cure *Mabs* infections and determining the  
404 susceptibility/resistance profile of clinical strains may greatly help the clinician to select optimal  
405 drug treatments. It is worth mentioning that for this particular application, no absolute  
406 requirement for the tested strains to carry pTEC27 is needed, as visualization of fluorescent  
407 bacteria is not necessary if assessing ZF survival only. Second, since the ZF is particularly  
408 amenable to mimic a CF-like micro-environment, by silencing the *cftr* expression level (40), this  
409 system would also allow to comparing the therapeutic efficacy of clarithromycin and imipenem  
410 (and perhaps other antibiotics) in a *cftr*-deficient environment as it remains to be established  
411 whether a defect in CFTR affects susceptibility to drugs. Third, this method could be further  
412 exploited to compare the intrinsic activity of antibiotics *in vivo* in embryos infected with the  
413 three species of the *M. abscessus* complex - *M. abscessus sensu stricto*, *M. massiliense*, and *M.*  
414 *bolletii* - which are known to respond differently to antibiotics *in vitro* (41, 42).

415 Finally, the ZF embryo is particularly suited for high throughput screening as shown recently  
416 for *M. marinum* (36, 43, 44). Work is currently in progress in our laboratory to develop an *in vivo*  
417 platform for high-throughput screening of molecules against *Mabs* in order to speed up the  
418 process of identifying promising drug candidates, particularly warranted due to the extreme  
419 resistance of *Mabs* to most current antibiotics.

420

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563  
564

565 **FIGURE LEGENDS**

566  
567 **Figure 1. Kinetics of colonization of *M. abscessus* in aerosolised or intravenously infected**  
568 **BALB/c mice. (A)** Mice were aerosolized by  $4 \times 10^7$  CFU/ml of *Mabs*. Animal were then  
569 sacrificed at days 1, 3, 8, 27 prior to CFU counting in the liver, spleen and lungs. Results are  
570 expressed as the log units of CFU. **(B)** Mice were challenged *i.v.*  $10^6$  CFU of *Mabs*. Animals were  
571 then sacrificed at days 1, 15 and 30 to determine the CFU counts in the different organs.  
572 Results are representative of 2-3 independent experiments and Log CFU are expressed as the  
573 mean  $\pm$  standard error (n=5-7 mice for each time point).

574  
575 **Figure 2. Experimental protocol to assess the *in vivo* drug activity on *M. abscessus* infection.**  
576 ZF embryos were *i.v.* infected with  $\approx 300$  CFU of *Mabs* expressing dtTomato and distributed and  
577 incubated into 96-wells plate (1). From 1dpi, embryos were exposed to the drugs of interest  
578 which were directly added to the wells. Drugs are then removed and daily renewed for 5 days  
579 (2). To determinate the *in vivo* antibacterial effects of the drugs, the embryo survival, the  
580 bacterial loads and the evolution of the infection process were monitored at a spatiotemporal  
581 level by videomicroscopy (3).

582  
583 **Figure 3. *In vivo* characterization of clarithromycin activity on *M. abscessus* infection. (A-F)**  
584 Embryos were soaked in clarithromycin at 1.7X, MIC 17X or 170X the MIC for 5 days. The red  
585 bar indicates the start and duration of treatment. **(A)** Survival of uninfected embryos treated  
586 with various doses of clarithromycin and compared to mock controls (DMSO 1%) (n=20 for  
587 each, representative of three independent experiments). Representative microscopy image of  
588 an untreated (inset, upper panel) or drug treated-embryo (inset, lower panel) at 8dpf.  
589 Clarithromycin appears toxic at the highest concentration as evidenced by the presence

590 development abnormalities and the increased mortality rate in the drug-exposed embryos  
591 compared to the mock control ( $p=0.028$ , log-rank test). **(B)** Survival of infected *Mabs* treated at  
592 various doses of clarithromycin and compared to untreated infected embryos ( $\approx 300$  CFU,  $n=20$ ,  
593 representative of three independent experiments). A significant increased survival was  
594 observed in the infected-embryos exposed to the highest drug concentration ( $p=0.029$ , log-rank  
595 test). **(C)** Bacterial loads of untreated or treated-embryos ( $\approx 400$  CFU). Results are expressed as  
596 mean  $\text{Log}_{10}$  CFU per embryo from three independent experiments. A significant reduction in  
597 bacterial burdens with 170X the MIC in drug treated-embryos is observed at 5dpi. **(D)**  
598 Spatiotemporal visualization of the infection by *Mabs* expressing dtTomato ( $\approx 300$  CFU) in  
599 untreated or drug treated-embryos. The representative fluorescence and transmission overlay  
600 of whole embryos are shown. The yolk is auto-fluorescent. **(E)** Frequency of abscesses in whole  
601 untreated or drug treated-embryos over 13dpi ( $\approx 300$  CFU; average of three independent  
602 experiments). Infected embryos developed significantly less abscesses in the presence  
603 clarithromycin at 170X the MIC than untreated infected-embryos. **(F)** Average localization of  
604 abscesses of the infected embryos in (E). *Mabs*-infected ZF developed significantly less  
605 abscesses within the brain and the spinal when exposed to the highest clarithromycin dose as  
606 compared to untreated infected-ZF. For (c) statistics were calculated using one-way ANOVA or  
607 for (e) and (f) with Fisher's exact test comparing each category of drug-treated embryos to  
608 untreated control. Error bars represent the standard errors.  $**p<0.01$ .

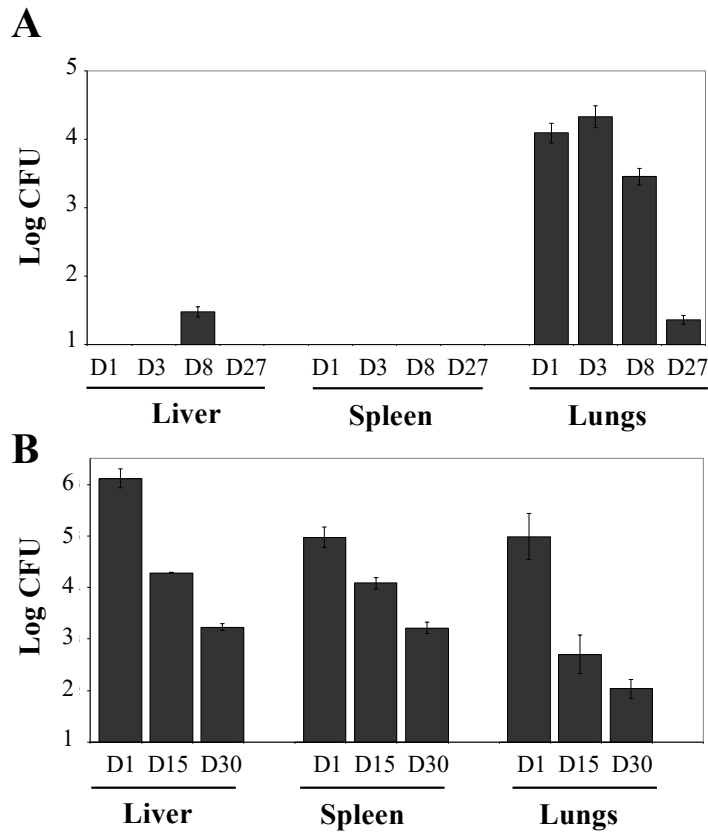
609  
610 **Figure 4. Imipenem treatment cures *M. abscessus*-infected embryos. (A-E)** From 1dpi,  
611 embryos were exposed for 5 days to imipenem concentrations corresponding to 0.5X, 5X or 28X  
612 the MIC. **(A)** Survival of infected *Mabs* R treated at various doses of imipenem and compared to  
613 untreated infected embryos ( $\approx 300$  CFU,  $n=20$ , representative of three independent

614 experiments). Survival of treated R-infected embryos is dose-dependent. Significant increased  
615 survival was observed in infected-embryos exposed to 5 X and 28X MIC of imipenem. The red  
616 bar indicates the start and duration of treatment. **(B)** Bacterial loads of untreated or imipenem  
617 treated-embryos ( $\approx 400$  CFU). Results are expressed as mean  $\text{Log}_{10}$  CFU per embryo from three  
618 independent experiments. A significant decreased of bacterial loads is already observed after  
619 3dpi in the 28X MIC imipenem treated-embryos. **(C)** Spatiotemporal visualization of the  
620 infection by *Mabs* expressing dtTomato ( $\approx 300$  CFU) in untreated or imipenem treated-embryos.  
621 The representative fluorescence and transmission overlay of whole embryos are shown. **(D)**  
622 Frequency of abscesses in whole untreated or imipenem-treated embryos over 13dpi ( $\approx 300$   
623 CFU, average of three independent experiments). Only the 28X MIC imipenem treated-embryos  
624 developed significantly fewer abscesses than untreated infected-embryos. **(E)** Average  
625 localization of abscesses of the infected embryos in (D). 5X and 28X MIC of imipenem treated-  
626 embryos infected by *Mabs* developed fewer abscesses within the brain than untreated  
627 infected-embryos. For (B) statistics were calculated using one-way ANOVA or for (D) and (E)  
628 with Fisher's exact test comparing each category of imipenem-treated embryos to untreated  
629 control. Error bars represent the standard error. \* $p=0.02$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ .

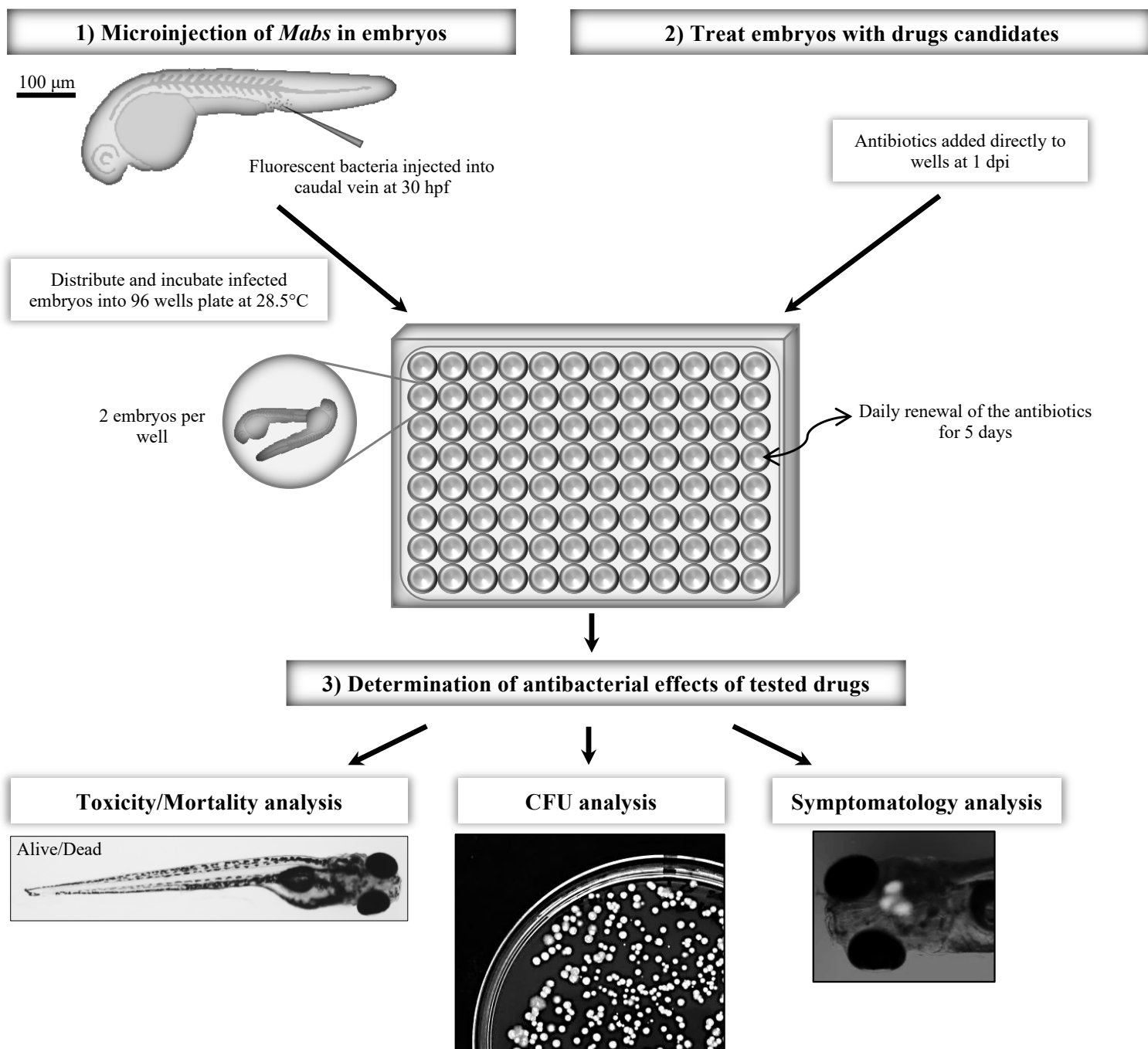
630  
631 **Figure 5. Imipenem treatment decreases the early pathophysiological signs within the CNS.**

632 **(A-D)**. dtTomato-expressing *Mabs* ( $\approx 300$  CFU) are injected in 30hpf embryos ( $n=15$ , average of  
633 three independent experiments). From 1dpi, embryos were exposed to imipenem at 0.5X, 5X or  
634 28X MIC during 5 days. **(A)** Fluorescence microscopy of a typical R serpentine cord. Scale bar,  
635 100 $\mu\text{m}$ . **(B)** Fluorescence and DIC overlay of whole heads of a 28X MIC imipenem-treated and  
636 untreated infected embryos with red fluorescent *M. abs* showing serpentine cord (white  
637 arrow). Scale bars, 100 $\mu\text{m}$ . **(C)** Percentage of embryos with cords in whole untreated and

638 imipenem-treated embryos at 4dpi. A significant reduction in the proportion of embryos with  
639 cords was observed when embryos were treated with the highest (28X MIC) imipenem  
640 concentration. **(D)** Average localization of cord of the infected embryos in (C). Infected embryos  
641 treated with the intermediate (5X MIC) and high (28X MIC) imipenem doses developed  
642 significantly fewer serpentine cords within the CNS compared to untreated infected-embryos.  
643 For (C) and (D), statistics were calculated using Fisher's exact test comparing each category of  
644 imipenem-treated embryos to untreated control. All results are expressed as the average from  
645 three independent experiments and error bars represent the standard errors.

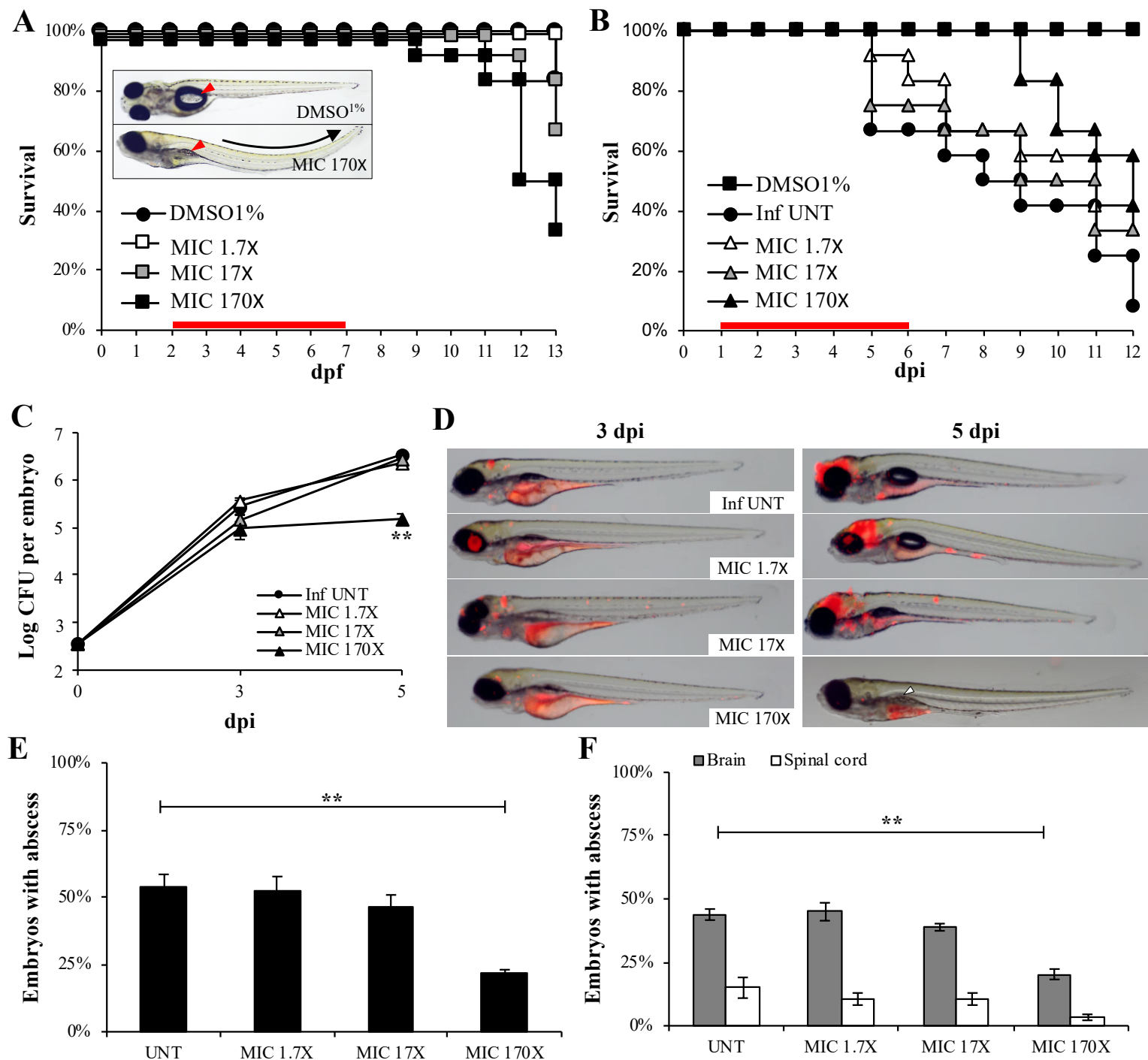


**Figure 1. Kinetics of colonization of *M. abscessus* in aerosolised or intravenously infected BALB/c mice.** (A) Mice were aerosolized by  $4 \times 10^7$  CFU/ml of *Mabs*. Animals were then sacrificed at days 1, 3, 8, 27 prior to CFU counting in the liver, spleen and lungs. Results are expressed as the log units of CFU. (B) Mice were challenged *i.v.*  $10^6$  CFU of *Mabs*. Animals were then sacrificed at days 1, 15 and 30 to determine the CFU counts in the different organs. Results are representative of 2-3 independent experiments and Log CFU are expressed as the mean  $\pm$  standard error (n=5-7 mice for each time point).

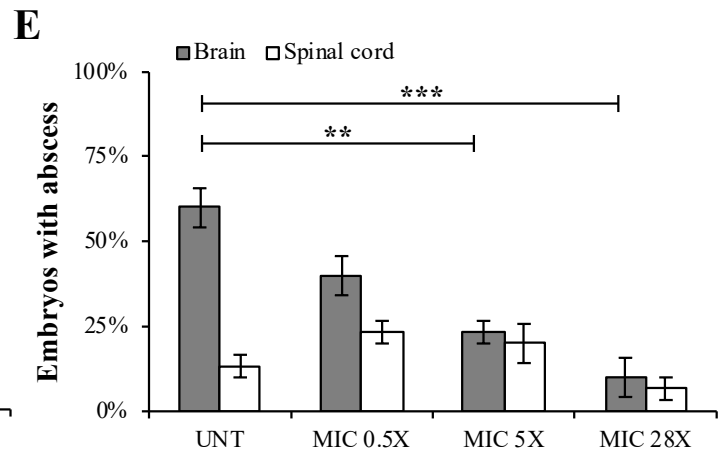
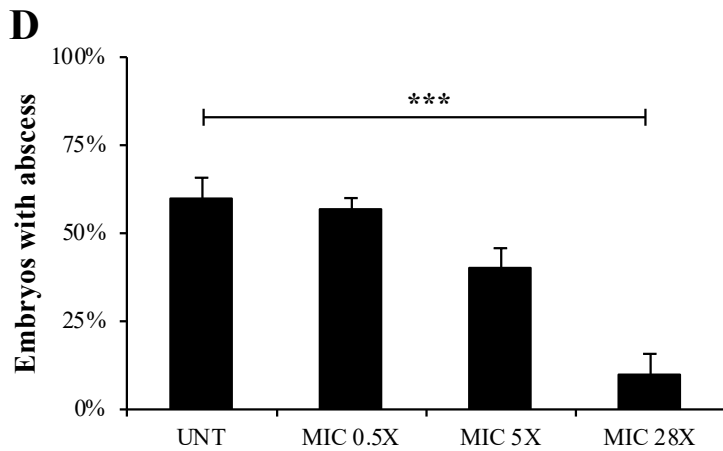
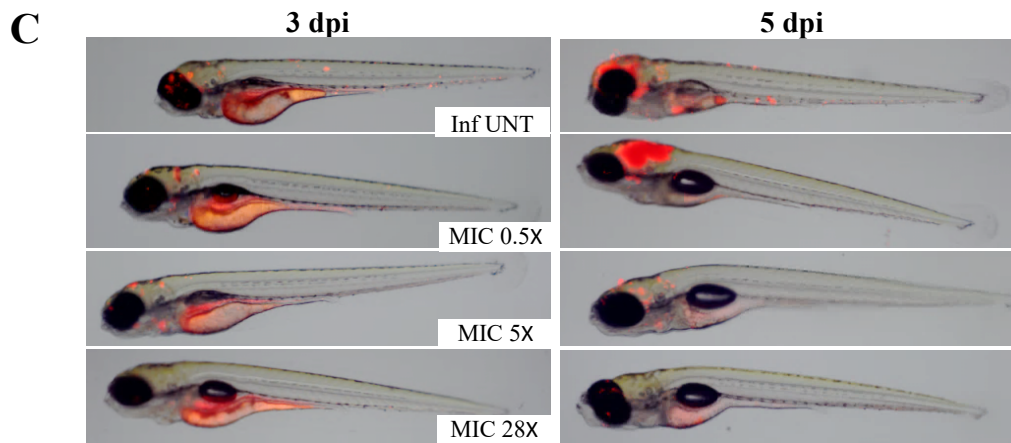
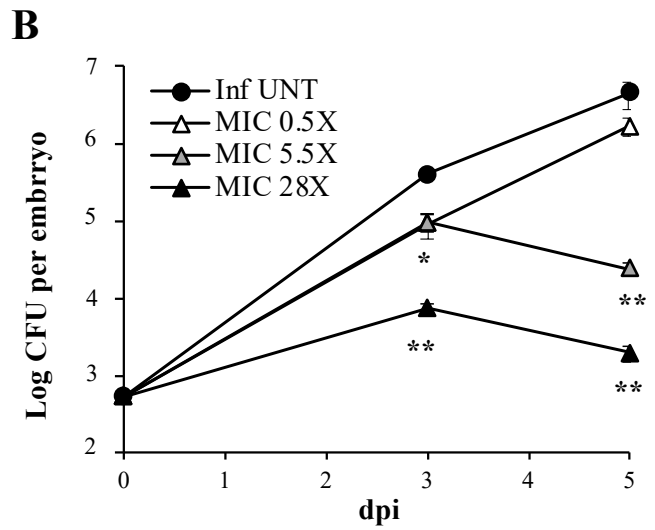
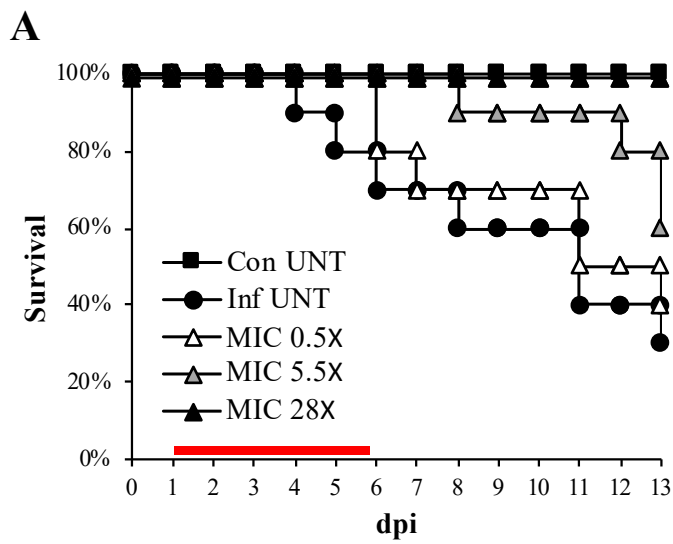


**Figure 2. Experimental protocol to assess the *in vivo* drug activity on *M. abscessus* infection.** ZF embryos were *i.v.* infected with  $\approx 300$  CFU of *Mabs* expressing dtTomato and distributed and incubated into 96-wells plate (1). From 1dpi, embryos were exposed to the drugs of interest which were directly added to the wells. Drugs are then removed and daily renewed for 5 days (2). To determinate the *in vivo* antibacterial effects of the drugs, the embryo survival, the bacterial loads and the evolution of the infection process were monitored at a spatiotemporal level by videomicroscopy (3).

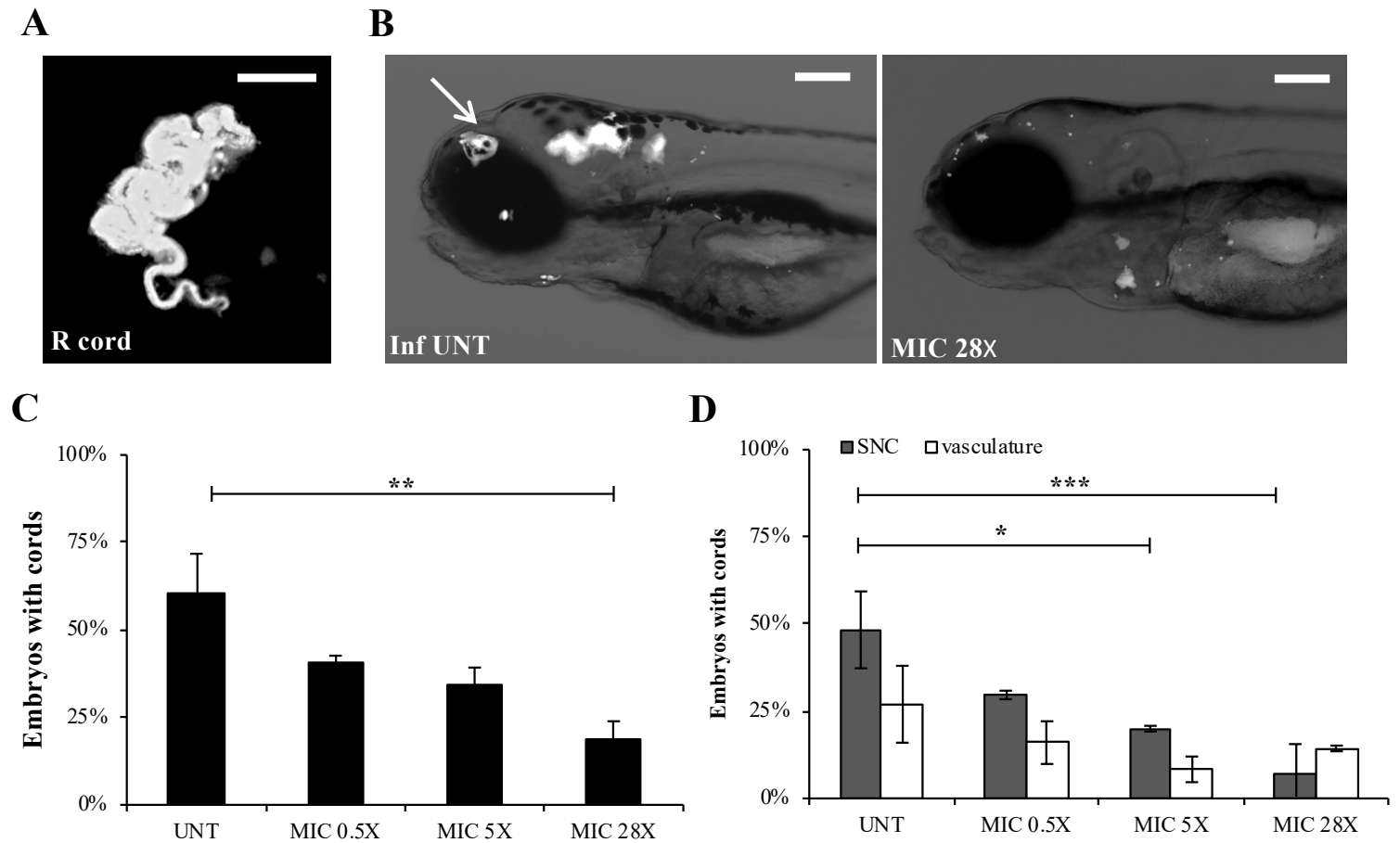




**Figure 3. *In vivo* characterization of clarithromycin activity on *M. abscessus* infection. (A-F)** Embryos were soaked in clarithromycin at 1.7X, MIC 17X or 170X the MIC for 5 days. The red bar indicates the start and duration of treatment. **(A)** Survival of uninfected embryos treated with various doses of clarithromycin and compared to mock controls (DMSO 1%) (n=20 for each, representative of three independent experiments). Representative microscopy image of an untreated (inset, upper panel) or drug treated-embryo (inset, lower panel) at 8dpf. Clarithromycin appears toxic at the highest concentration as evidenced by the presence development abnormalities and the increased mortality rate in the drug-exposed embryos compared to the mock control (p=0.028, log-rank test). **(B)** Survival of infected *Mabs* treated at various doses of clarithromycin and compared to untreated infected embryos ( $\approx 300$  CFU, n=20, representative of three independent experiments). A significant increased survival was observed in the infected-embryos exposed to the highest drug concentration (p=0.029, log-rank test). **(C)** Bacterial loads of untreated or treated-embryos ( $\approx 400$  CFU). Results are expressed as mean Log<sub>10</sub> CFU per embryo from three independent experiments. A significant reduction in bacterial burdens with 170X the MIC in drug treated-embryos is observed at 5dpi. **(D)** Spatiotemporal visualization of the infection by *Mabs* expressing dtTomato ( $\approx 300$  CFU) in untreated or drug treated-embryos. The representative fluorescence and transmission overlay of whole embryos are shown. The yolk is auto-fluorescent. **(E)** Frequency of abscesses in whole untreated or drug treated-embryos over 13dpi ( $\approx 300$  CFU; average of three independent experiments). Infected embryos developed significantly less abscesses in the presence clarithromycin at 170X the MIC than untreated infected-embryos. **(F)** Average localization of abscesses of the infected embryos in (E). *Mabs*-infected ZF developed significantly less abscesses within the brain and the spinal when exposed to the highest clarithromycin dose as compared to untreated infected-ZF. For (c) statistics were calculated using one-way ANOVA or for (e) and (f) with Fisher's exact test comparing each category of drug-treated embryos to untreated control. Error bars represent the standard errors. \*\*p<0.01.



**Figure 4. Imipenem treatment cures *M. abscessus*-infected embryos. (A-E)** From 1dpi, embryos were exposed for 5 days to imipenem concentrations corresponding to 0.5X, 5X or 28X the MIC. **(A)** Survival of infected *Mabs* R treated at various doses of imipenem and compared to untreated infected embryos ( $\approx 300$  CFU,  $n=20$ , representative of three independent experiments). Survival of treated R-infected embryos is dose-dependent. Significant increased survival was observed in infected-embryos exposed to 5 X and 28X MIC of imipenem. The red bar indicates the start and duration of treatment. **(B)** Bacterial loads of untreated or imipenem treated-embryos ( $\approx 400$  CFU). Results are expressed as mean  $\text{Log}_{10}$  CFU per embryo from three independent experiments. A significant decreased of bacterial loads is already observed after 3dpi in the 28X MIC imipenem treated-embryos. **(C)** Spatiotemporal visualization of the infection by *Mabs* expressing dtTomato ( $\approx 300$  CFU) in untreated or imipenem treated-embryos. The representative fluorescence and transmission overlay of whole embryos are shown. **(D)** Frequency of abscesses in whole untreated or imipenem-treated embryos over 13dpi ( $\approx 300$  CFU, average of three independent experiments). Only the 28X MIC imipenem treated-embryos developed significantly fewer abscesses than untreated infected-embryos. **(E)** Average localization of abscesses of the infected embryos in (D). 5X and 28X MIC of imipenem treated-embryos infected by *Mabs* developed fewer abscesses within the brain than untreated infected-embryos. For (B) statistics were calculated using one-way ANOVA or for (D) and (E) with Fisher's exact test comparing each category of imipenem-treated embryos to untreated control. Error bars represent the standard error. \* $p=0.02$ , \*\* $p<0.01$ , \*\*\* $p<0.001$ .



**Figure 5. Imipenem treatment decreases the early pathophysiological signs within the CNS. (A-D).** dtTomato-expressing *Mabs* ( $\approx 300$  CFU) are injected in 30hpf embryos ( $n=15$ , average of three independent experiments). From 1dpi, embryos were exposed to imipenem at 0.5X, 5X or 28X MIC during 5 days. **(A)** Fluorescence microscopy of a typical R serpentine cord. Scale bar,  $100\mu\text{m}$ . **(B)** Fluorescence and DIC overlay of whole heads of a 28X MIC imipenem-treated and untreated infected embryos with red fluorescent *M. abs* showing serpentine cord (white arrow). Scale bars,  $100\mu\text{m}$ . **(C)** Percentage of embryos with cords in whole untreated and imipenem-treated embryos at 4dpi. A significant reduction in the proportion of embryos with cords was observed when embryos were treated with the highest (28X MIC) imipenem concentration. **(D)** Average localization of cord of the infected embryos in (C). Infected embryos treated with the intermediate (5X MIC) and high (28X MIC) imipenem doses developed significantly fewer serpentine cords within the CNS compared to untreated infected-embryos. For (C) and (D), statistics were calculated using Fisher's exact test comparing each category of imipenem-treated embryos to untreated control. All results are expressed as the average from three independent experiments and error bars represent the standard errors.

**SUPPLEMENTAL MATERIAL**

***In vivo* assessment of drug efficacy against *Mycobacterium abscessus*  
using the embryonic zebrafish test system**

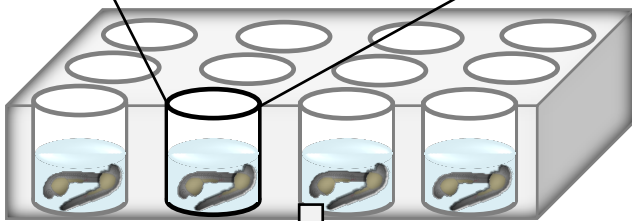
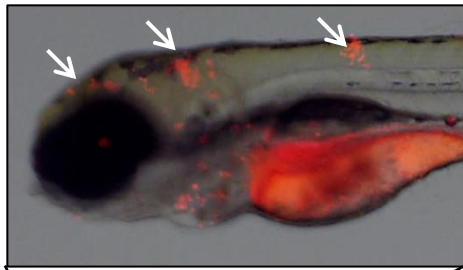
Audrey Bernut<sup>1</sup>, Vincent Le Moigne<sup>3</sup>, Tiffany Lesne<sup>1</sup>, Georges Lutfalla<sup>1</sup>,  
Jean-Louis Herrmann<sup>3</sup> and Laurent Kremer<sup>1,2,#</sup>

**A**

Microinjection of fluorescent bacteria  
into caudal vein at 30hpf



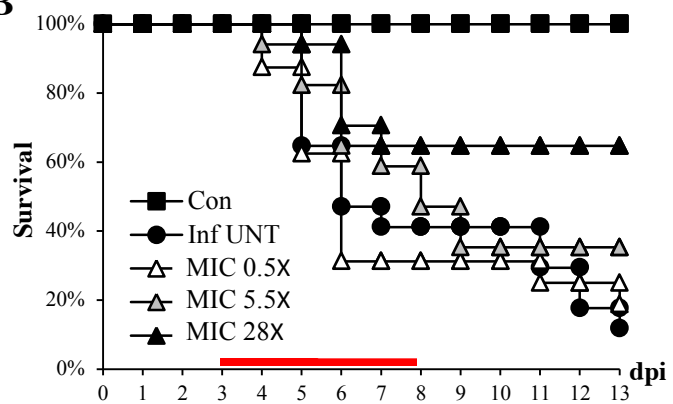
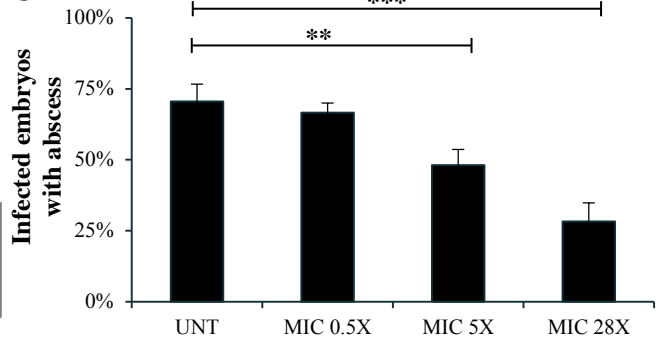
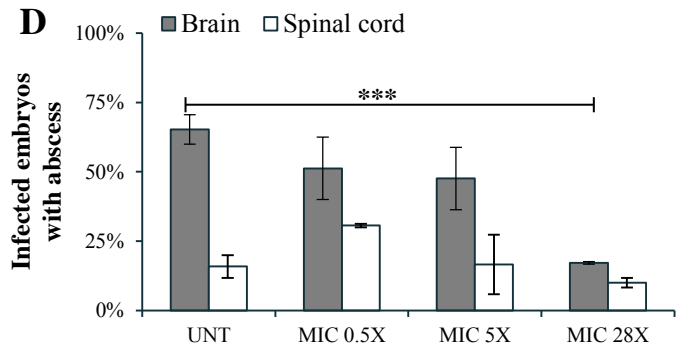
Incubate infected embryos  
for 3 days at 28.5 C



Add imipenem into wells  
Daily drug renewal for 5 days



Drug activity assessment  
(monitoring survival and  
abscess evolution)

**B****C****D**

**Figure S1. Exposure to imipenem overcomes and protects against severe *M. abscessus* infections. (A-C)** dtTomato expressing *Mabs* ( $\approx 300$  CFU) were injected in 30hpf embryos. From 3dpi, embryos were exposed during 5 days to imipenem at 0.5X, 5X or 28X MIC. **(A)** Schematic representation illustrating the “curing” protocol used as well as the infection status of the embryos at 3dpi (numerous abscesses within the CNS) when the drug treatment is applied. **(B)** Survival of infected *Mabs* treated at various doses of imipenem and compared to untreated infected embryos ( $n=20-30$ ), representative of three independent experiments). A significant increased survival was observed in embryos exposed to the highest (28X MIC) imipenem dose. The red bar indicates the start and duration of treatment. **(C)** Frequency of *Mabs* abscesses in whole untreated or imipenem-treated embryos over 13dpi. Data are expressed as the average of three independent experiments. MIC 5X and MIC 28X imipenem treated-embryos infected by *Mabs* developed significantly fewer abscesses than untreated infected-embryos. **(D)** Average localization of abscesses of the infected embryos in (C). 28X MIC imipenem treated-embryos infected by *Mabs* developed significantly fewer abscesses within the brain than untreated infected-embryos. For (C) and (D) statistics were calculated using Fisher’s exact test comparing each category of imipenem-treated embryos to untreated control. All results are expressed as the average from two or three independent experiments and error bars represent the standard errors. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

**Table S1.** Minimal inhibitory concentrations of several drugs against *M. abscessus* using the microdilution method in cation-adjusted Mueller-Hinton (MH) broth or on LB agar. Antibiotics used in infected ZF are shown in bold. Results are expressed in  $\mu\text{M}$  and  $\mu\text{g}/\text{mL}$ .

Antibiotic	Molecular weight	Solvent	MIC MH broth		MIC LB agar	
			$\mu\text{M}$	$\mu\text{g}/\text{mL}$	$\mu\text{M}$	$\mu\text{g}/\text{mL}$
<b>Clarithromycin</b>	<b>748</b>	<b>DMSO</b>	<b>4</b>	<b>3.0</b>	<b>0.7</b>	<b>0.5</b>
Cefoxitin	427	DMSO	60	25.6	35	15
Amikacin	586	H <sub>2</sub> O	125	73.25	26	15
Isoniazid	137	H <sub>2</sub> O	1000	137	365	50
Erythromycin	734	DMSO	125	91.75	10	7.5
<b>Imipenem</b>	<b>299</b>	<b>H<sub>2</sub>O</b>	<b>60</b>	<b>17.94</b>	<b>3.3</b>	<b>1</b>
Thiacetazone	236	DMSO	1000	236	42	10