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TITLE:

Phage Therapy Application to Counteract *Pseudomonas aeruginosa* Infection in Cystic Fibrosis Zebrafish Embryos

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KEYWORDS:

bacteriophage, bacteria, zebrafish, cystic fibrosis, *Pseudomonas aeruginosa*, infection, phage therapy, immunity

SUMMARY:

Presented here is a protocol for *Pseudomonas aeruginosa* infection and phage therapy application in cystic fibrosis (CF) zebrafish embryos.

ABSTRACT:

Antimicrobial resistance, a major consequence of diagnostic uncertainty and antimicrobial overprescription, is an increasingly recognized cause of severe infections, complications, and mortality worldwide with a huge impact on our society and on the health system. In particular, patients with compromised immune systems or pre-existing and chronic pathologies, such as cystic fibrosis (CF), are subjected to frequent antibiotic treatments to control the infections with the appearance and diffusion of multidrug resistant isolates. Therefore, there is an urgent need to address alternative therapies to counteract bacterial infections and bacteriophages. Use of bacteriophages, the natural enemies of bacteria, can be a possible solution. The protocol detailed in this work describes the application of phage therapy against *Pseudomonas aeruginosa* infection in cystic fibrosis (CF) zebrafish embryos. Zebrafish embryos were infected with *P. aeruginosa* to demonstrate that phage therapy is effective against *P. aeruginosa* infections as it reduces lethality, bacterial burden and pro-inflammatory immune response in CF embryos.

INTRODUCTION:

Phage therapy, the use of the natural enemies of bacteria to fight bacterial infections, is garnering renewed interest as bacterial resistance to antibiotics becomes widespread[1,2]. This therapy, used for decades in Eastern Europe, could be considered a complementary treatment to antibiotics in curing lung infections in patients with CF and a possible therapeutic alternative for patients infected with bacteria that are resistant to all the currently in use antibiotics[2,3]. Advantages of antibiotic therapy are that bacteriophages multiply at the infection site, whereas antibiotics are metabolized and eliminated from the body[4,5]. Indeed, the administration of cocktails of virulent phages isolated in different laboratories has proven to be effective in treating *Pseudomonas aeruginosa* infections in animal models as different as insects and mammals[6–8]. Phage therapy was also shown to be able to reduce the bacterial burden in burn wounds infected with *P. aeruginosa* and *Escherichia coli* in a randomized clinical trial[9].

Zebrafish (*Danio rerio*) has recently emerged as a valuable model to study infections with several pathogens, including *P. aeruginosa*[10,11], *Mycobacterium abscessus* and *Burkolderia cepacia*[12,13]. By microinjecting bacteria directly into the embryo blood circulation[14] it is easy to establish a systemic infection that is counteracted by the zebrafish innate immune system, which is evolutionary conserved with neutrophils and macrophage generation similar to the human counterpart. Moreover, during the first month of life, zebrafish embryos lack the adaptive immune response, making them ideal models for studying the innate immunity, which is the critical defense mechanism in human lung infections[15]. Zebrafish recently emerged as a powerful genetic model system to better understand the CF onset and to develop new pharmacological treatments[10, 16-17]. The CF zebrafish model of *cftr* knock-down generated with morpholino injection in zebrafish presented a dampened respiratory burst response and reduced neutrophil migration[10], while the *cftr* knock-out leads to impaired internal organ position and the destruction of the exocrine pancreas, a phenotype that mirrors human disease[16,17]. Of greatest interest was the finding that the *P. aeruginosa* bacterial burden was significantly higher in *cftr*-loss-of-function embryos than in controls at 8 hours post-infection (hpi), which parallels the results obtained with mice and human bronchial epithelial cells[2,18].

In this work, we demonstrate that phage therapy is effective against *P. aeruginosa* infections in zebrafish embryos.

PROTOCOL:

Adult zebrafish (*Danio rerio*) from the AB strain (European Zebrafish Resource Center EZRC) are maintained according to international (EU Directive 2010/63/EU) and national guidelines (Italian decree 4th March 2014, n. 26) on the protection of animals used for scientific purposes. Standard conditions are set in the fish facility with a 14 h of light/10 h dark cycle and tank water temperature at 28° C.

1. Preparation of solutions and tools

1.1. Prepare a 50x stock solution and 1x working solutions for E3 embryo medium for growing zebrafish embryos (see **Table 1**).

1.2. Make pigmentation blocking solution, to block the embryo pigmentation 24 hours post fertilization (24 hpf) (see **Table 1**).

1.3. Prepare 25x stock and 1x working Tricane anesthetic solution as described in **Table 1**.

NOTE: To avoid the repeated freezing and thawing of the solution that could damage the stock solution, make aliquots of 2 mL of 25x tricaine and store them at -20 °C.

1.4. Prepare tracks for embryo microinjection by dissolving 1 g of agarose in 100 mL of distilled H_2O in a microwavable flask or bottle. Microwave for 1-3 min until the agarose is completely dissolved.

1.5. Position zebrafish microinjection molds (see **Table of Materials**) turned upside down in a Petri dish (90 mm x 15 mm) and cover the entire surface by pouring the liquid agarose gel with a width of approximately 10 mm.

1.6. Remove the mold once the agarose has solidified. Fill the Petri dish with 1x E3 embryo medium. This can be stored at 4 °C for approximately one week. Before use, replace with the fresh E3 medium containing Tricaine.

NOTE: Older molds might develop fungi or bacterial contaminations.

1.7. Use fire polished 10 cm borosilicate capillaries to prepare needles for microinjection and secure them to the micropipette puller by tightening the handles. Set the puller with the following settings: heat 500, velocity 100, time 150, pull 150.

2. *P. aeruginosa* (PAO1) inoculum preparation

2.1. Inoculate 1 colony of GFP^+ *P. aeruginosa* strain PAO1[19] in 5 mL of LB broth (see **Table 1**) and grow overnight at 37 °C with shaking (200 rpm).

2.2. Dilute the above overnight culture to OD_{600} = 0.1 in 10 mL of LB broth and grow to OD_{600} = 0.5 at 37 °C with shaking (200 rpm).

2.3. Centrifuge 2 mL of the culture for 2 min at 4 °C at 16,100 *x g* and resuspend the cell pellet to OD_{600} = 1, equivalent to about 1 x 10^9 cfu/mL, in 1 mL of the physiological solution (see **Table 1**).

2.4. Dilute the cell suspension to about 5 x 10^7cfu/mL in physiological solution and store at 4 °C.

3. Phage stock preparation

3.1. Inoculate 1 colony of *P. aeruginosa* strain PAO1 in 5 mL of LB broth and incubate overnight at 37 °C with shaking. Dilute the overnight culture to OD_{600} = 0.01 in 500 mL of LB broth and grow at 37 °C with shaking to OD_{600} = 0.05, equivalent to about 2.5 x 10^7 cfu/mL.

3.2. To the diluted culture of *P. aeruginosa*, add about 1.25 x 10^7 phages, to get the multiplicity of infection (M.O.I.) of 10^{-3}. Incubate at 37 °C with shaking until the OD_{600} drops to about 0.1-0.3 (in about 3 to 5 h, depending on the phage used).

NOTE: Four phages were selected for this experiment that infect PAO1 strain: Two Podoviridae, GenBank accession numbers vB_PaeP_PYO2, MF490236, and vB_PaeP_DEV, MF490238, and two Myoviridae, vB_PaeM_E215, MF490241, and vB_PaeM_E217, MF490240. Perform the steps below for each phages individually.

3.3. Incubate the lysate with 1 μg/mL of DNase and RNase for 30 min at 37 °C. Centrifuge at 5,000 *x g* for 30 min at 4 °C and carefully recover the supernatant (SN). Filter the SN with a filter having 0.8 μm pore diameter.

3.2. To the SN, add 58 g/L of NaCl and 105 g/L of PEG6000. Dissolve with stirring at RT 20 min and then keep it overnight at 4 °C. Centrifuge at 20, 000 *x g* for 30 min at 4 °C for phage precipitation. Remove the SN and carefully dissolve the phage pellet in 15 mL of TN buffer (see **Table 1**).

3.3 Purify the phages with CsCl density gradient as described in steps below.

3.3.1. In a polyallomer ultracentrifuge tubes for SW41 rotor, prepare a CsCl step density gradient by stratifying 2 mL of the four CsCl solutions described in **Table 1**. Add 3.5 mL of phage suspension in each of the 4 tubes.

NOTE: CsCl is toxic, adopt proper safety procedures to handle and discard it.

3.3.2. Introduce the tubes into the rotor and ensure that the tubes are balanced (the weight difference between the two facing tubes must be ≤ 0.01g).

3.3.3. Centrifuge for 2 h at 4 °C and 100, 000 *x g* (25,000 rpm for SW41 rotor). After centrifugation, slowly remove the tubes and carefully immobilize them on a support. Using a syringe with 19 G needle, draw up the white/opalescent band that is located usually between the d=1.5 and the d=1.4 density region.

3.3.4. Transfer the suspensions from the syringe into new polyallomer tubes for SW60 rotor and use the d=1.4 solution to precisely balance the tubes.

3.3.5 Centrifuge the suspension at 4 °C and 150,0000 *x g* (38,000 rpm for the SW60 rotor) for at least 16 h.

3.3.6 Collect the visible band as above (see step 3.3.3) and transfer them into dialysis tubes with 6,000 Da cut-off. Dialyze the obtained visible band 2x for 20 min against 500 mL of water and overnight against 500 mL of TN buffer. Filter with a 0.22 µm pore filter and store at 4 °C.

4. Phage cocktail preparation

4.1. Make a mix of four phages that infect the strain, PAO1, previously isolated and characterized[8]. Before mixing, estimate the titer of each phage stock by plaque assay[20].

4.2. Assemble the phage cocktail, with an overall titer of 5×10^8 pfu/mL, by equally mixing four phage preparations at the same pfu/mL immediately before each experiment, to ensure accurate phage titers.

5. Collection and preparation of zebrafish embryos for *cftr* morpholinos microinjection

NOTE: Collect 1-2 cell embryos from wild type zebrafish for *cftr* morpholinos (*cftr*-MOs) microinjection.

5.1. Two days before the bacterial microinjection experiment, set up breeding pairs and collect embryos as described previously[21]. Adult zebrafish (*Danio rerio*) of the AB strain were purchased from the European Zebrafish Resource Center, EZRC.

5.2. The day after, collect the embryos immediately after fertilization with a plastic pipette or using a fine-mesh strainer. Place the collected embryos in a Petri dish containing E3 embryo medium and carefully remove the debris with a plastic pipette to avoid contamination that might damage the embryo development.

5.3. Prepare 5 µL of morpholino injection solution by diluting the two morpholino stock solutions (1 mM) in sterile water to a final concentration of 0.25 pmol/embryo ATG-MO + 0.25 pmol/embryo splice-MO. To trace the embryo injection, add 0.5 µL of phenol red to the morpholino injection solution mix.

5.4. Load a microinjection needle with approximately 5 µL of the morpholino mix solution using a 20 µL micropipette with a fine gel loading tip. Secure the needle to the micromanipulator connected to a stand and position it under a stereomicroscope.

NOTE: It is not necessary that the solution reaches the tip of the needle as the pressure of the microinjector pump will push it.

5.5. Clip off the tip of the needle by cutting the needle tip with fine sterile tweezers. Measure the diameter of the drop using a scale bar in the ocular of the microscope. Alternatively, create a drop of mineral oil onto a micrometer slide and set the microinjection volume by injecting the

solution into the oil-drop to evaluate the droplet size. Use a drop with a 156 µm diameter to inject a volume of 2 nL.

5.6. Set the microinjector by adjusting the compensation pressure to 15 hPa, injection time to 0.5 s, and injection pressure between 300 and 600 hPa to obtain the correct injection volume depending on the needle used.

5.7. With a plastic pipette arrange the embryos from step 5.2 onto a glass positioned in a 96 mm diameter Petri dish.

NOTE: Too much E3 medium will prevent the proper penetration of the microinjection needle into the chorion of the embryos.

5.8. Penetrate the chorion and then the yolk with the needle to inject 2 nL into the embryo as described previously[22].

5.9. Place injected embryos into a Petri dish with E3 medium and put them in an incubator at 28 °C to let them develop until the day after.

6. Microinjection of zebrafish embryos with bacteria and phage cocktail

NOTE: To perform a systemic infection, the embryo must have blood circulation that usually starts after 26 hpf.

6.1. After 26 hpf (for zebrafish developmental stages refer to Kimmel[21]) dechorionate embryos with pronase solution prepared by dissolving pronase powder in E3 medium at a concentration of 2 mg/mL. Gently pipette the embryos to break the chorion. Discard the pronase/E3 medium and rinse the dish several times with fresh E3 to remove all pronase.

6.2. Anesthetize zebrafish embryos in E3 medium containing Tricaine approximately 5 min prior to injections.

6.3. Pipette the anesthesized embryos into the track prepared in step 1. Use a fine gel loading tip to line the embryos in the lateral position.

6.4. Load a microinjection needle with approximately 5 µL of the *P. aeruginosa* preparation as described in part 5.

6.5. Insert the needle dorsally to the starting point of the duct of Cuvier where it starts spreading over the yolk sac. Inject a volume of 1-3 nL and ensure that the volume expands directly within the duct and enters into the circulation.

6.6. Approximately after every 50 injected-embryo, inject a drop of the bacteria into a 1.5 mL centrifuge tube with 100 µL of sterile PBS to check if the injection volume remains the same. Plate the inoculum on LB agar (see **Table 1**) at 37 °C overnight to assess the CFU.

6.7. Transfer the microinjected embryos in two clean Petri dishes with fresh E3 medium + PTU and incubate them at 28 °C.

6.8. At two selected time points: 30 min or 3 h after the bacterial injection, take out one of the Petri dishes with bacterial-injected embryos from the incubator and align them in the track as described in 6.4 for the phage cocktail injection.

6.9. Load a microinjection needle with approximately 5 µL of the phage cocktail (prepared in step 4) with a fine gel loading tip and fix it to the microinjector (see step 5).

6.10. Inject the phage cocktail in the duct of Cuvier of the embryos previously injected with bacteria.

6.11. Transfer the microinjected embryos in two clean Petri dishes with fresh E3 medium + PTU and incubate them at 28 °C.

7. Evaluation of the bacterial burden of embryos injected with PAO1 and phages

7.1. At 8 hpi, pipette 15 anesthetized embryos from the Petri dish to a 1.5 mL centrifuge tube.

7.2. Replace the anesthetic solution with 300 µL of 1% Triton X-100 in PBS (PBSTritonX) and homogenize the embryos by passing them at least 15x through an insulin syringe with a sterile 27-G needle.

7.3. Prepare serial dilutions of homogenates in sterile PBS by transferring 100 µL of the diluted homogenate into 900 µL of sterile PBS.

7.4. To select for the naturally amp-resistant PAO1 strain, plate 100 µL of the dilutions on LB agar added with ampicillin (100 µg/mL), and incubate at 37 °C over-night.

7.5. The day after, count the number of colonies, multiply by the dilution factor to determine the total number of CFU, and divide by the number of embryos homogenized to obtain the average number of CFU/infected embryo.

8. Evaluation of the lethality of embryos injected with PAO1 and phages

8.1. To evaluate the lethality of the PAO1 infection, score the injected embryos at 20 hpi under a stereomicroscope and count for the dead embryos (white/not transparent).

8.2. Calculate the half-maximal lethal concentration 50 (LD50) dose of PAO1 that determines the death of 50% of injected embryos at 20 hpf.

9. Embryo preparation for stereomicroscope time-lapse imaging of GFP$^+$ PAO1 infection

9.1. Preparate the mold for live-embryo imaging by dissolving 1.5% low melting agarose in 100 mL of E3 solution.

9.2. Add 1% Tricaine (see **Table 1**) to anesthetize the embryos.

9.3. At 4 hpi transfer one embryo with a plastic pipette in a glass bottom dish and add the warm low-melting-point agarose solution.

9.4. Position the glass bottom dish under a stereomicroscope and with a tip position the embryo in the desired orientation. Use a plastic pipette to carefully add a drop of agarose on the embryo.

9.5. Let the agarose cool for 5-10 min and gently fill the glass bottom dish with E3 containing the anesthetic solution to keep the embryo moist.

9.6. Place the glass bottom dish with the embryo under the fluorescent stereomicroscope with a fluorescent filter (channel 488 nm) for GFP$^+$ bacteria. Do not remove the Petri dish until further acquisitions with the same parameters at 9, 14 and 18 hpi.

10. Expression analyses of pro-inflammatory cytokines

10.1. At 20 hpi anesthetize the embryos with tricaine 1x solution and transfer them from the Petri dish to a 1.5 mL centrifuge tube.

10.2. Under a fume hood remove the anesthetic solution and replace with 200 µL of guanidium hydrochloride reagent.

10.3. Homogenize embryos by pipetting them repetitively through a 200 µL pipette first and then at least 15x with an insulin syringe with a sterile 27 G needle.

10.4. Extract total RNA from homogenized zebrafish embryos using a commercially available guanidium hydrochloride reagents as per the manufacturer's instructions.

10.5. Treat 1 µg of each sample of RNA with 2 µL of 1U/µL of DNase I (RNase-free), following the manufacturer's instructions.

10.6. Use 1 µg of DNase I treated RNA for reverse-transcription reaction (RT) using commercially available kit (see **Table of Materials**), in a total volume of 20 µL according to manufacturer's instructions.

10.7. Perform RT qPCR reaction in a total volume of 10 µL containing 1x SYBR Green mastermix using 1.5 µL of a 1:6 dilution of RT reaction. For normalization purposes, use the primers for the house-keeping genes *rpl8* and for pro-inflammatory cytokines, use primers for *TNF-α* and *IL-1β* (see **Table 2**). qPCR protocol was: cycle 1 step 1: 95 °C, 2 min, repeat once; cycle 2: step 1 95 °C, 10 s, step 2 55 °C, 30 s, repeat 40x; cycle 3: step 1 72 °C, 15 min.

REPRESENTATIVE RESULTS:

Results and figures presented here are referred to CF embryos generated through the injection of *cftr* morpholinos as described previously[10] and in step 5. To validate the CF phenotype, the impaired position of internal organs such as heart, liver, and pancreas as previously described[17] (**Figure 1**) were considered. Similar results were obtained in case of the WT embryos as reported in our previous publication[19].

Bacterial burden was reduced by phage therapy in CF embryos infected with PAO1. Furthermore, we evaluated the bacterial burden at 8 hpi by homogenizing groups of 15 embryos: the average number of bacteria (cfu/embryo) present in the PAO1 infected embryos was reduced to about 20% after phage administration treatment, thus confirming a less severe infection in the presence of the phage cocktail (**Figure 2**).

Lethality was reduced by phage therapy in CF embryos infected with GFP^+ bacteria PAO1. CF zebrafish embryos at 48 hpf were injected with GFP^+ bacteria of the PAO1 strain at a dose that caused 50% lethality after 20 hpi (30 cfu/embryo, **Figure 3A**). The site of injection was the yolk or the Duct of Cuvier to generate a systemic infection. Phage therapy against PAO1 infection was tested by injecting 2 nL of the equally mixed phage cocktail (300-500 pfu/embryo). The injection was performed at two different time points: 30 min (early) and 7 hours (late) after bacterial injection. In both cases, lethality was reduced at 20 hpi, indicating that phage therapy is effective (**Figure 3B**).

Time lapse analyses of bacterial progression in CF embryos infected with GFP^+ PAO1 and bacterial burden reduction following phage therapy. With live imaging, using a fluorescent stereomicroscope, we also followed the progression of the infection in CF embryos injected with GFP^+ PAO1 and showed the efficacy of phage therapy in reducing the spread of fluorescent bacteria over the yolk sac. The CF+PAO1 injected embryo with GFP+ bacteria multiplication at 4, 9, 14 and 18 hpi is shown in the upper side of **Figure 4**, whereas the CF+PAO1+phages embryo with reduced fluorescence due to phage action against bacteria is shown in the bottom part (**Figure 4**).

Phage therapy reduced the inflammatory response generated by PAO1 infection in CF embryos. We, also, evaluated the immune response generated by PAO1 and PAO1 + phages injection at 8 hpi. As expected, the expression of the pro-inflammatory cytokines TNF-α and IL-1β analyzed by qPCR techniques was significantly increased following PAO1 injection in comparison to controls, while it was reduced with the co-injection of the phage cocktail (**Figure 5A,B**).

FIGURE LEGENDS:

Figure 1: Generation and validation of CF embryos upon *cftr* morpholinos (*cftr*-MOs) injection. (**A**) Impaired position and looping of the heart in CF injected embryos in comparison to wild-type (WT) embryos. Heart is visualized with *cmlc2* expression by in situ hybridization technique**. (B)** Impaired position of the liver (arrows) and pancreas in CF embryos in comparison to WT. Liver and pancreas are visualized with *prox1a* expression by in situ hybridization techniques. Scale bars indicate 100 μm. liv: liver; p: pancreas.

Figure 2: Bacterial burden in CF embryos infected with PAO1 or PAO1+phages. The relative percentage of cfu/embryo in PAO1+phages vs PAO1 embryos are given. The mean and SD of three independent experiments is reported. The figure is reprinted from [19].

Figure 3: Lethality of CF zebrafish embryos infected with PAO1 and with PAO1+phages. (**A**) Determination of LD50 in 48 hpf zebrafish embryos microinjected with *cftr*-MO at 1-cell stage (CF embryos) and infected at 48 hpf with 2 nL of a culture of PAO1 containing increasing number of bacteria (cfu/embryo). Lethality of the embryos was observed at 20 hpi. (**B**) Lethality at 20 hpi of CF embryos infected with PAO1 at 48 hpf and treated with the phage cocktail (PAO1+ ϕ). The mean and SD reported are from six and four experiments, respectively, each with 25-40 embryos. Angular transformation was applied to the percentage of lethality and one-way ANOVA followed by Duncan's test was used. The figure is reprinted from [19].

Figure 4: Imaging of the efficacy of phage therapy in zebrafish. Progression of the infection in CF embryos following PAO1 injection (upper embryo) and efficacy of the phage therapy in PAO1+phages injected embryos (bottom embryo) at 4, 9, 14 and 18 hpi. Scale bar indicates 100 microns. The figure is reprinted from [19].

Figure 5: Expression of pro- and anti-inflammatory cytokines following PAO1 and PAO+phage administration. Expression levels of the TNF-α (A) and IL-1β genes measured by RT-qPCR at 8 hpi in CF embryos injected with PAO1 and PAO1+ϕ at 48 hpf and normalized using the expression of *rpl8*. The mean and SD of four experiments are reported. Statistical significance was assessed by ANOVA followed by Duncan's test: for TNF- α (CF) vs (CF+PAO1) p = 0.015*; (CF) vs (CF+PAO1+ϕ) p = 0.019*; (CF+PAO1) vs (CF+PAO1+ ϕ) p = 0.77 n.s.; for IL-1β (CF) vs (CF+PAO1) p = 0.00014***; (CF) vs (CF+PAO1+ ϕ) p = 0.00068***; (CF+PAO1) vs (CF+PAO1+ ϕ) p = 0.031*. The figure is reprinted from [19].

Table 1: Preparation of solutions.

Table 2: Primers used for RT-qPCR.

DISCUSSION:

In this manuscript, we described the protocol to perform *P. aeruginosa* (PAO1) infection in

zebrafish embryos and how to apply phage therapy with a cocktail of phages previously identified as able to infect PAO1 to resolve it. The use of bacteriophages as an alternative to antibiotic treatments has been of increasing interest since the last few years. This is mainly due to the diffusion of multi-drug resistant (MDR) bacterial infections, which constitute a serious issue for public health. Of course, the scope of this work is limited to the application of phage therapy to an animal model and not to humans. However, we generated a cystic fibrosis zebrafish model with the injection of a morpholino targeting the *cftr* gene, demonstrating the efficacy of phage therapy also in a pathogenetic model particularly susceptible to *P.aeruginosa* infection.

It is to note that we could easily achieve phage therapy, as in a previous work, we isolated and characterized different phages able to infect *P. aeruginosa* both in vitro and in vivo[8]. This step is fundamental to obtain the antimicrobial activity of the phages. The isolation and characterization of phages able to infect a specific bacterial strain are critical steps of phage therapy application. Indeed, to avoid adverse effects of phages on the animal/human host, it is necessary to use lytic phages instead of those lysogenic. Most importantly, it is necessary to check the phage genomes for the presence of harmful genes as those for antibiotic resistance, virulence and gene transfer.

Phage therapy has already been successfully used in other animal models. We are conscious that zebrafish is not a mammalian model and some effects of phages might be different. However, since zebrafish possess an innate immune system comparable to the human one with a conserved population of neutrophils and macrophages[23], we speculate that the data on the immune response could be reproducible in humans. Moreover, zebrafish is well assessed for bacterial infection studies, its use as a tester for phage therapy efficacy might be promising for therapeutic approaches. Indeed, the infection can be systemic if bacteria are injected into the circulation through the Duct of Cuvier, or localized as reported[17]. We performed both systemic and localized infections and demonstrated that they similarly generated increased lethality and bacterial burden that were both decreased following phage therapy application. Moreover, since GFP^+ fluorescent PAO1 bacteria was injected, it was easy to follow the infection at different time points of embryo development confirming the reduction of fluorescent bacteria when phages were injected.

To our knowledge, this is the first description of phage therapy application in zebrafish, with the added value for the demonstration of phages antimicrobial activity against *P. aeruginosa* in a CF background, that is particularly susceptible to this bacterial infection.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

Click here to access/download;Figure;Figure 1_V2 jove.tif

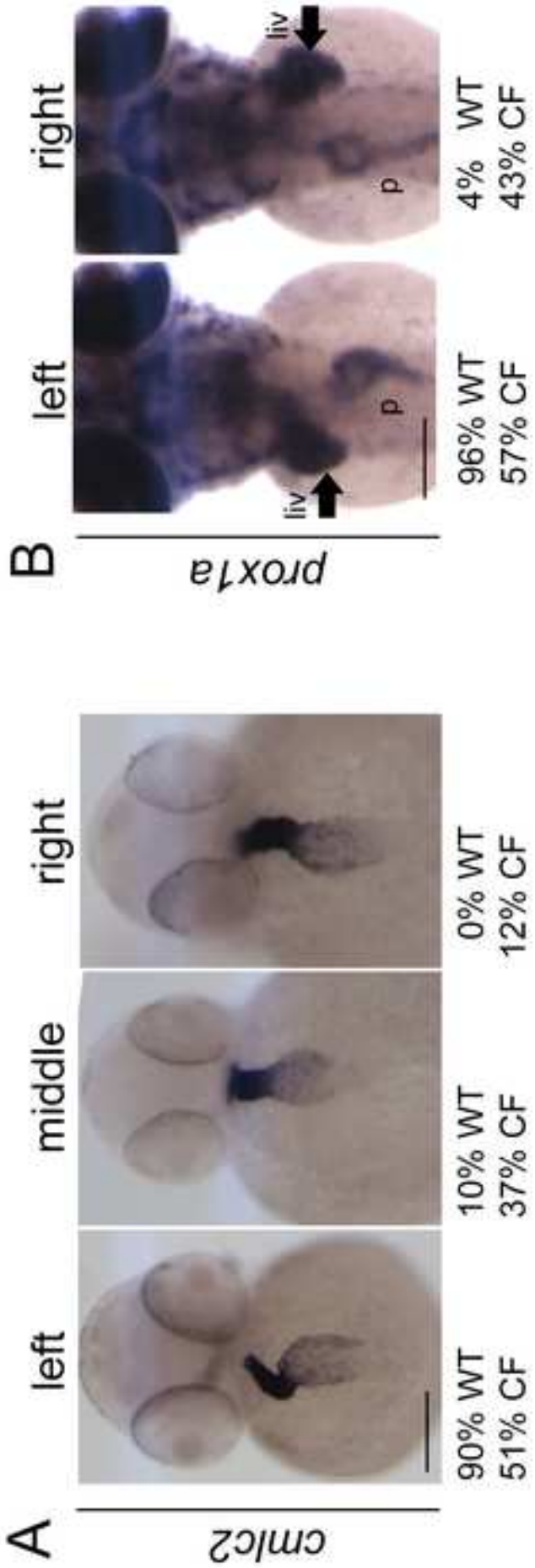

Figure 2

Click here to access/download;Figure;Figure 2_V2 jove (2).tif

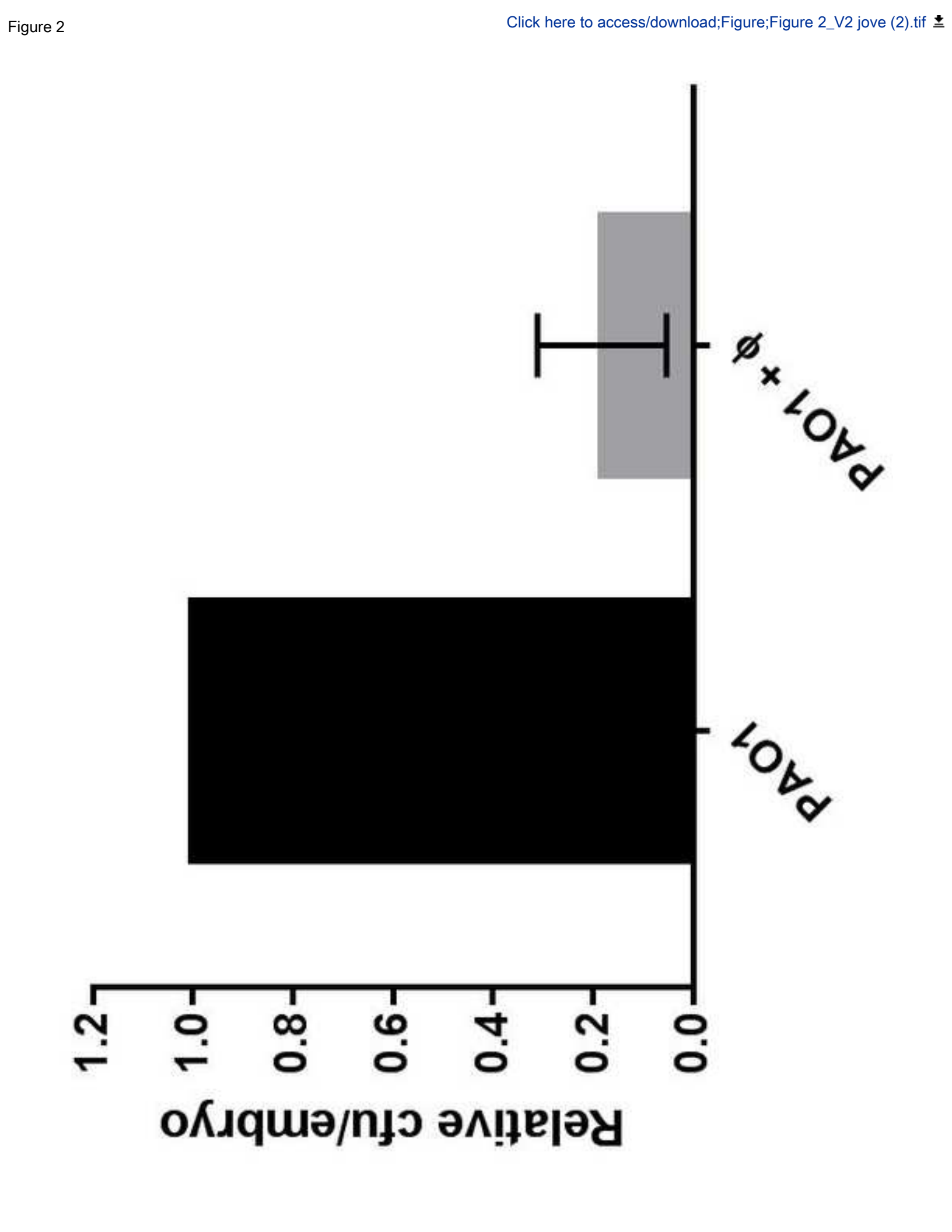

Figure 3

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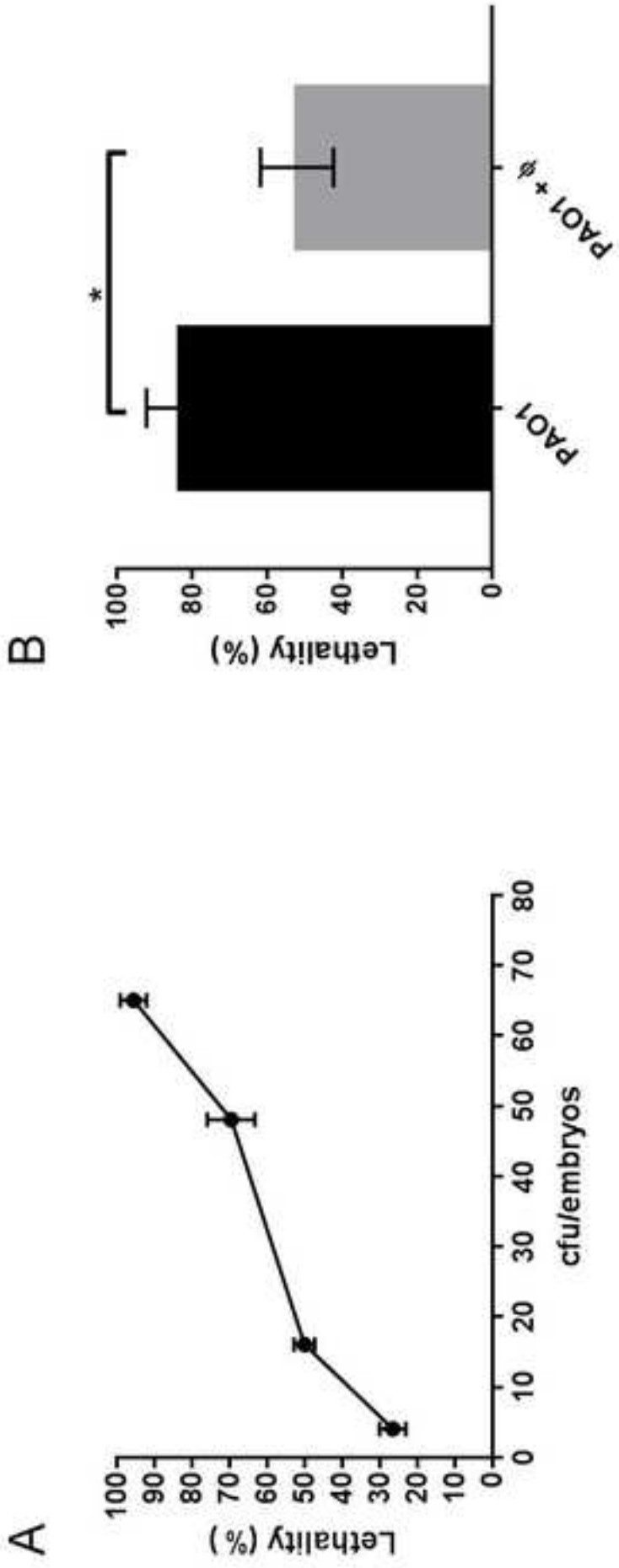

Figure 4

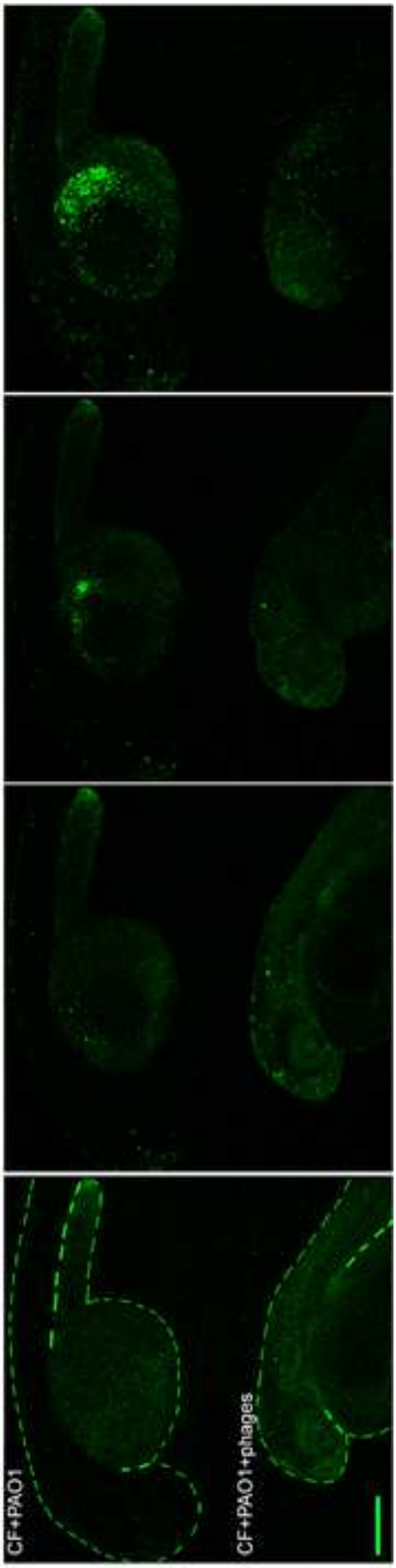

Figure 5

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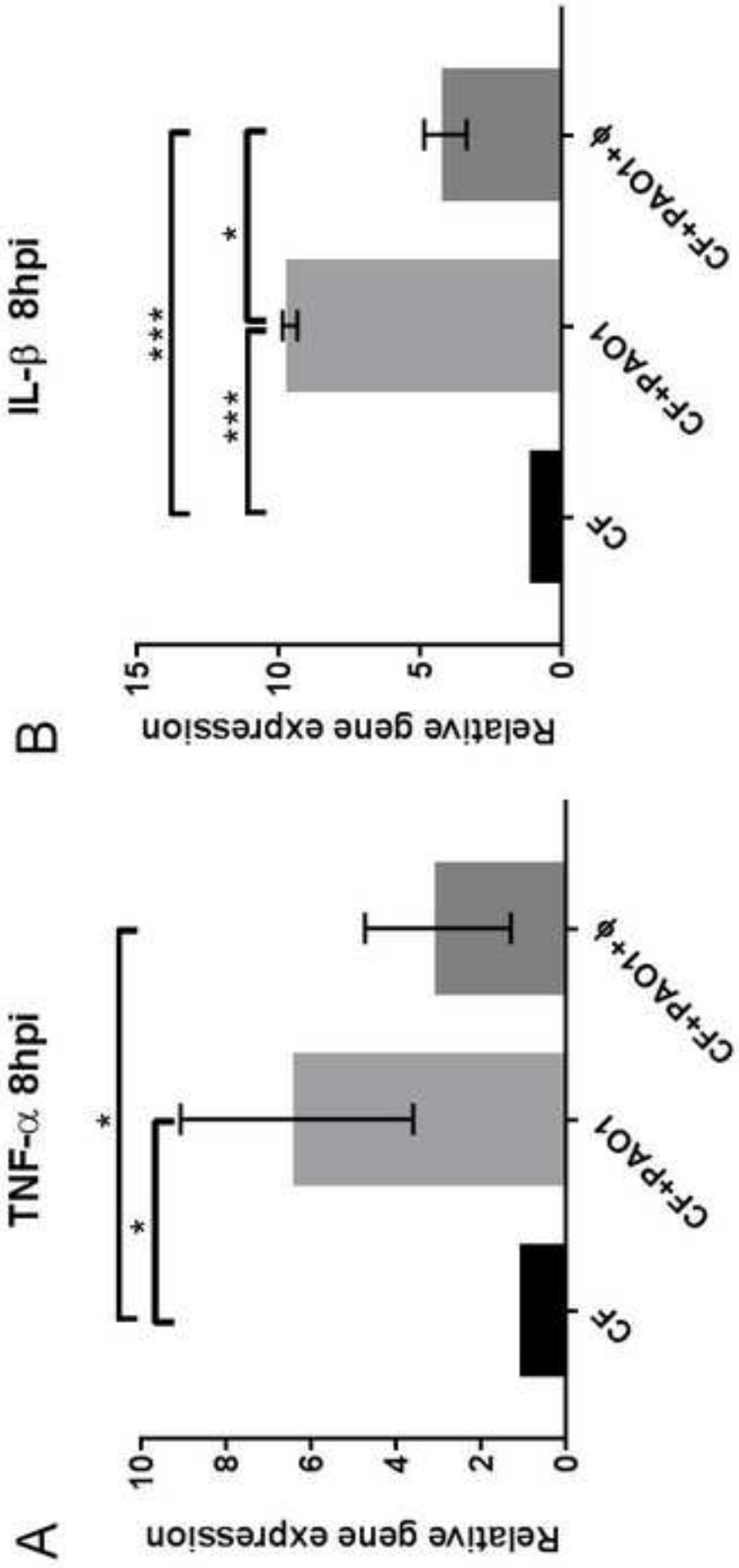

Table 1

Solutions

Anaesthetic stock solution 25X

Anaesthetic working solution 1X

CsCl d=1.3

CsCl d=1.4

CsCl d=1.5

CsCl d=1.6

E3 embryo medium for zebrafish embryo

E3 embryo medium stock solution (50X)

LB agar

LB broth

PBST

Physiological solution

Pigmentation blocking stock solution 10X

Pronase stock solution 5X

TN buffer

Preparation

4 mg/mL of Tricaine in distilled H_2O.

dilute in distilled H_2O the Tricaine stock solution 25X preparation to reach the 1X concentration (0.16 mg/m

20.49 g in 50 mL TN

20.28 g in 50 mL TN

34.13 g in 50 mL TN

41.2 g in 50 mL of TN

1 L 1of E3 (dilute the 50X stock with distilled H_2O) + 200 µl of 0.05% methyl blue . Store at RT.

73.0 g NaCl, 3.15 g KCl , 9.15 g $CaCl_2$, and 9.95 g $MgSO_4$ in 5 L of distilled H_2O. Store at RT.

10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 10 g/L agar

for 1L: 950 mL H_2O, 10 g Tryptone, 10 g NaCl, 5 g Yeast extract

PBS 1X + Triton X 1%

0.9% NaCl

0.3 mg/mL phenyl thiourea (PTU) powder in E3 embryo medium for zebrafish embryo

5 mg/mL pronase powder in E3 embryo medium for zebrafish embryo

10 mM Tris HCl pH 8.0, 150 mM NaCl

L) Tricaine of distilled H_2O.

Table 2

Gene name	Primer sequence
TNF-alpha Fw	5’-TGCTTCACGCTCCATAAGACC-3’
TNFalpha Rev	5’-CAAGCCACCTGAAGAAAAGG-3’
IL1-beta Fw	5’-TGGACTTCGCAGCACAAAATG-3’
IL1-beta Rev	5’-CGTTCACTTCACGCTCTTGGATG-3’
rpl8 Fw	5’-CTCCGTCTTCAAAGCCCAT-3’
rpl8 Rev	5’-TCCTTCACGATCCCCTTGAT-3’

Table of Materials

Name of Material/ Equipment	Company	Catalog Number
Bacto Agar	BD	214010
Calcium chloride	Sigma-Aldrich	10043-52-4
CsCl	Sigma-Aldrich	289329
Dulbecco's phospate buffered saline PBS	Sigma-Aldrich	D8537
Ethyl 3-aminobenzoate methanesulfonate	Sigma-Aldrich	886-86-2
Femtojet Micromanipulator	Eppendorf	5247
Fleming/brown P-97	Sutter Instrument Company	P-97
LE-Agarose	Sigma-Aldrich	11685660001
Low Melting Agarose	Sigma-Aldrich	CAS 9012-36-6
Magnesium sulfate	Sigma-Aldrich	7487-88-9
Methyl Blue	Sigma-Aldrich	28983-56-4
Microinjection needles	Harvard apparatus	
N-Phenylthiourea >=98%	Aldrich-P7629	103-85-5
Oligo Morpholino	Gene Tools	
PEG6000	Calbiochem	528877
Phenol Red Solution	Sigma-Aldrich	CAS 143-74-B
Potassium chloride	Sigma-Aldrich	7447-40-7
Pronase	Sigma-Aldrich	9036-06-0
Sodium chloride ACS reagent, ≥99.0%	Sigma-Aldrich	S9888
Stereomicroscope	Leica	S9I
Tris HCl	Sigma-Aldrich	T5941
Triton X	Sigma-Aldrich	T9284
Tryptone	Oxoid	LP0042B
Yeast extract	Oxoid	LP0021B
Z-MOLDS Microinjection	Word Precision Instruments	

Comments/Description

common name tricaine

designed by the researcher

Point by point rebuttal V1

1) The manuscript needs a thorough proofreading.
We carefully checked the entire manuscript

2) Please check authors
We checked for the authors affiliations

3) Wild type results not shown.
We modified accordingly

4) We cannot have paragraphs of text in the protocol section.
We moved the paragraph concerning fish strain in the introduction

5) Only Protocol section needs 2.75 pages of highlights. The total length of the protocol can be 10 pages. Please highlight complete sentences thorughout.
We generated a filmable protocol of 2.75 pages

6) Please remove the redundancy and make the steps crisps and in the order or performing experiments.
We modified accordingly

7) Please ensure that there are no commercial terms in the text. Please use generic terms instead. All commercial terms must be moved to the Table of materials.
We checked for commercial terms

8) Please use complete sentences throughout. Please bring out the link between each section of the protocol so as to make a complete story.
We modified accordingly

9) we checked for all the parts moved into the text.

10) Drops to ?
We added the information

11) Please reword for clarity
We reworded all the sentences for clarity

12) Any specific amount/ number of phages to be added?
It is a quantity, 3.5 mL

13) From step 3.3.3? Significance of using this?
As explained before the band with the phages has a density between 1.5 and 1.4

14) Please check this? Is this 150, 000?
We corrected with 150.000

15) Please include the step number.
We included all the step numbers

16) How do you check this? Citations
We added the citation n. 23

17) So for the morpholino you use 1-2 cell days and bacterial/phage injection it is 26hpf? Is this the same which was injected with MO? Please bring out the clarity and the link between step 5 and 6.
We modified accordingly

18) Volume and concentration added?
We added the percentage of tricaine

19) Please include the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We modified accordingly