Frontiers in Bioscience Landmark

Front. Biosci. (Landmark Ed) **2023**; 28(5): 99
https://doi.org/10.31083/j.fbl2805099

Original Research

Zebrafish Larvae as an *in vivo* Model for Antimicrobial Activity Tests against Intracellular *Salmonella*

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Academic Editors: Lucia Rocco and Filomena Mottola
Submitted: 23 February 2023 Revised: 12 April 2023 Accepted: 26 April 2023 Published: 25 May 2023

Abstract

Introduction: Blood infections from multi-drug-resistant *Salmonella* pose a major health burden. This is especially true because *Salmonella* can survive and replicate intracellularly, and the development of new treatment strategies is dependent on expensive and time-consuming *in vivo* trials. The aim of this study was to develop a *Salmonella*-infection model that makes it possible to directly observe *Salmonella* infections of macrophages *in vivo* and to use this model to test the effect of antimicrobials against intra- and extracellular *Salmonella* in order to close the gap between *in vitro* and rodent-infection models. **Methods**: We established suitable *Salmonella*-infection conditions using genetically engineered zebrafish and *Salmonella*-expressing fluorescent proteins (*green fluorescent protein* (*GFP*) and/or *mCherry*). **Results**: We detected *Salmonella* inside and outside zebrafish larvae macrophages. Administration of the cell-impermeable antibiotic tobramycin removed *Salmonella* residing outside macrophages but did not affect *Salmonella* in macrophages, whereas ceftriaxone successfully cleared both types of *Salmonella*. *Salmonella* inside and outside macrophages experienced substantial DNA damage after administration of fluoroquinolones consistent with the excellent cell penetration of these antibiotics. **Conclusions**: The zebrafish-larvae model enables testing of antimicrobials for efficacy against extra- and intracellular *Salmonella* in a complex *in vivo* environment. This model thus might serve for antimicrobial lead optimization prior to using rodent models.

Keywords: *Salmonella enterica*; infections; macrophages; zebrafish larvae; antibiotic resistance; drug screening

1. Introduction

Salmonella is a major cause of systemic infections with a high fatality rate in low- and middle-income countries. As such, it is an significant health burden in those countries [1]. *Salmonella* can survive and replicate intracellularly in many cellular types, including macrophages [2]. This is particularly problematic because a number of regularly used antibiotics are unable to cross cell membranes and therefore cannot reach intracellular *Salmonella* [3–5]. After termination of the antibiotic intervention, these surviving intracellular pathogens can reemerge and lead to a reinfection of the organism [6].

Increasing resistance to currently used antibiotics [7, 8] and the low number of promising novel antibiotics pose a major threat to modern medicine and human health worldwide. This threat has the potential to become one of the most serious challenges in modern medical practice [9–13]. However, the development of anti-invective treatment regimens is expensive and the return of investment is relatively small, as efficient antibiotics rarely make it onto the market [14].

Due to poor biodistribution and pharmacokinetic-model properties, *in vitro* tests often do not mirror the efficiency of antibiotic compounds and formulations *in vivo* [15]. Thus, in addition to *in vitro* testing, novel antibiotics need to show activity in expensive and time-consuming *in vivo* trials. Several *in vitro* and *in vivo* models have been described, suitable to investigate *Salmonella* infections including underlying molecular mechanisms and pathogen-host interactions. The wide variety of described models includes *in vitro* models like enteroids and organoids, and *in vivo* models like *C.elegans*, insect larvae, zebrafish larvae, chicken embryos, rodents and calves [16–20].

In this work, we describe a zebrafish-larvae-(ZFL)-based vertebrate model, optimized for the screening of antibiotics. We can distinguish between their intra- and extracellular efficiency against *Salmonella*. The proposed model is an alternative to commonly used rodent models, enabling researchers to reduce the costs for the development of novel anti-infectives or treatment regimens and to increase the throughput of compound screenings. The advantages of ZFL include the ease of nursing and drug injection, their transparency, which allows for fluorescence-based imaging, and a much higher throughput compared to other animal models due to their short reproductive cycle and high number of offspring [21]. In contrast to *in vitro* screening models, a ZFL-based model makes it possible to obtain pharmacological information, to identify the toxicity of

compounds, and to observe the effects of compounds that only work *in vivo* (e.g., prodrugs, effects on pathogen–host interactions, etc.). Due to its unique properties, the ZFL animal model was already proposed as an early-stage screening tool for studying pharmacological aspects like the circulation behavior and the renal and macrophage clearance of a variety of drugs [22–25]. Early macrophages in ZFL appear 22 hours post fertilization (hpf). *Macrophages* are capable of phagocytosing bacteria within the ZFL blood 30 hpf [26,27]. ZFL have previously been used as infection models including for *Salmonella* [28–30] and in antibiotic activity testing against extracellular pathogens [29,31–33]. Here, we established ZFL as a model for testing antimicrobials against intracellular *Salmonella* by infecting genetically engineered zebrafish selectively expressing *KAEDE* within macrophages [34] with *Salmonella* expressing fluorescent reporter proteins (*GFP* and/or *mCherry*).

2. Materials and Methods

2.1 Materials

Peptone (No. 8952.2), yeast extract (No. 2904.4), and agar (No. 5210.2) were obtained from Carl Roth GmbH, Karlsruhe, Germany. Methanol (Art. No. 34860), triethylamine (No. 471283), triton X-100 (No. 648466), N-phenylthiourea (No. P7629), tricaine (ethyl-3-aminobenzoate methane sulfonate) (No. E10521), ceftriaxone (No. C5793), tobramycin (No. T4014), ciprofloxacin (No. 17850), moxifloxacin (No. SML1581), and gemifloxacin (No SML1625). were purchased from Merck & Cie, Buchs, Switzerland. Ethanol (No. 24562) was obtained from Honeywell, Riedel-de-Haën, Seelze, Germany. Sodium chloride (No. A2942) was obtained from AppliChem GmbH, Darmstadt, Germany. LB Lennox medium: peptone (20 g), yeast extract (10 g), and sodium chloride (10 g) were dissolved in 2 liters of water, and the pH was adjusted to 7. Agar plates: microbiological grade agar (15 g) was dissolved in 1 liter of water and autoclaved for 20 min at 15 psi liquid cycle; 50 µg/mL kanamycin was added, and the medium was distributed to 10 cm petri plates (25 mL per plate) and allowed to cool down to 25 °C. An attenuated *Salmonella* SDB15 SL1344 Δ*aroA* strain was used for the infection models [35]. For constitutive *mCherry* expression, *Salmonella* were transformed with a pSC101-derived episomal construct expressing *mCherry* under the constitutively active PybaJ promoter [36,37]. For the DNA damage reporter system, *Salmonella* were transformed with a pSC101 backbone containing the Pcad promoter of colicin D, fused to *gfp-ova* encoding an unstable variant of the *green fluorescent protein* (*GFP*) and the PrecA promoter fused to *mCherry* [38–42].

2.2 Methods

2.2.1 Growth Curve of Salmonella

Bacteria from a glycol stock kept at –80 °C were streaked on LB agar containing 50 µg/mL kanamycin at 37 °C o/n. A single colony of bacteria was taken the next day, inoculated in 3 mL of LB medium with 50 µg/mL kanamycin, and incubated overnight at 37 °C and 250 rpm. Then 0.5 mL of the culture was added to 50 mL fresh LB medium with 50 µg/mL kanamycin and incubated at 37 °C and 200 rpm. The optical density at 600 nm was measured, and samples were taken from the culture every 15 min for 3 h. The samples were each diluted in 1:10 steps in D-PBS until a final dilution of 10^{-7} and plated. The plates were incubated at 37 °C o/n, and colonies were counted the next day. Average colony-forming units (CFUs) were plotted against OD_{600} values of each time point.

2.2.2 Preparing Salmonella for Injection

Bacteria were prepared for injections with an overnight culture followed by inoculation of a 50 mL culture as described above. Then 1 mL samples were taken in 30 min steps until the OD_{600} was between 0.8 and 1 (experimentally validated exponential growth phase; data not shown). From then on, the bacteria were kept on ice. Next 6 mL of the 50 mL culture were centrifuged at 2000 G for 10 min. The supernatant was discarded. The amount of CFU was calculated according to the calibration described in 2.2.1, and the pellet was resuspended in Dulbecco's phosphate-buffered saline to get the desired CFU concentration.

2.2.3 Zebrafish Husbandry and Larvae Collection

Zebrafish (*Danio rerio*) larvae (kindly provided by Prof. Dr. M. Affolter and Dr. H. Belting, University of Basel, Switzerland) were obtained from adult Tg(mpeg1:Gal4;UAS:Kaede) fish [34,43] with macrophages expressing EGFP or wild-type (AB/TU) fish [44] and were kept at 28 °C in a zebrafish-culture medium supplemented with 30 μg/mL 1-phenyl-2-thiourea (PTU). All fish were kept in accordance with Swiss animal-welfare regulations [22,23].

2.2.4 Zebrafish-Larvae Experiments

The ZFL were mechanically dechorionized 2 days post fertilization (dpf) using two jeweler's forceps (Dumont No. 5, L 4 ¼ in., Inox alloy), anaesthetized with 0.01% tricaine, embedded in 0.3% agarose containing tricaine and PTU, and injected with the indicated amounts of the selected *Salmonella* strain into the duct of Cuvier (if not stated differently) using a micromanipulator (Wagner Instrumentenbau KG, Schöffengrund, Germany), a pneumatic PicoPump PV830 (WPI, Sarasota, Florida, USA), and a Leica S8APO microscope (Leica, Wetzlar, Germany). From then on, the ZFL were kept at 35 °C until the end of the experiment unless stated differently. At the indicated time points, the infected ZFL were injected with the given amounts of antibiotics.

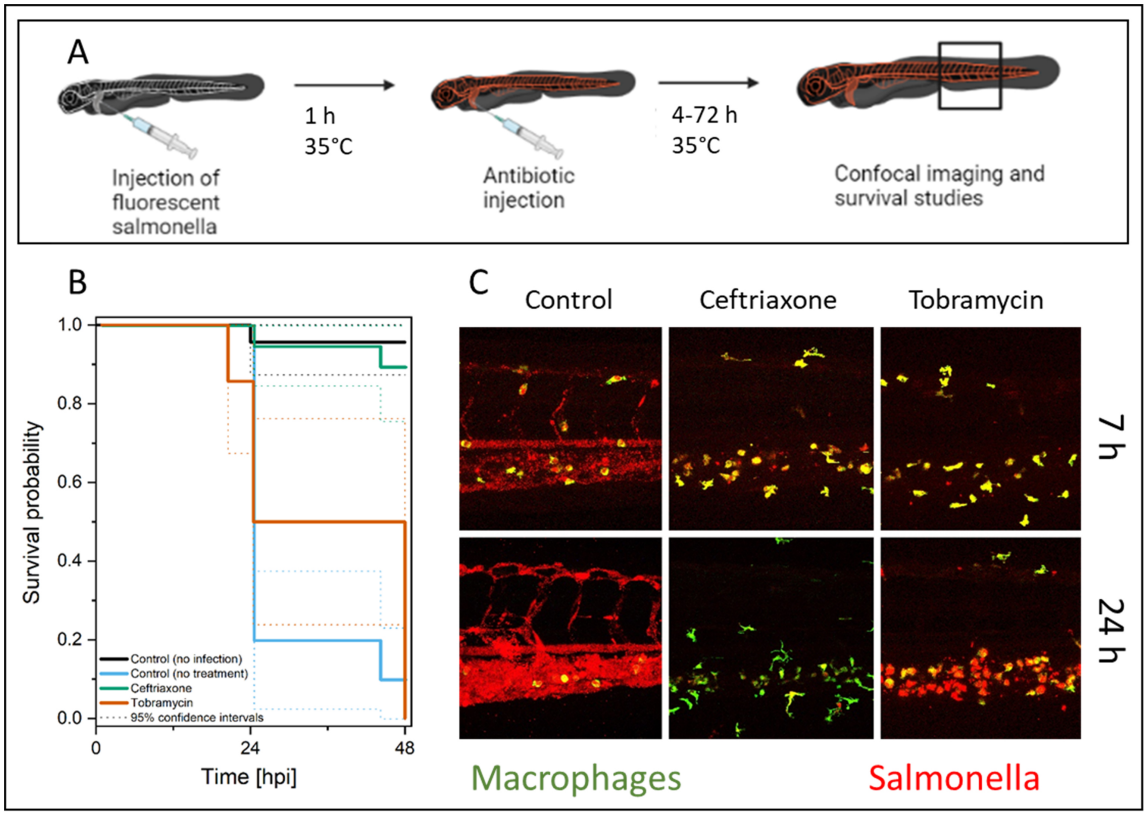

Fig. 1. Infection-model validation: Treatment of systemic *Salmonella* infection with ceftriaxone or tobramycin. (A) Experimental procedure. ZFL expressing *KAEDE* (green) under macrophage-specific promoter mpeg, were injected intra venously (i.v.) with *mCherry*-expressing *Salmonella* (300 CFU) and incubated at 35 °C. Then 1 h after *Salmonella* injection, the fish were injected i.v. with either PBS (control), ceftriaxone, or tobramycin (600 pg per antibiotic per fish). Confocal images were taken 7 h and 1 day after *Salmonella* injection, and survival studies were carried out until 2 days post *Salmonella* injection. (B) Kaplan–Meier curve of survival studies 0–48 h after *Salmonella* injection. Survival probability including 95% confidence intervals (dotted lines, n > 10). Observable heartbeat served as the survival criterium. (C) Confocal images of ZFL tail region 7 h and 24 h post *Salmonella* injection. Green: *KAEDE* (macrophages), red: *mCherry* (*Salmonella*).

2.2.5 Imaging and Data Analysis

At the indicated time points, the fish were imaged using either a confocal-laser scanning microscope (either an SP5-II-MATRIX, Leica, Wetzlar, Germany, equipped with a 25x HCX IRAPO L (NA 0.95) objective or an Olympus FV 1000 inverted microscope, Olympus Ltd, Tokyo, Japan, with a 20x UPLSAPO (NA 0.75) objective) and, where indicated, the number of surviving fish was counted. Visual inspection to discriminate between living and dead larvae was done with a Leica S8APO microscope (Leica, Wetzlar, Germany). The presence of a heartbeat was chosen as the survival criterion. Only living larvae (with an observable heartbeat) were used for confocal imaging. The image analysis was carried out using Fiji ImageJ v. 1.52n (U.S. National Institutes of Health, Bethesda, Maryland, USA) and OMERO.web v. 5.9.1 (https://www.openmicroscopy.org/). A minimum of 3 ZFL were imaged in all settings. The area for the quantification of the fluorescent area fraction was chosen based on a phase-contrast image of the tail region of one ZFL. Subsequent measurements in the same experiment were done based on the same region. The statistical analysis and data plotting was carried out using OriginPro 2018 (64-bit) SR1 b9.5.1.195 (Academic) Software (OriginLab Corporation, Northampton, MA, USA). A minimum of 10 ZFL per group were used for survival analysis.

3. Results

3.1 Model Optimization

To evaluate the appropriate number of colony-forming units (CFU) that need to be injected into the ZFL for drug screening, we injected either 30,000, 20,000, 10,000, 7500, 5000, 3000, or 1500 CFU *Salmonella* into the ZFL and kept them at 28 °C. At each inoculum size, the *Salmonellae* were easily detectable within the whole ZFL using confocal imaging (**Supplementary Figs. 1,2**). Some colocalization of the green (*KAEDE* of the macrophages) and the red (*mCherry* of *Salmonella*) fluorescence could be observed 2 h post injection of 3000 and 1500 CFU, indicating a fraction of intracellular *Salmonella*, while other *Salmonella* seemed to remain in the vasculature. After in-

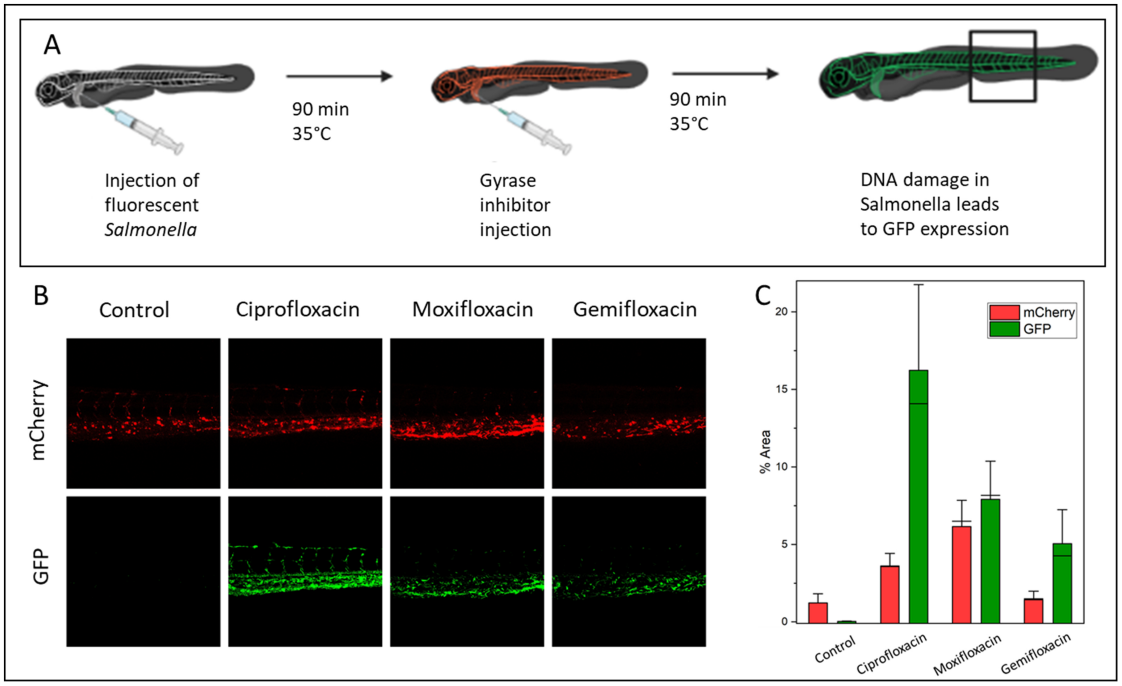

Fig. 2. The effect of gyrase inhibitors on SOS-DNA damage-reporter *Salmonella* in ZFL blood vessels. (A) ZFL were injected i.v. with SOS-DNA damage-reporter *Salmonella* (300 CFU) and incubated at 35 °C. Reporter *Salmonella* constitutively express *mCherry* with an increased *mCherry* expression after DNA damage. *gfp* is exclusively expressed after DNA damage. Ninety min after *Salmonella* injection, the fish received either no treatment (control), or an i.v. injection of either ciprofloxacin, moxifloxacin, or gemifloxacin (600 pg of antibiotic per fish). Confocal images were taken 90 min after the antibiotic injection. (B) Confocal images of *GFP* and *mCherry* fluorescence. Green: *GFP* (*cda* promoter), red: *mCherry* (*recA* promoter). (C) Percentages of areas with *GFP* or *mCherry* fluorescence, respectively, 90 min after antibiotic injection. Bars: average; horizontal line: median; whiskers: SE; n $\geq$ 3.

jections lower than 5000 CFU, the red *mCherry* signal of *Salmonella* largely vanished from ZFL circulation within 24 h. Survival was clearly observable to be dependent on the dose: doses higher than 5000 CFU led to a rapid death of the fish within 24 h.

We tested infections at 35 °C, which lies within the optimal temperature range for *Salmonella* growth (35–37 °C) [45]. While 35 °C is still well tolerated by ZFL, higher temperatures (nearer to 37 °C) lead to an unsatisfactory survival rate [46]. Keeping the model as close as possible to human body temperature is important since the efficacy of some antibiotics is highly temperature dependent. For example, the EC_{50} of ciprofloxacin decreases by >75% with a 10 °C temperature increase [47]. Injections higher than 300 CFU at 35 °C led to rapid deaths of the ZFL. An injection of 300 CFU led to a survival rate comparable to 3000 or 1500 CFU in fish maintained at 28 ºC, but a bacterial spread more comparable to an injection of 10,000 CFU or more when maintained at 28 °C (**Supplementary Fig. 3**), thus providing a more informative setting.

3.2 Validation of the ZFL Blood-Infection Model

We determined the impact of ceftriaxone and tobramycin treatment on ZFL infected with 300 CFU of *Salmonella*. Differentiating between intra- and extracellular efficiency is a major strength of the proposed model. Ceftriaxone and tobramycin were chosen to validate the model because of their known distinct efficiencies in killing intracellular *Salmonella* [48,49]. Ceftriaxone is used to treat salmonellosis in humans [50].

The ED_{50} of tobramycin was 600 pg and at higher doses 80–90% of ZFL survived 24 h post infection (Fig. 1, **Supplementary Fig. 4)**. Based on an larval blood volume of ~80 nL [51], this corresponds to a dose of 7.5 mg/kg as recommended for treating a “serious infection” in human patients [52]. Confocal imaging of tobramycin-treated ZFL revealed a large number of remaining *Salmonella*, but these bacteria were all confined to macrophages, consistent with the poor cell penetration of tobramycin and other aminoglycosides [48]. At later time points, a reemergence of an *mCherry* signal in the vasculature indicated a release of surviving *Salmonella* from macrophages and a restarting of proliferation. In contrast to tobramycin, ceftriaxone-treated ZFL showed a high survival rate and almost no residual bacteria in the circulation and macrophages. These results were consistent with the poor cell penetration of tobramycin and excellent cell penetration and clinical efficacy of ceftriaxone [48,49], and they validate ZFL as a model for determining extra- and intracellular antimicrobial activity in live animals.

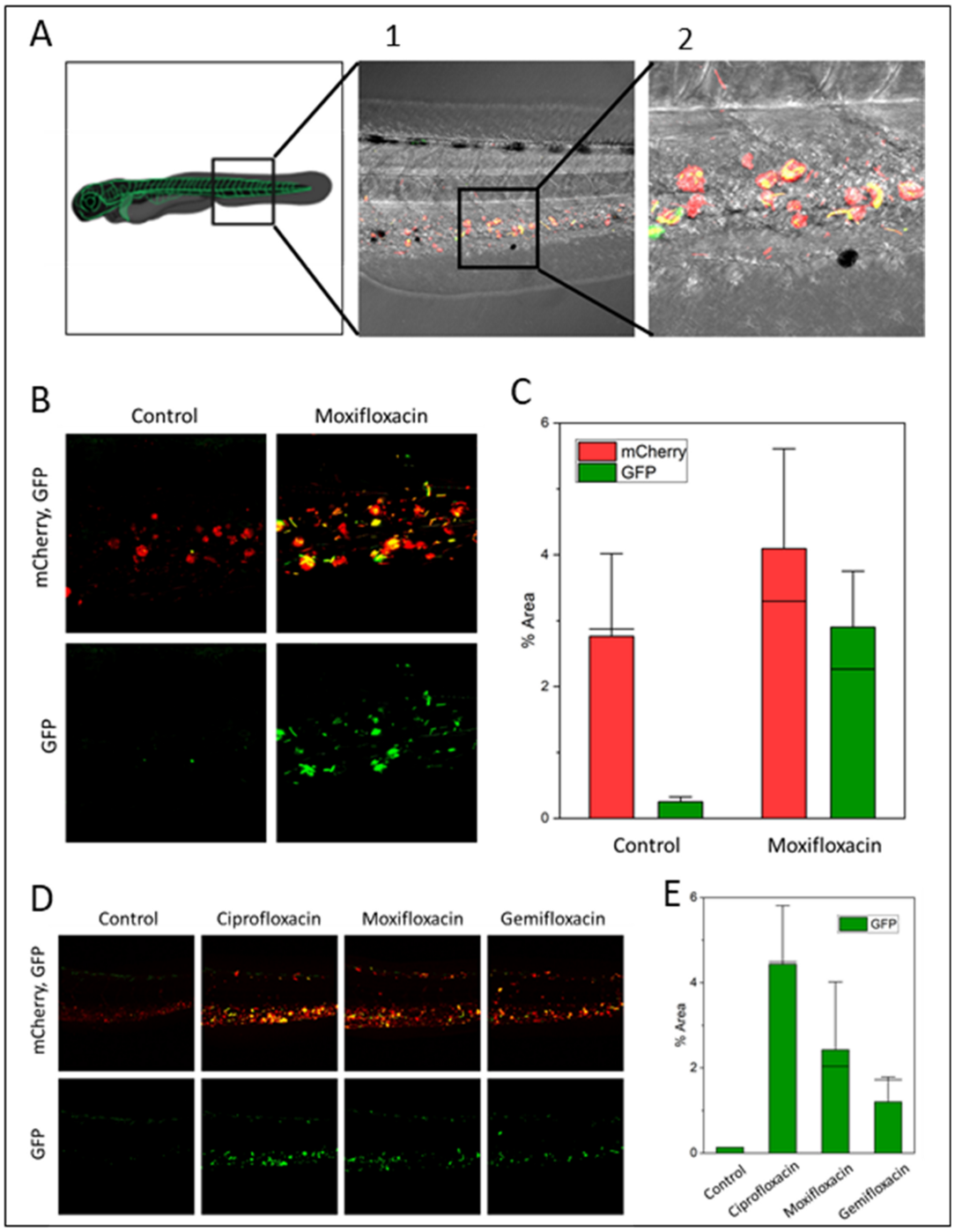

Fig. 3. The effect of gyrase inhibitors on SOS-DNA damage-reporter *Salmonella* within *Salmonella* containing vacuoles (SCVs). ZFL were injected i.v. with SOS-DNA damage-reporter *Salmonella* (300 CFU) and incubated at 35 °C. Reporter *Salmonellae* constantively express *mCherry*, with an increased *mCherry* expression after DNA damage. *gfp* is exclusively expressed after DNA damage. (A) Sites of imaging. Imaging of (B): A2 and of (D): A1. (B) *GFP* and *mCherry* fluorescence (DNA damage response within SCVs) 4 h after moxifloxacin injection. Ninety min after *Salmonella* injection, fish were injected i.v. with either 600 pg tobramycin only or 600 pg tobramycin and 600 pg moxifloxacin. Confocal images were taken 4 h after antibiotic injection. (C) Percentages of areas with *GFP* or *mCherry* expression, respectively, 4 h after antibiotic injection. (D) *GFP* and *mCherry* expression (DNA damage response within SCVs) 1 day after gyrase inhibitor treatment. 90 min after *Salmonella* injection, fish were injected i.v. with 600 pg tobramycin to eradicate free *Salmonella* outside of SCVs. Ninety min after tobramycin injection, ZFL received either no second treatment (control), or a second injection of either ciprofloxacin, moxifloxacin, or gemifloxacin (600 pg per antibiotic per fish). Confocal images were taken 1 day post *Salmonella* injection. (E) Percentages of total areas with *GFP* signal 1 day after gyrase inhibitor treatment. (B) and (D): Green: *GFP* (*cda* promoter), red: *mCherry* (*recA* promoter). (C) and (E): Bars: average; horizontal line: median; whiskers: SE; $n \geq 3$.

3.3 DNA-Damage-Reporter Model

We also tested a model using ZFE embryos infected with DNA-damage reporter *Salmonella*, in which genotoxic stress induces red and green fluorescence. These reporter *Salmonella* carry the following two promoter fusions responding to DNA damage on a pSC101 backbone: The Pcad promoter of colicin D fused to *gfp-ova* encoding an unstable variant of the green fluorescent protein and the PrecA promoter fused to *mCherry* [38–42]. PrecA has moderate activity even in absence of genotoxic stress resulting in baseline red fluorescence of the reporter strain, with an increasing *mCherry* expression and red fluorescence after genotoxic stress. A detectable *GFP* signal is expected to only appear after induction of a DNA damage response. Infection of ZFE with these bacteria without any additional treatment yielded detectable *mCherry* but no *GFP*signals, as expected (Fig. 2).

To validate the capability of this model to assess the *in vivo* efficiency of antibiotic compounds targeting bacterial DNA in- and outside host Macrophages, we tested the effect of 3 distinct fluroquinolones (which inhibit DNA gyrase and cause DNA damage), known to be effective against *Salmonella*, namely ciprofloxacin, moxifloxacin and gemifloxacin [53–55].

After administering all three of the tested fluoroquinolones, *GFP* and intensified *mCherry* fluorescence appeared, indicating that the antibiotics had reached their *Salmonella* targets (Fig. 2). To evaluate the effectiveness of gyrase inhibitors on intracellular *Salmonella*, extracellular *Salmonella* were eradicated by co-administering tobramycin. The remaining intracellular *Salmonella* still reported substantial DNA damage consistent with excellent cell penetration of the fluoroquinolones (Fig. 3).

4. Conclusions

Testing antimicrobials *in vivo* in rodents is resource consuming and raises ethical concerns. Efficacy data are usually end-point measurements only. ZFL are easy to generate in large numbers, and their transparency enables real-time monitoring of infections and treatment responses using fluorescence microscopy. We established suitable infection conditions and localized *Salmonella* inside macrophages using fluorescent zebrafish lines and fluorescent *Salmonella* strains. Using these methods, we could demonstrate differential access of tobramycin and ceftriaxone to intra- versus extracellular *Salmonella*. Using DNA damage-reporter, we could even directly monitor the action of fluoroquinolones on extra- and intracellular *Salmonella*. The results from this ZFL model were entirely consistent with the well-characterized permeability properties of the three antibiotics and their suitability for treating systemic *Salmonella* infections in human patients [56–59]. Even the effective doses of tobramycin were comparable to recommendations for extracellular infections in humans. Our ZFL model thus appeared to be suitable as an informative and predictive *in vivo* model for antimicrobial-activity testing against intra- and extracellular pathogens *in vivo*, closing the gap between *in vitro* assays and rodent models.

Availability of Data and Materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

PH, JB, DB, and JH designed the research study. PH, JB, RP, CLA, and SS performed the research. JF, BC, and DB provided help and advice on bacteria cultivation and the design of the infection models. PH, JB, and RP analyzed the data. PH, DB, and JH wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

All fish were kept in accordance with Swiss animal-welfare regulations [22,23].

Acknowledgment

We thank the group of Prof. Markus Affolter, Biozentrum, University of Basel, and particularly the group member Dr. Heinz-Georg Belting for providing us with zebrafish larvae and their valuable inputs. Illustrations were created with https://www.biorender.com/.

Funding

This research was funded by a grant (SCAHT-GL 21-09) from the Swiss Centre for Applied Human Toxicology – SCAHT and by the Swiss Nanoscience Institute (SNI-P1801).

Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/j.fbl2805099.

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