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To the Editors,

On behalf of my colleagues and me, I hereby submit our revised manuscript entitled '*Mycobacterium marinum* infection in the adult zebrafish as a model of human tuberculosis' to JoVE for consideration of publication. We have addressed the reviewers' concerns and proposals and we feel that this has improved and clarified the manuscript.

One quarter of the world's population has been estimated to be infected with *Mycobacterium tuberculosis*. According to the World Health Organization, tuberculosis caused 1.7 million deaths in 2016 and is the leading cause of death by a single pathogen worldwide. A total of 10.4 million tuberculosis cases were reported along with an increasing number of multidrug resistant strains (WHO, 2017). As the current prevention and treatment regimens have proven insufficient, new treatment strategies are needed.

M. tuberculosis infection includes a wide range of disease outcomes with different pathologies in humans. The variation in the infection outcomes caused by this bacterium is hard to model experimentally. *Mycobacterium marinum* causes a very similar disease spectrum in adult zebrafish. By using this natural fish-pathogen, it possible to study tuberculosis in a vertebrate model with both the functional innate and adaptive immune systems, which is not possible in the commonly used zebrafish larva, since they do not have a fully functional adaptive immunity. In this study, we show how adult wild-type and *rag-/-* mutant zebrafish are infected with an intraperitoneal *M. marinum* injection and how the mycobacterial loads are measured with qPCR. In addition, the expression of *il4* and *ifnγ* are analyzed with RT-qPCR 4 weeks post infection. The results of increasing mycobacterial loads and limited induction of *il4* in *rag-/-* mutant fish compared to wild-type fish emphasize the importance of adaptive immunity in the control of mycobacterial infection.

Our work provides a valuable platform for studying tuberculosis. The understanding of the mechanisms leading to different disease outcomes will benefit the scientific community in the search of new vaccines and treatment regimens against tuberculosis. We wish to thank the reviewers for their valuable comments that have helped us improving the study. We hope that the revised manuscript is now suitable for publication.

Thank you for your consideration.

Hanna Luukinen

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TITLE:

Modeling Tuberculosis in *Mycobacterium marinum* Infected Adult Zebrafish

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KEYWORDS:
Zebrafish; tuberculosis; *Mycobacterium marinum*; *Mycobacterium tuberculosis*; mycobacterial infection; qPCR; immune system

SUMMARY:

Here, we present a protocol to model human tuberculosis in an adult zebrafish using its natural pathogen *Mycobacterium marinum*. Extracted DNA and RNA from the internal organs of infected zebrafish can be used to reveal the total mycobacterial loads in the fish and the host's immune responses with qPCR.

ABSTRACT:

Mycobacterium tuberculosis is currently the deadliest human pathogen causing 1.7 million deaths and 10.4 million infections every year. Exposure to this bacterium causes a wide disease spectrum in humans ranging from a sterilized infection to an actively progressing deadly disease. The most common form is the latent tuberculosis, which is asymptomatic, but has the potential to reactivate into a fulminant disease. Adult zebrafish and its natural pathogen *Mycobacterium marinum* have recently proven to be an applicable model to study the wide disease spectrum of tuberculosis. Importantly, spontaneous latency and reactivation as well as adaptive immune responses in the context of mycobacterial infection can be studied in this model. In this article, we describe methods for the experimental infection of adult zebrafish, the collection of internal organs for the extraction of nucleic acids for the measurement of mycobacterial loads and host immune responses by quantitative PCR. The in-house-developed, *M. marinum*-specific qPCR assay is more sensitive than the traditional plating methods as it also detects DNA from non-dividing, dormant or recently dead mycobacteria. As both DNA and RNA are extracted from the same individual, it is possible to study the relationships between the diseased state, and the host and pathogen gene-expression. The adult zebrafish model for tuberculosis thus presents itself as a highly applicable, non-mammalian *in vivo* system to study host-pathogen interactions.

INTRODUCTION:

Zebrafish (*Danio rerio*) is a widely used animal model in biomedical research and it is an accepted model for common vertebrate biology. The zebrafish has been adapted to many fields of research modeling human diseases and disorders ranging from cancer[1] and cardiac disease[2] to infection and immunological studies of several bacterial [3] and viral infections[4,5]. In addition, the *ex utero* development of zebrafish embryos has made the zebrafish a popular model in developmental biology[6] and toxicology[7,8].

In many fields of research, including infection biology, the optically transparent zebrafish larvae are commonly used. The first immune cells appear within 24 hours post fertilization (hpf), when primitive macrophages are detected[9]. Neutrophils are the next immune cells to appear around 33 hpf[10]. Zebrafish larvae are thus feasible for studying the early stages of infection and the role of innate immunity in the absence of adaptive immune cells[11]. However, the adult zebrafish with its fully functional adaptive immune system provides an additional layer of complexity for infection experiments. T cells can be detected around 3 days post fertilization[12], and B cells are able to produce functional antibodies by 4 weeks post fertilization[13]. The adult zebrafish has all the main counterparts of the mammalian innate and adaptive immune system. The main differences between the immune systems of fish and humans are found in antibody isotypes as well as in the anatomy of lymphoid tissues. The zebrafish has only three antibody classes[14], whereas humans have five[15]. In the absence of bone marrow and lymph nodes, the primary lymphoid organs in the fish are the kidney and the thymus[16] and the spleen, the kidney and the gut serve as secondary lymphoid organs[17]. Despite these differences, with its full immune arsenal of innate and adaptive cells, the adult zebrafish is a highly applicable, easy-to-use, non-mammalian model for host-pathogen interaction studies.

The zebrafish has lately been established as a feasible model to study tuberculosis[18-22]. Tuberculosis is an airborne disease caused by *Mycobacterium tuberculosis*. According to the World Health Organization, tuberculosis caused 1.7 million deaths in 2016 and is the leading cause of death by a single pathogen worldwide[23]. Mice[24,25], rabbits[26] and non-human primates[27] are the best-known animal models in tuberculosis research but each face their limitations. The non-human primate model of *M. tuberculosis* infection resembles the human disease most closely, but using this model is limited due to serious ethical considerations. Other animal models are hindered by the host-specificity of *M. tuberculosis* that affects the disease pathology. Probably the biggest issue in modeling tuberculosis is the wide spectrum of infection and disease outcomes in the human disease: tuberculosis is a very heterogeneous disease ranging from sterilizing immunity to latent, active and reactivated infection[28], which can be hard to reproduce and model experimentally.

Mycobacterium marinum is a close relative of *M. tuberculosis* with ~3,000 orthologous proteins with 85% amino acid identity[29]. *M. marinum* naturally infects zebrafish producing granulomas, the hallmarks of tuberculosis, in its internal organs[19,30]. Unlike other animal models used in tuberculosis research, zebrafish produces many offspring, it requires only a limited space and importantly, it is neurophysiologically the least developed vertebrate tuberculosis model available. Additionally, the *M. marinum* infection causes latent infection, active disease or even sterilization of mycobacterial infection in adult zebrafish closely mimicking the spectrum of disease outcomes of human tuberculosis[19,31,32]. Here, we describe methods for the experimental tuberculosis model of adult zebrafish by injecting *M. marinum* into the abdominal cavity and using quantitative PCR to measure the mycobacterial loads and immune responses from zebrafish tissue samples.

PROTOCOL:

All zebrafish experiments have been approved by the Animal Experiment Board in Finland (ESAVI/8245/04.10.07/2015). Methods are performed according to the act (497/2013) and the government decree (564/2013) on the protection of animals used for scientific or educational purposes in Finland.

1. Culturing of *Mycobacterium marinum*

Note: Since *Mycobacterium marinum* is a pathogen capable of causing superficial infections in humans, find out the local guidelines for personal safety and biohazard waste disposal before starting to work with this bacterium.

1.1 Culture *M. marinum* on a 7H10 plate supplemented with OADC (oleic acid, albumin, dextrose, catalase) enrichment and 0.44% v/v of glycerol at 29 °C for at least 5 days. Maintain *M. marinum* cultures by taking fresh bacteria from the freezer every two weeks and transferring onto a new plate every other week.

Note: *M. marinum* is a natural fish pathogen infecting aquatic species and it is important to take precautions not to contaminate zebrafish stocks with the bacteria. Infected zebrafish and items contaminated with bacteria have to be kept separate from the breeding facilities.

1.2 Use a sterile 1-µL inoculation loop to aseptically transfer a loopful of *M. marinum* bacterial mass into a cell culture flask containing 10 mL of 7H9 medium with 10% ADC (albumin, dextrose, catalase) enrichment, 0.2% v/v polysorbate 80 and 0.17% v/v of glycerol. Culture for 3-4 days to approximately an OD_{600} (optical density) of 0.7 at 29 °C in the dark without shaking. Leave the cap loose to allow for the sufficient exchange of gases.

Note: Polysorbate 80 is added to the medium to prevent the aggregation of bacteria. In addition, exposure to light leads to phenotypic changes in bacterial colonies (*e.g.*, the color changes from white to yellow). To avoid this, keep the cultures in the dark.

1.3 Measure the OD_{600} value with a spectrophotometer. Dilute the liquid culture to an OD_{600} of 0.07-0.09 and continue culturing for 2 days at 29 °C in the dark without shaking. Leave the cap loose.

Note: During these two days, the bacterial suspension will reach an OD_{600} of approximately 0.5

corresponding to an early log-phase.

2. Preparation of Bacterial Solution for Infecting Adult Zebrafish

2.1 Transfer the bacterial suspension into a big sterile cuvette or a 15 mL tube and place it in the dark at room temperature for 15 min to allow the biggest clumps to settle.

2.2 Transfer the top 5–7 mL of the suspension into a clean tube or a cuvette and measure the OD_{600}. Use this top phase of the suspension for the infections.

2.3 Collect 1 mL of *M. marinum* culture into a fresh tube and centrifuge for 3 min at 10,000 x g. Remove the supernatant and resuspend the pellet in 1 mL of sterile 1x PBS.

2.4 Dilute to reach the desired bacterial concentration by using sterile 1x PBS with 0.3 mg/mL phenol red as a tracer. Divide the diluted suspension into three aliquots.

Note: Use a predetermined OD_{600} vs. CFU (colony-forming units)/µL curve to estimate the dilution required to get the wanted number of bacteria in the 5 µL injection volume[34]. The correlation between OD_{600} and concentration of the bacterial suspension needs to be validated before starting the actual infection experiments. Reserve two weeks for collecting this validation data.

2.5 Using a 1 mL syringe, slowly pull the suspension through a 27G needle 3x. For each aliquot, perform this step just before use.

Note: Do not use the same bacterial solution for more than 2 h.

3. Experimental *M. marinum* Infection with Intraperitoneal Injection

3.1 Pipette a 5 µL droplet of the diluted bacterial solution onto a piece of parafilm film and pull the droplet into a 30G insulin needle.

3.2 Use 5–8-month-old wild-type fish and rag1−/− hu1999 mutant fish for the experiment. Anesthetize adult zebrafish in the tank water with 0.02% 3-aminobenzoic acid ethyl ester (pH 7.0). Position the fish ventral side up into a slit on a moist foamed plastic.

Note: *Rag-/-* mutant fish are not able to undergo somatic recombination and produce functional T and B cells.

3.3 Inject the needle between the pelvic fins at an approximately 45° angle. Keep the needle opening upwards to observe that the entire opening is inside the abdominal cavity. Slowly inject the bacterial suspension and carefully remove the needle.

Note: In case the red tracer is leaking out of the fish upon injection, exclude the fish from the experiment.

3.4 Immediately after injection, transfer the fish into a recovery tank with fresh tank water.

3.5 Take samples of the bacterial suspension on 7H10 plates every 15 min from the bacterial aliquot in use and incubate the bacteria at 29 °C for 5 days and verify the infection dose by counting the colonies on the plates.

3.6 Check the well-being of the fish regularly and euthanize any fish with symptoms of the infection with over 0.02% concentration of 3-aminobenzoic acid ethyl ester (pH 7.0).

Note: Approximately, 7% of the adult zebrafish infected with 34 ± 15 CFU and 30% of zebrafish infected with 2029 ± 709 CFU will have had symptoms by 8 weeks [19]. The symptoms may include abnormal swimming, lack of response to touch, gasping, edema or observable wasting.

3.7 Maintain the zebrafish according to the common standards[35].

4. Collection of Internal Organs

4.1 Euthanize the zebrafish with an overdose of 3-aminobenzoic acid ethyl ester (over 0.02% concentration, pH 7.0) in the tank water.

4.2. Insert one pin posterior to the branchiostegal rays and another through the tail to tack the fish onto the platform.

4.3 Open the whole abdominal cavity with a scalpel and collect the internal organs by using a small spoon and sharp-ended tweezers. Start from the heart and work along the spine towards the tail to detach all internal organs in one block.

Note: Be sure to collect all kidney tissue by scraping along the spine with the spoon.

4.4 Finally, use tweezers to detach the gut next to the cloaca and transfer the organs into a 1.5 mL homogenization tube with six 2.8 mm ceramic beads. Immediately place on dry-ice to freeze the sample. The sample can be stored at – 80 °C until homogenized.

4.5 Rinse the instruments with 70% ethanol between individuals.

5. Homogenization and RNA extraction from an organ block.

Note: The method is modified from the Stanford University protocols [36].

5.1 Add guanidine thiocyanate-phenol solution used for nucleic acid extraction (**Table of Materials**) on the top of the sample to a total volume of 1,500 µL. Ensure that the sample covers a maximum of 10% of the total volume.

CAUTION: The expected volume of the harvested tissue is 100 µL. Guanidine thiocyanate-phenol solution contains toxic and irritating compounds and requires protective clothing, nitrile gloves and working in a fume hood. Do not combine with bleach as this will cause formation of toxic gases. Read the material safety data sheet (MSDS) before use.

5.2 Homogenize samples using a bead-beating homogenizer 3 times for 40 s at 3,200 rpm. Cool on ice for 30 s between the cycles. Sonicate the homogenized samples in a water bath for 9 min.

5.3 Centrifuge the samples at 12,000 x g for 10 min at 4 °C and move 1,000 µL of the cleared homogenate into a fresh microcentrifuge tube.

5.4 Add 200 µL of chloroform, immediately mix by vortexing for 15 s and incubate for 2 min at room temperature.

CAUTION: Chloroform is a toxic and irritant compound if inhaled, swallowed or contacted with skin or eyes. Use necessary safety equipment for personal protection and work in a fume hood. Read the material safety data sheet (MSDS) before use.

5.5 Centrifuge at 12,000 x g for 15 min at 4 °C to separate the aqueous and organic phases.

5.6 Carefully, transfer 500 µL of the top phase to a fresh tube to avoid contaminating the RNA. Remove and discard the rest of the aqueous phase (~100 µL) and store the interphase and organic phase at 4 °C for DNA extraction.

Note: The top phase contains the RNA.

5.7 Add 500 µL of 2-propanol and immediately mix by vortexing for 15 s. Incubate for 10 min at room temperature to precipitate the RNA.

5.8 Centrifuge at 12,000 x g for 10 min at 4 °C to pellet the RNA. Remove the supernatant by pipetting.

5.9 Add 1 mL of 75% ethanol and vortex for 10 s.

Note: The protocol can be paused here, and the samples kept overnight at 4 °C.

5.10 Centrifuge at 7,500 x g for 5 min at 4 °C. Remove the supernatant by pipetting.

5.11 Repeat the wash steps 5.9-5.10. Remove the supernatant carefully by pipetting and let the pellet air-dry in a fume hood.

5.12 Dissolve the RNA pellet in 500 µL of nuclease-free water and keep the samples on ice. Measure the concentrations with a microvolume spectrophotometer or with equivalent equipment. Store the RNA at -80 °C.

6. Purification of co-extracted zebrafish and mycobacterial DNA

6.1 Prepare a back-extraction buffer (BEB) by dissolving 118.2 g of guanidine thiocyanate (final concentration 4 M), 3.68 g of sodium citrate (final concentration 50 mM) and 30.29 g of Tris free base (final concentration 1 M) in 120 mL of nuclease-free water (this may require stirring overnight). Add nuclease-free water to a final total volume of 250 mL and filter to sterilize the solution.

Note: This buffer can be stored at room temperature for up to 6 months. Do not combine BEB with bleach as they react to produce toxic gases such as hydrogen chloride and hydrogen cyanide.

6.2 Use the interphase and organic phase of the sample to extract mycobacterial DNA. Add 500 µL of BEB to each tube. Mix extensively for 10 min by inversion at room temperature.

6.3 Centrifuge the tubes at 12,000 x g for 30 min at room temperature and carefully transfer 500 µL of the upper aqueous phase containing the DNA to a new tube.

6.4 Add 400 µL of 2-propanol. Mix by inverting and incubate for 10 min at room temperature.

6.5 Centrifuge the samples at 12,000 x g for 15 min at 4 °C. A pellet containing the DNA should be visible at this point. Carefully remove the supernatant by pipetting.

6.6 Add 800 µL of 70% ethanol. Wash the pellet by inversion. Do not vortex the samples at this point, as genomic DNA breaks down easily.

6.7 Centrifuge the samples at 12,000 x g for 15 min at 4°C and remove the supernatant by pipetting. Repeat the ethanol wash (steps 6.6 and 6.7).

6.8 Remove the ethanol by carefully pipetting. Let the samples air-dry for 5-10 min. Dissolve the pellet in 200 µL of nuclease-free water.

6.9 Measure DNA concentrations with a microvolume spectrophotometer or with equivalent equipment. DNA can be stored at 4 °C or at -20 °C for long-term storage.

7. Quantitative PCR for Measuring Mycobacterial Loads

7.1 Prepare qPCR reaction mixes with no-ROX (carboxy-X-rhodamine) against the *M. marinum* internal transcribed spacer (ITS) between 16S-23S ITS according to the manufacturer's instructions with MMITS1 primers (**Table 1**). Pipette the reaction mix and sample dilutions as duplicates on a 96-well plate suitable for qPCR. Include a DNA standard dilution series of a known amount of bacteria in each run.

Note: The expected *M. marinum* load per fish can range from 0 CFU to 1,000,000 CFU at 4 wpi.

The qPCR assay can be also performed with other qPCR kits but the annealing temperature for the primers has to be re-optimized.

7.2 Seal the plate with an optically transparent film and centrifuge the plate at 2,000 x g for 2 min at 4 °C.

7.3 Run the qPCR program shown in the **Table 2**.

7.4 Using the standard curve, calculate the number of bacteria in the entire fish sample.

8. DNase Treatment of the RNA Samples

8.1 To remove any possible remaining traces of genomic DNA from the RNA, carry out DNase I treatment. Thaw the RNA samples on ice.

Note: Be sure to use only RNase-free equipment and solutions and wipe the working surface and pipettes with a decontamination reagent eliminating RNases (**Table of Materials)** before starting to work. Wear a long-sleeved lab coat and gloves to protect your samples.

8.2 Prepare 10 µL DNase I reaction mixes on ice according to the manufacturer's instructions. Mix 1 µL of DNase I, 1 µL of 10x DNase buffer and 8 µL of RNA sample including a maximum of 1 µg of RNA.

8.3 Gently mix the reactions (no vortexing) and incubate for 30 min at 37 °C.

8.4 Prior to heat-inactivation, add 1 µL of 50 mM EDTA to each 10-µL sample. If EDTA is not added, the RNA will undergo chemical degradation when heated.

8.5 Incubate for 10 min at 65 °C to heat-inactivate DNase I. Continue directly to cDNA synthesis or store the DNase-treated RNA at -80 °C.

9. cDNA Synthesis

9.1 Keep all reagents and samples on ice and prepare the reaction mixes according to the manufacturer's instructions. For a 5-µL reaction mix, include 1 µL of Reverse Transcription Master Mix, 3 µL of nuclease-free water and 1 µL of DNase treated RNA.

9.2 Gently mix the reverse transcription reactions and briefly spin the tube, if needed.

9.3 Place samples in a PCR machine and use the program shown in **Table 3**.

9.4 Dilute the cDNA in nuclease-free water for qPCR to a maximum concentration of 2.5 ng/µL, if needed. cDNA can be stored at -20 °C.

10. Measuring Zebrafish Gene Expression by Quantitative PCR

10.1 Prepare a qPCR master mix on ice according to the manufacturer's instructions and protect from light. Use the primers introduced in **Table 1.**

Note: To calculate the fold of induction for each gene, measure the expression also from a pooled baseline sample extracted from 6 healthy zebrafish.

10.2 Prepare replicates of each sample and pipette the reaction mixes onto a qPCR plate. Seal the plate with an optically transparent film and centrifuge the plate at 2,000 x g for 2 min at 4 °C before starting the run.

10.3 Run the qPCR program shown in the **Table 4** with the annealing temperature depending on the primer pair used (**Table 1**).

10.4 Analyze the gene expression ratio compared to a house-keeping gene (*loopern4*[37]) with the ΔCt method using the equation:

$$\mathrm{ratio} = \frac{2_{gene}^{Ct(healthy)-Ct(infected)}}{2_{loopern4}^{Ct(healthy)-Ct(infected)}}$$

REPRESENTATIVE RESULTS:

The natural fish pathogen *Mycobacterium marinum* infects the internal organs of the zebrafish and produces a systemic infection with histologically visible granulomas[19]. Adult zebrafish are infected with *M. marinum* by an intraperitoneal injection. The DNA and RNA are extracted, and the mycobacterial load is measured by quantitative polymerase chain reaction (qPCR) using DNA as the template. The outline of the method is shown in **Figure 1**.

The initial number of mycobacteria used for infecting the fish is a critical determinant for the outcome of infection. A high infection dose of *M. marinum* (~2000 CFU) leads to a progressive disease in which the mycobacterial loads continue to increase until the average bacterial load reaches around five million bacteria (**Figure 2A**) ultimately killing the fish. A low dose (~20-90 CFU) of *M. marinum* leads to the development of a disease spectrum similar to that seen in human tuberculosis (**Figure 2B**). The bacterial load continues to increase until around 4-7 weeks (**Figures 2A & 3A)**, after which in the majority of the fish the disease reaches a steady-state. **Figure 2B** shows an example of the distribution of disease outcomes with a low dose infection: About 7% of the infected zebrafish were unable to restrict the bacterial growth. These individuals developed a primary progressive disease and they died within two months after the infection. Around 10% of the individuals cleared the mycobacterial infection by 4 weeks. The remaining 65% of the fish population developed a latent mycobacterial infection with steady bacterial burdens. However, between 8 and 32 weeks of infection, in 18%, the latent infection spontaneously reactivated leading to the progression of the disease.

By using *rag-/-* mutant fish, it is possible to study the role of adaptive immune responses in the adult fish. *Rag-/-* mutant fish cannot sufficiently limit the growth of mycobacteria leading to higher bacterial loads (**Figure 3A)** and increased morbidity (**Figure 3B**), clearly demonstrating the importance of adaptive immunity in controlling mycobacterial infection. Also, the importance of adaptive responses in evoking certain cytokine responses in mycobacterial infection can be studied in this model. Here, we show that the adaptive response is required for the efficient induction of interleukin 4 (IL4) (**Figure 3C)** but is dispensable for the induction of interferon-γ (IFNγ) at 4 wpi (**Figure 3D**). Interferon-γ is a cytokine driving the response against intracellular pathogens whereas interleukin 4 is a common mediator in the adaptive immune response against extracellular pathogens. The significantly higher expression levels of *il4* in the wild-type group compared to *rag-/-* mutant fish refers to important adaptive humoral responses in the mycobacterial infection (**Figure 3C**).

FIGURE AND TABLE LEGENDS:

Figure 1. Workflow of studying the development of mycobacterial loads in the adult zebrafish. Adult zebrafish are infected with an intraperitoneal injection of *M. marinum*. DNA and RNA are extracted from the internal organs of the fish and the *M. marinum* load and host's immune responses are analyzed with quantitative polymerase chain reaction (qPCR).

Figure 2. Injection of *M. marinum* into adult zebrafish causes a spectrum of disease states. A. Zebrafish were injected with a low (34±15 CFU) or a high dose (2029±709 CFU) of *M. marinum*. Average loads for 5 fish (except 32 weeks (wk) high dose, n = 2) are shown with SD. Low-dose statistics: * $p<0.05$ compared with 1 wk, ** $p<0.05$ compared with 1 and 2 wk. High-dose statistics: *** $p<0.05$ compared with 1, 2, 8, 11 and 20 wk. ¤ low dose vs. high dose $p<0.05$. Modified from Parikka *et al.* 2012[19]. B. Typical distribution of disease outcomes within a wild-type zebrafish population infected with a low-dose of *M. marinum*.

Figure 3. Adaptive immunity affects the course of mycobacterial infection in the adult zebrafish. Adult wild-type (wt) and rag1 (−/−) zebrafish were infected with a low dose (n = 30) of *M. marinum*. A. The average mycobacterial loads were measured by qPCR at 2, 4, and 7 weeks post infection (wpi) (n = 10) *$P<0.05$. B. The fish were euthanized upon the development of symptoms of the disease and survival plots were created. C. The expression levels of *il4* were measured at 4 wpi. D. The expression levels of *IFNγ* were measured at 4 wpi. A&B modified from Parikka *et al.* 2012[19]. C&D Modified from Hammarén *et al.* 2014[38].

Table 1. Primer sequences and annealing temperatures. The sequences of the primers used and their optimized annealing temperatures. The primers for the *M. marinum* 16S-23S rRNA transcript have been optimized for a No-ROX qPCR kit and the other primers for a ROX including qPCR kit.

Table 2. qPCR program for measuring *M. marinum* DNA. A qPCR protocol designed according to the manufacturer's instructions and optimized for measuring *M. marinum* DNA from zebrafish samples.

Table 3. cDNA synthesis program. Protocol for synthesizing cDNA from the extracted RNA of an

infected zebrafish according to the manufacturer's instructions.

Table 4. qPCR program for measuring host's gene expression. A qPCR protocol designed according to the manufacturer's instructions and optimized for measuring the expression of different zebrafish genes.

DISCUSSION:

Here we describe a qPCR-based application to measure mycobacterial loads from DNA extracted from experimentally infected adult zebrafish tissues. This application is based on primers designed around the 16S-23S rRNA internal transcribed spacer sequence[40]. The total mycobacterial load in a fish sample is estimated using a standard curve prepared from DNA extracted from a known number of cultured mycobacteria and assuming that one bacterium has one copy of its genome at any given moment. The detection limit of the *M. marinum* -qPCR is approximately 100 colony forming units[18]. A clear advantage of the method compared to traditional plating is that both active and non-dividing dormant bacteria can be detected. In addition, a common problem of contaminating growth on culture plates from zebrafish tissues is circumvented by this approach. However, as DNA is used as the template, it is possible that some of the copies measured can be derived from the DNA of bacteria that have died very recently. A significant advantage of the nucleic acid-based protocol is that as both DNA and RNA (as well as proteins, not described here) can be extracted from the same individual, the mycobacterial load of the individual can be combined with gene expression data of both the host and the bacteria.

The dosing of *M. marinum* is a critical determinant of the outcome of infection. The low *M. marinum* (~20-90 CFU) infection dose produces a spectrum of disease states with latency as the most common form. If the infection dose is in the order of thousands, a more progressive disease develops in the majority of individuals. As the natural infectious dose is known to be low in human tuberculosis[41], using a low dose of *M. marinum* in the zebrafish model is likely to produce a more natural infection. To reach the correct dose, make sure to validate the relation between the OD600 and colony-forming units prior to starting any experiments. The normal variation of the plated infection doses is approximately 30% and is not considered a problem. However, it is important to verify that the entire 5 µL volume of the bacterial suspension remains inside the fish. Leaking of the injection solution will cause extra variation in the infection dose. In addition to the infection dose, the strain of the bacteria can affect the disease progression. It has been shown that the virulence of the different *M. marinum* strains can alter between the strains. The two most commonly used strains are ATCC927 (fish isolated strain used in these experiments) and the M strain. However, these strains differ greatly in their virulence. The human-isolated M strain develops a more progressive disease whereas the fish-isolated strain produces a milder disease well-suitable for studying the latent form of the mycobacterial infection[33]. The virulence of the bacteria within a strain may also alter if the bacteria is serially transferred from one culture to another, which can be prevented by taking fresh mycobacteria from a freezer stock often enough.

In vitro culturing of slowly dividing *M. marinum* without antibiotics is prone to contaminations. Therefore, the handling of the bacteria requires strict aseptic approach carried out in a laminar flow hood. Possible contamination in the culture can be detected as too high an OD_{600} value, odd-looking bacterial suspension or colonies. *M. marinum* colonies are normally fuzzy-edged, flat and matt white in color. *M. marinum* cultures are sensitive to light and they start producing yellow pigment when exposed to light. This yellow pigment can be used to distinguish *M. marinum* colonies from other bacteria after the infections. Contaminants can cause the fish to die soon after infection. Usually, the first symptoms of a low-dose infection do not appear before 3 weeks post infection and any mortalities before this time point are likely due to contamination in the bacterial suspension or trauma induced during injection.

Adult zebrafish are very sensitive to 3-aminobenzoic acid ethyl ester and do not survive if the exposure time is too long or the concentration of the anesthetic is too high. Therefore, when infecting, the zebrafish should be exposed to the anesthetic only for the minimum of time to achieve good anesthesia (~1-2 minutes). After infection, adult zebrafish are kept in groups at water temperature of 26-28 °C. If the temperature is higher or lower than this, it might affect the *M. marinum* growth rate and kinetics of the infection. Also, the tank water quality (microbiological quality, salt concentration, pH, oxygen saturation) is an important part ensuring a successful experiment. Health monitoring of the fish needs to be carried out daily and fish that show symptoms of infection need to be removed from the group and euthanized. If the fish die in the tank, the other fish may become re-infected through the gut, which will affect the progression of infection.

The commonly used zebrafish larval model of tuberculosis is applicable to study innate immunity, which directs the experiments to a limited subset of host-microbe interactions. The use of adult zebrafish makes it possible to study both innate and adaptive immune responses in a versatile model[18,32,39]. Adult zebrafish presents itself as a convenient vertebrate model to study the entire spectrum of tuberculosis. With a low-dose exposure of *M. marinum*, 7% of the fish population develop primary progressive disease, 10% are able to sterilize the infection, 65% develop latent disease and in 18% spontaneous reactivation occurs (**Figure 2**). The disease spectrum closely resembles that seen in human tuberculosis, in which the vast majority develop a latent disease, approximately 4–14% produce primary active infection within the first five years after the infection[42], 10–20% of heavily exposed persons seem to be able to sterilize the infection[43] and 5–10% of the latent infections reactivate[44]. Differences in hosts' genetics affects the susceptibility to tuberculosis and the disease progression[45]. This is also seen in the zebrafish population that is genetically very heterogeneous unlike many other laboratory animals[46,47]. The natural genetic variation makes it a highly applicable model in the search for optimal immune responses in this multifactorial disease.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Zhao, S., Huang, J. & Ye, J. A fresh look at zebrafish from the perspective of cancer research. *Journal of Experimental & Clinical Cancer Research.* **34**, 80, doi:10.1186/s13046-015-0196-8 (2015).

2. Bournele, D. & Beis, D. Zebrafish models of cardiovascular disease. *Heart failure reviews.* **21** (6), 803-813, doi:10.1007/s10741-016-9579-y (2016).

3. Torraca, V. & Mostowy, S. Zebrafish Infection: From Pathogenesis to Cell Biology. *Trends in cell biology.* **28** (2), 143-156, doi:10.1016/j.tcb.2017.10.002 (2018).

4. Varela, M., Figueras, A. & Novoa, B. Modelling viral infections using zebrafish: Innate immune response and antiviral research. *Antiviral Research.* **139**, 59-68, doi:10.1016/j.antivira1.2016.12.013 (2017).

5. Goody, M.F., Sullivan, C. & Kim, C.H. Studying the immune response to human viral infections using zebrafish. *Developmental and comparative immunology.* **46** (1), 84-95, doi:10.1016/j.dci.2014.03.025 (2014).

6. Thisse, C. & Zon, L.I. Development - Organogenesis - Heart and wood formation from the zebrafish point of view. *Science.* **295** (5554), 457-462, doi:10.1126/science.1063654 (2002).

7. Eimon, P.M. & Rubinstein, A.L. The use of in vivo zebrafish assays in drug toxicity screening. *Expert Opinion on Drug Metabolism & Toxicology.* **5** (4), 393-401, doi:10.1517/17425250902882128 (2009).

8. Sukardi, H., Chng, H.T., Chan, E.C.Y., Gong, Z. & Lam, S.H. Zebrafish for drug toxicity screening: bridging the in vitro cell-based models and in vivo mammalian models. *Expert Opinion on Drug Metabolism & Toxicology.* **7** (5), 579-589, doi:10.1517/17425255.2011.562197 (2011).

9. Wittamer, V., Bertrand, J.Y., Gutschow, P.W. & Traver, D. Characterization of the mononuclear phagocyte system in zebrafish. *Blood.* **117** (26), 7126-7135, doi:10.1182/blood-2010-11-321448 (2011).

10. Harvie, E.A. & Huttenlocher, A. Neutrophils in host defense: new insights from zebrafish. *Journal of leukocyte biology.* **98** (4), 523-537, doi:10.1189/jlb.4MR1114-524R (2015).

11. Yoshida, N., Frickel, E. & Mostowy, S. Macrophage-Microbe interactions: Lessons from the Zebrafish Model. *Frontiers in Immunology.* **8**, 1703, doi:10.3389/fimmu.2017.01703 (2017).

12. Langenau, D.M., *et al*. In vivo tracking of T cell development, ablation, and engraftment in transgenic zebrafish. *Proceedings of the National Academy of Sciences of the United States of America.* **101** (19), 7369-7374, doi:10.1073/pnas.0402248101 (2004).

13. Lewis, K.L., Del Cid, N. & Traver, D. Perspectives on antigen presenting cells in zebrafish. *Developmental and comparative immunology.* **46** (1), 63-73, doi:10.1016/j.dci.2014.03.010 (2014).

14. Hu, Y., Xiang, L. & Shao, J. Identification and characterization of a novel immunoglobulin Z isotype in zebrafish: Implications for a distinct B cell receptor in lower vertebrates. *Molecular immunology.* **47** (4), 738-746, doi:10.1016/j.molimm.2009.10.010 (2010).

15. Danilova, N., Bussmann, J., Jekosch, K. & Steiner, L.A. The immunoglobulin heavy-chain locus in zebrafish: identification and expression of a previously unknown isotype, immunoglobulin Z. *Nature immunology.* **6** (3), 295-302, doi:10.1038/ni1166 (2005).

16. Zapata, A., Diez, B., Cejalvo, T., Frias, C.G. & Cortes, A. Ontogeny of the immune system of fish. *Fish & shellfish immunology.* **20** (2), 126-136, doi:10.1016/j.fsi.2004.09.005 (2006).

17. Traver, D., Paw, B.H., Poss, K.D., Penberthy, W.T., Lin, S. & Zon, L.I. Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. *Nature immunology.* **4** (12), 1238-1246, doi:10.1038/ni1007 (2003).

18. Hammaren, M.M., *et al*. Adequate Th2-Type Response Associates with Restricted Bacterial Growth in Latent Mycobacterial Infection of Zebrafish. *Plos Pathogens.* **10** (6), e1004190, doi:10.1371/journal.ppat.1004190 (2014).

19. Parikka, M., *et al*. Mycobacterium marinum Causes a Latent Infection that Can Be Reactivated by Gamma Irradiation in Adult Zebrafish. *PLoS Pathog.* **8** (9), 1-14 (2012).

20. Tobin, D.M., *et al*. Host Genotype-Specific Therapies Can Optimize the Inflammatory Response to Mycobacterial Infections. *Cell.* **148** (3), 434-446, doi:10.1016/j.cell.2011.12.023 (2012).

21. Lesley, R. & Ramakrishnan, L. Insights into early mycobacterial pathogenesis from the zebrafish. *Current opinion in microbiology.* **11** (3), 277-283, doi:10.1016/j.mib.2008.05.013 (2008).

22. Berg, R.D. & Ramakrishnan, L. Insights into tuberculosis from the zebrafish model. *Trends in molecular medicine.* **18** (12), 689-690, doi:10.1016/j.molmed.2012.10.002 (2012).

23. World Health Organization. WHO Global tuberculosis report 2017 ., http://www.who.int/tb/publications/global_report/en/ (2017).

24. Ordonez, A.A., *et al*. Mouse model of pulmonary cavitary tuberculosis and expression of matrix metalloproteinase-9. *Disease Models & Mechanisms.* **9** (7), 779-788, doi:10.1242/dmm.025643 (2016).

25. Kramnik, I. & Beamer, G. Mouse models of human TB pathology: roles in the analysis of necrosis and the development of host-directed therapies. *Seminars in Immunopathology.* **38** (2), 221-237, doi:10.1007/s00281-015-0538-9 (2016).

26. Manabe, Y.C., *et al*. The aerosol rabbit model of TB latency, reactivation and immune reconstitution inflammatory syndrome. *Tuberculosis.* **88** (3), 187-196, doi:10.1016/j.tube.2007.10.006 (2008).

27. Pena, J.C. & Ho, W. Monkey Models of Tuberculosis: Lessons Learned. *Infection and immunity.* **83** (3), 852-862, doi:10.1128/IAI.02850-14 (2015).

28. Cadena, A.M., Fortune, S.M. & Flynn, J.L. Heterogeneity in tuberculosis. *Nature Reviews Immunology.* **17** (11), 691-702, doi:10.1038/nri.2017.69 (2017).

29. Stinear, T.P., *et al*. Insights from the complete genome sequence of Mycobacterium marinum on the evolution of Mycobacterium tuberculosis. *Genome research.* **18** (5), 729-741, doi:10.1101/gr.075069.107 (2008).

30. Swaim, L.E., Connolly, L.E., Volkman, H.E., Humbert, O., Born, D.E. & Ramakrishnan, L. Mycobacterium marinum infection of adult zebrafish causes caseating granulomatous tuberculosis and is moderated by adaptive immunity. *Infection and immunity.* **74** (11), 6108-6117, doi:10.1128/IAI.00887-06 (2006).

31. Myllymaki, H., Bauerlein, C.A. & Ramet, M. The Zebrafish Breathes new Life into the Study of Tuberculosis. *Frontiers in Immunology.* **7**, 196, doi:10.3389/fimmu.2016.00196 (2016).

32. Luukinen, H., *et al*. Priming of Innate Antimycobacterial Immunity by Heat-killed Listeria monocytogenes Induces Sterilizing Response in Adult Zebrafish Tuberculosis Model . *Disease Models and Mechanisms.* **11**, 1754-8403 (2018).

33. van der Sar, A M, Abdallah, A.M., Sparrius, M., Reinders, E., Vandenbroucke-Grauls, C. & Bitter, W. Mycobacterium marinum strains can be divided into two distinct types based on genetic diversity and virulence. *Infection and immunity.* **72** (11), 6306-6312, doi:10.1128/IAI.72.11.6306-6312.2004 (2004).

34. Madigan, M. & Martinko, J. *Brock Biology of Mircoorganisms.* (2016).

35. Nüsslein-Volhard, C. & Dahm, R. *Zebrafish:a practical approach.* Oxford University Press, Oxford (2002).

36. Anonymous Stanford University Protocols ., http://med.stanford.edu/labs/vanderijn-west/Protocols.html (2018).

37. Vanhauwaert, S., *et al*. Expressed Repeat Elements Improve RT-qPCR Normalization across a Wide Range of Zebrafish Gene Expression Studies. *Plos One.* **9** (10), e109091, doi:10.1371/journal.pone.0109091 (2014).

38. Hammaren, M.M., *et al*. Adequate Th2-Type Response Associates with Restricted Bacterial Growth in Latent Mycobacterial Infection of Zebrafish. *Plos Pathogens.* **10** (6), e1004190, doi:10.1371/journal.ppat.1004190 (2014).

39. Oksanen, K.E., *et al*. An adult zebrafish model for preclinical tuberculosis vaccine development. *Vaccine.* **31** (45), 5202-5209, doi:10.1016/j.vaccine.2013.08.093 (2013).

40. Roth, A., Fischer, M., Hamid, M.E., Michalke, S., Ludwig, W. & Mauch, H. Differentiation of phylogenetically related slowly growing mycobacteria based on 16S-23S rRNA gene internal transcribed spacer sequences. *Journal of clinical microbiology.* **36** (1), 139-147 (1998).

41. Rajararna, M.V.S., Ni, B., Dodd, C.E. & Schlesinger, L.S. Macrophage immunoregulatory pathways in tuberculosis. *Seminars in immunology.* **26** (6), 471-485, doi:10.1016/j.smim.2014.09.010 (2014).

42. Vynnycky, E. & Fine, P. The natural history of tuberculosis: the implications of age-dependent risks of disease and the role of reinfection. *Epidemiology and infection.* **119** (2), 183-201, doi:10.1017/S0950268897007917 (1997).

43. Cobat, A., *et al*. Two loci control tuberculin skin test reactivity in an area hyperendemic for tuberculosis. *Journal of Experimental Medicine.* **206** (12), 2583-2591, doi:10.1084/jem.20090892 (2009).

44. Delogu, G. & Goletti, D. The Spectrum of Tuberculosis Infection: New Perspectives in the Era of Biologics. *Journal of Rheumatology.* **41**, 11-16, doi:10.3899/jrheum.140097 (2014).

45. Abel, L., *et al*. Genetics of human susceptibility to active and latent tuberculosis: present knowledge and future perspectives. *Lancet Infectious Diseases.* **18** (3), E75, doi:10.1016/S1473-3099(17)30623-0 (2018).

46. Guryev, V., *et al*. Genetic variation in the zebrafish. *Genome research.* **16** (4), 491-497, doi:10.1101/gr.4791006 (2006).

47. Brown, K.H., *et al*. Extensive genetic diversity and substructuring among zebrafish strains revealed through copy number variant analysis. *Proceedings of the National Academy of Sciences of the United States of America.* **109** (2), 529-534, doi:10.1073/pnas.1112163109 (2012).

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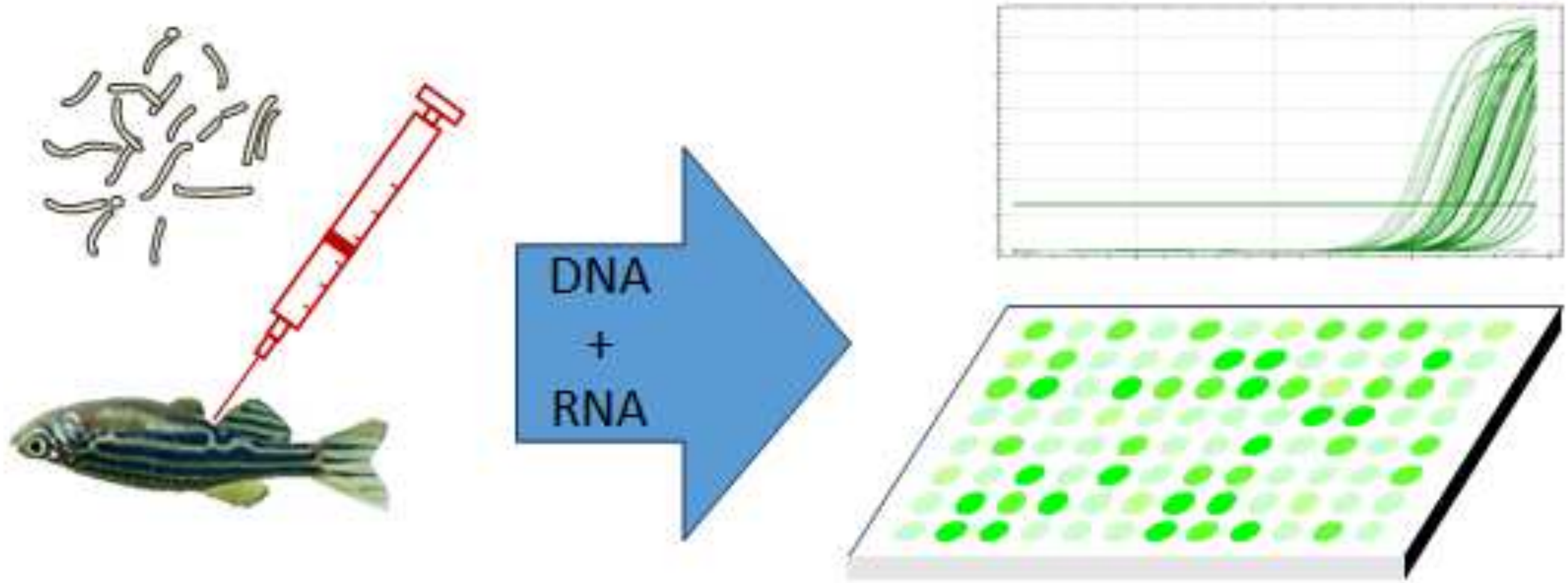

M. marinum
infection with
intraperitoneal
injection

Quantification of
M. marinum load
and host immune
response with
qPCR

Figure2

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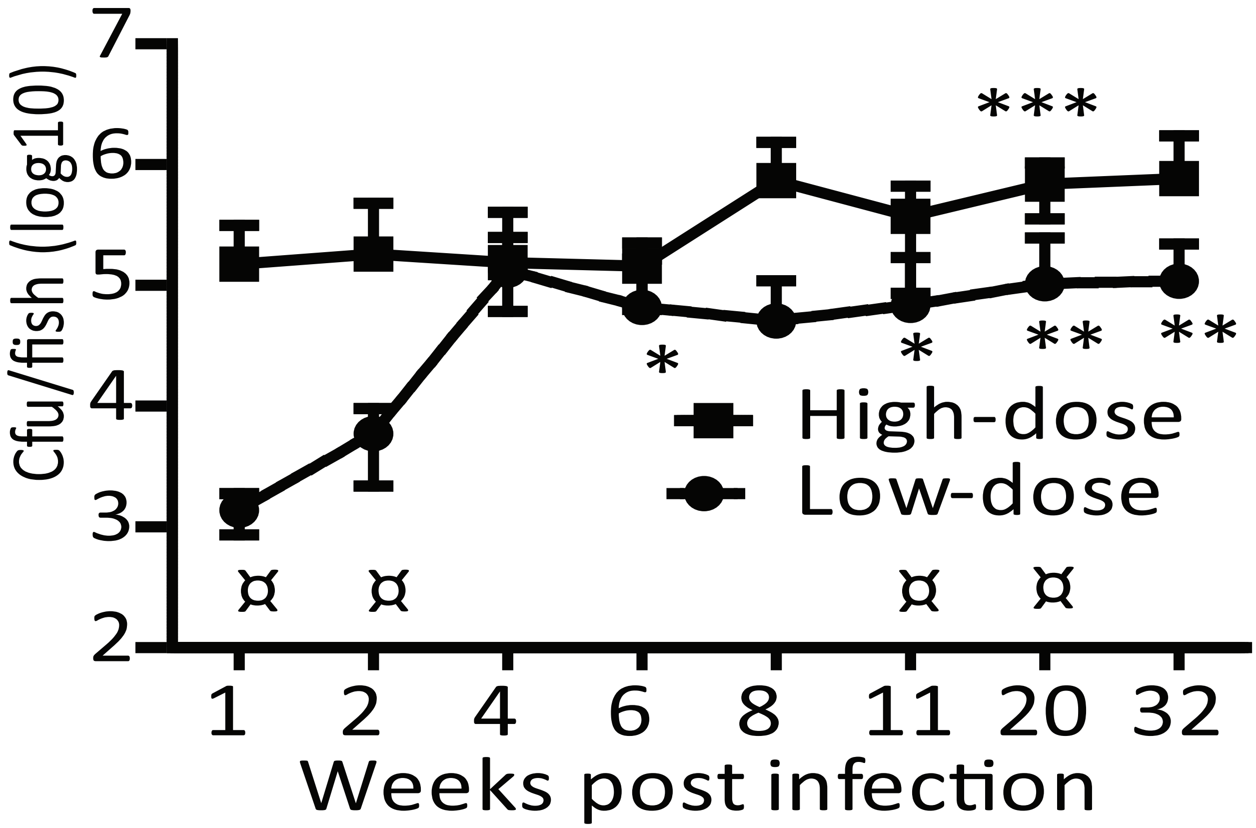
A



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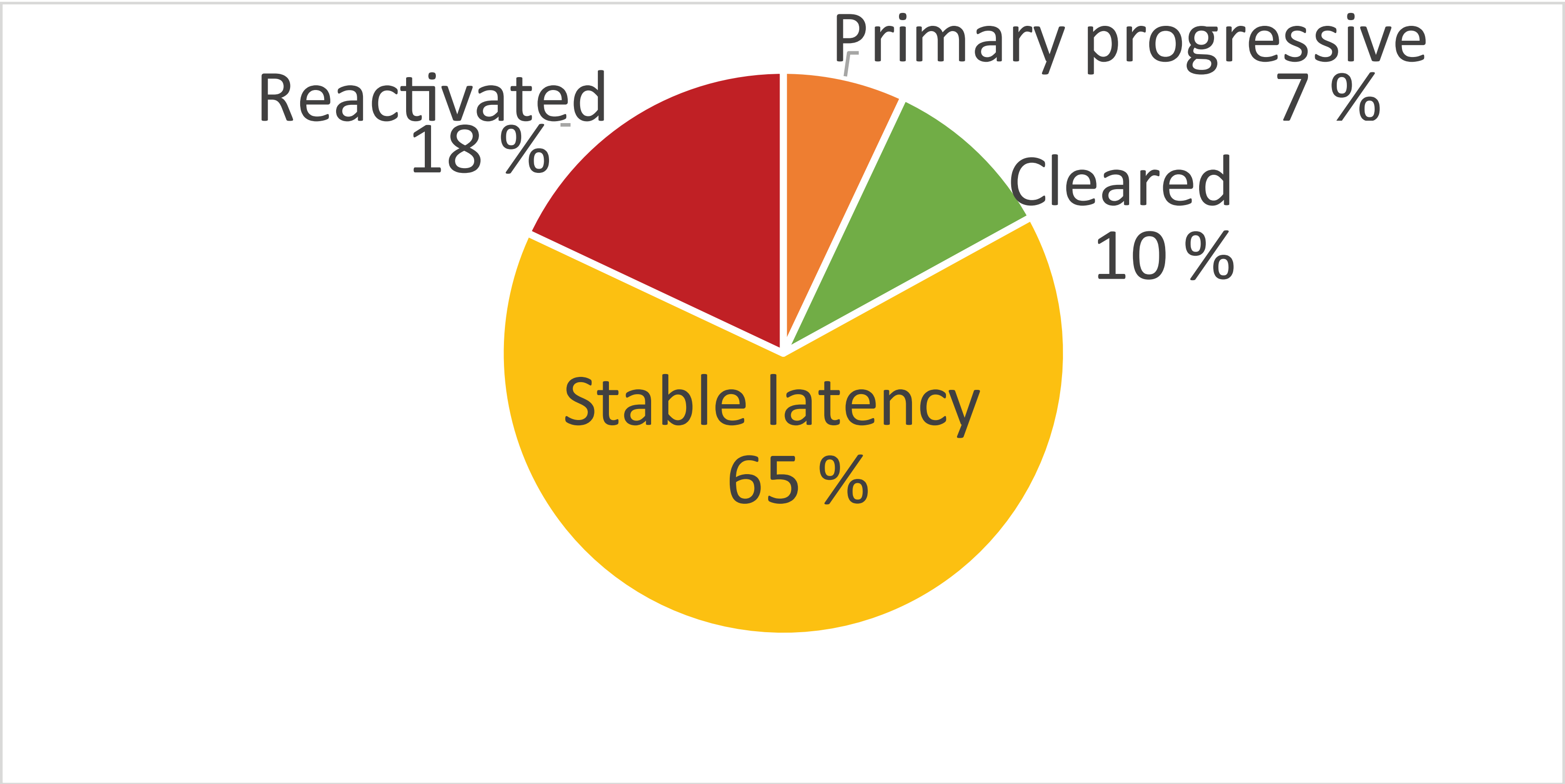

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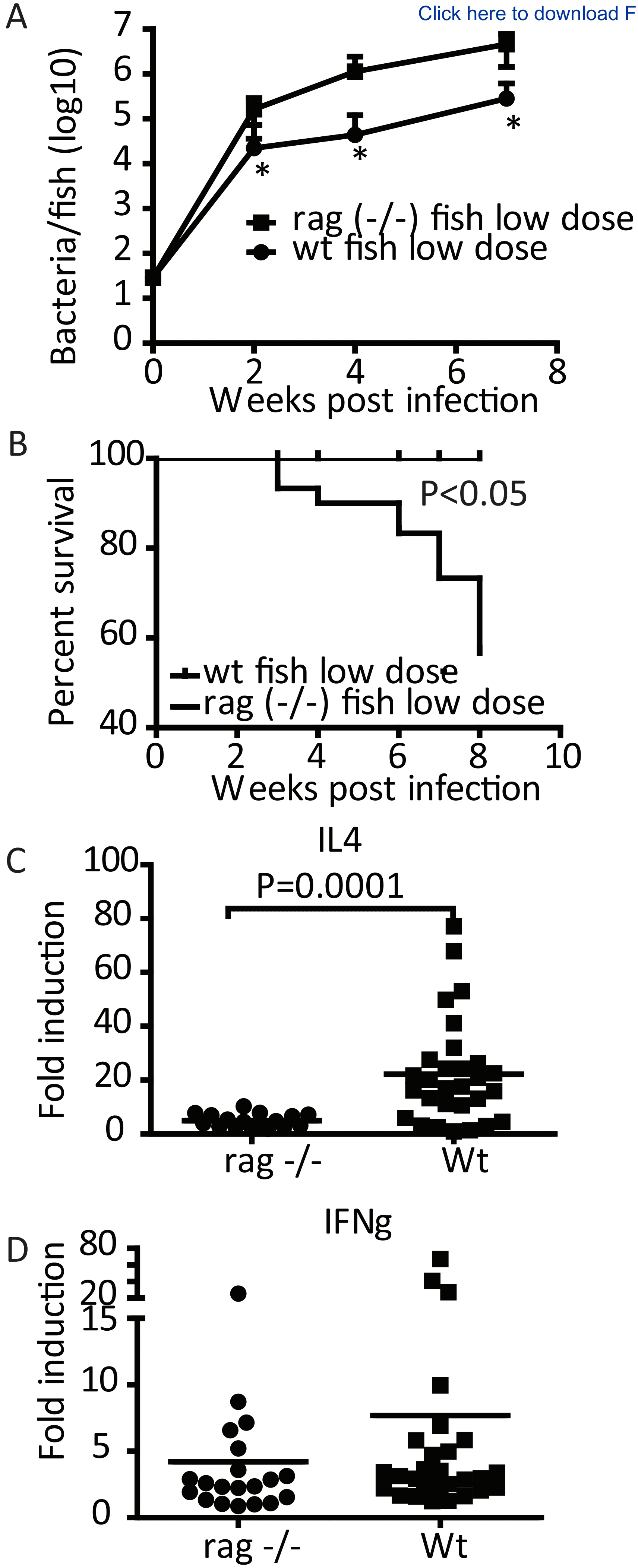

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Gene	Primer sequence
MMITS1 16S–23SITS Locus AB548718 for *M. marinum* quantification	F: CACCACGAGAAACACTCCAA R: ACATCCCGAAACCAACAGAG
loopern4 Expressed repetitive elements	F: TGAGCTGAAACTTTACAGACACAT R: AGACTTTGGTGTCTCCAGAATG
il4 ZDB-GENE-100204-1	GCAGGAATGGCTTTGAAGGG GCAGTTTCCAGTCCCGGTAT
ifnγ1-2 ZDB-GENE-040629-1	F: GGGCGATCAAGGAAAACGACCC, R: TAGCCTGCCGTCTCTTGCGT

Annealing temperature
65
61
59.5
61

Table2

Click here to download Table Table 2. qPCR program for marinum.xlsx

Step	Time	Temperature
1	3 min	95 °C
2	5 s	95 °C
3	10 s	65 °C
4	5 s	72 °C (fluorescence detection)
5	Go to step 2. 39 times	
6	Melting curve analysis 55-95°C with 0.5°C intervals	
7	Forever	4 °C

Table3

Click here to download Table Table 3. cDNA synthesis.xlsx

Time	Temperature
5 min	25°C
30 min	42°C
5 min	85°C
forever	4°C

Click here to download Table Table 4. qPCR program for hosts gene expression.xlsx

Step	Time	Temperature
1	30 s	95°C
2	12 s	95°C
3	30 s	Annealing °C
4	Go to step 2. for 39 times	
5	Melting curve analysis 65–95°C with 0.5°C intervals	
6	Forever	4°C

Click here to download Table of Materials Table JoVE_Materials.xls

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Mycobacterium marinum	American Type Culture Collection	ATCC 927	
Middlebrock 7H10 agar	BD, Thermo Fisher Scientific	11799042	
Middlebrock OADC enrichment	BD, Thermo Fisher Scientific	11718173	
Middlebrock 7H9 medium	BD, Thermo Fisher Scientific	11753473	
Middlebrock ADC enrichment	BD, Thermo Fisher Scientific	11718173	
Tween 80	Sigma-Aldrich	P1754	
Glycerol	Sigma-Aldrich	G5516-500ML	
GENESYS20 Spectrophotometer	Thermo Fisher Scientific		
Phosphate buffered saline tablets (PBS)	Sigma-Aldrich	P4417-50TAB	
Phenol red	Sigma-Aldrich	P3532	
27G needle	Henke Sass Wolf	4710004020	
1 ml syringe	Henke Sass Wolf	4010.200V0	
Omnican 100 30G insulin needle	Braun	9151133	
3-aminobenzoic acid ethyl ester (pH 7.0)	Sigma-Aldrich	A5040	
1.5 ml homogenization tube	Qiagen	13119-1000	
2.8 mm ceramic beads	Qiagen	13114-325	
Ethanol, ETAX Aa	Altia		
2-propanol	Sigma-Aldrich	278475	
Chloroform	VWR	22711.290	
Guanidine thiocyanate	Sigma-Aldrich	G9277	FW 118.2 g/mol
Sodium citrate	Sigma-Aldrich	1613859	FW 294.1 g/mol
Tris (free base)	Sigma-Aldrich	TRIS-RO	FW 121.14 g/mol
TRI reagent	Molecular Research Center	TR118	Guanidine thiocyanate-phe
PowerLyzer24 homogenizator	Qiagen		
Sonicator m08	Finnsonic		
Nanodrop 2000	Thermo Fisher Scientific		
SENSIFAST No-ROX SYBR, Green Master Mix	Bioline	BIO-98005	
qPCR 96-well plate	BioRad	HSP9601	
Optically transparent film	BioRad	MSB1001	
C1000 Thermal cycler with CFX96 real-time syste	BioRad		
RNase AWAY	Thermo Fisher Scientific	10666421	decontamination reagent e
DNase I	Thermo Fisher Scientific	EN0525	

Reverse Transcription Master Mix	Fluidigm	100-6298
SsoFast Eva Green master mix	BioRad	172-5211

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liminating RNases

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Editorial comments and responses:

1. The editor has formatted the manuscript to match the journal's style. Please retain the same.

Formation checked.

2. Please address specific comments in the manuscript.

The comments and responses are visible in the manuscript and under these comments.

3. Please reword the title to more clearly reflect the protocol.
The title has been reword.

4. Please remember that the highlighted steps are the steps which are shown in the video. Please ensure these are the core steps of the protocol and reflective of the title and manuscript. The manuscript emphasizes the identification of microbial loads by qPCR while none of the RNA, DNA, qPCR steps are highlighted.
The extraction of nucleic acids and qPCR was left out because these steps are very familiar to anyone doing molecular techniques and it is not interesting to follow someone pipeting from one tube to another. The idea was to explain these steps verbally. However, you have a good point and a step where the qPCR reactions are pipetted is now added. In this step, also the homogenization of the sample and nucleic acid extraction could be briefly mentioned.

5. Please remember that the highlight should not be more than 2.75 pages including heading and spacings.
The highlighted text does not exceed 2.75 pages.

6. The protocol needs more clarity.
The specific clarity comments has been addressed in the manuscript file.

7. Please proofread the manuscript for any spelling and grammar issues.

Checked

Comments within the manuscript:

Please change the title to reflect the protocol clearly. Please be more specific. Please do not use the term model of Human tuberculosis … please tone this down a bit.

Title modified

Citation?

Citations added

Do you work under BL-2 guideline? Please mention the same as well, if yes.

There is no ethical approval needed for handling BSL2 pathogen. In this case, the ethical permissions are needed only when animals are involved. This part refers to biosafety levels (not laboratory animals). The handling of pathogens is regulated locally. Please note, that the Finnish guidelines, that we are following, may not be the same in other laboratories.

Please mention if you needed any local ethics committee approval for your work. Please do not use personal pronouns in the protocol section.

The permits have been mentioned at the beginning of the protocol section. No other ethical permits were required. The text has been combined with the note below.

Converted to imperative tense, please check.

Checked, OK

We cannot have commercial language in the manuscript. Please move this part to the table of materials.

Commercial language removed and added to the table of materials

We cannot have paragraphs of text in the protocol section. Please consider moving some parts to the discussion.

Some text was moved to discussion

Till O.D what?

Added

We cannot have one note after another. So I have combined both. Please check.

Checked, OK

Since not in imperative tense, this is converted to a note.

OK

What is the day of infection- you grow bacteria for 3-4 days in liquid medium till O.D .., then dilute to 0.07 O.D, then grow again for two days to reach early log phase…. Then when is the day of infection?
Please write steps as if you the doing the protocol.

You followed the protocol correctly. The beginning of the sentence has now been removed

So, bacteria form clumps in the steps above? What is the O.D. at this time?

Many bacteria have a natural tendency to form aggregates and so does M. marinum. The bacteria form clumps even when tween is added to the culture medium (as it is in this protocol). The settling step does not change the OD-value much but gives a better correlation between OD600 and cfu/ul. So, the OD-value remains approximately at 0.5.

Please provide the desired concentration used by you in your experiment. This is important for the person who reads to have a sense of what concentrations and volumes are used throughout.

The infection dose depends on the experimental setup. In this article, we show results that have been produced by a low-dose infection (35 cfu) and high-dose infection (2000 cfu). The infection outcome is very different with these infection doses and has to be chosen by the reader. Also, adding a range from 35 to 2000 cfu would give a false impression that any infection dose can be used.

Notes cannot be filmed. Please convert this to steps in imperative tense if this needs to be filmed.

Not necessary to film

Needs citation. Or else how do you predetermine this?

A citation added

How do you do this.

The correlation between OD and cfu/ul has been determined by first measuring OD600-value of the bacterial suspension and then plating a sample of the same suspension on 7H10 plate and counting the bacterial colonies formed on the plate. The measurements and platings have been repeated in different OD-values with a minimum of three replicates.

What does AB wild-type represent and rag1-/- stands for. Either provide a reference or provide details in the materials table.

AB removed and rag explained in the note below

This detail should be included either in the introduction or in the protocol section (as a note) where the term is introduced.

Moved from the results

How do you maintain sterility in this case.

The injection of the bacterial suspension is not sterile. It would be technically hard to wipe a small zebrafish with ethanol. Also, it would do extra harm to the fish.

Please do not use personal pronouns in the protocol text.
Also, at which step did you add the red tracer to the bacterial solution?

Red tracer is added at step 2.4., as mentioned
The sentence has been modified

Take samples of bacterial suspension from where? Do you take samples from the fish?

The samples are taken from the bacterial suspension used for infecting the fish. We do not take samples from the fish at this point.

How do you verify the infection dose? What is the infection dose in this case.

We count the colonies that form on the plates. This has been now explained in the protocol. The infection dose has to be confirmed with this plating method, we cannot know the exact infection dose beforehand.

What are the symptoms of infection?
The symptoms are listed in the following sentence.

Is there a specific concentration used for euthanisa

Unfortunately, there is not. It is unethical to test, which concentration of the anesthetic is enough for euthanizing the fish. Therefore, we cannot say more specifically.

Please clarify? What symptoms what 30% ?

This has now been clarified.

Needs more clarity. Please reword better.

Sentence modified

Please do not use commercial language and use generic term instead. Also use imperative tense throughout.

Tri reagent changed to guanidine thiocyanate-phenol solution

Please make this a note or rewrite in imperative tense.

Added as a note

Please do not use commercial language in the manuscript. Please use generic terms instead. Please refer to the commercial language in the table of materials.

ROX is an abbreviation of carboxy-X-rhodamine which is now mentioned in the text

Wouldn't this section 8 and 9 come before DNA isolation after step 5?

No, this is in the correct place. We want to remove any possible traces of contaminating genomic DNA in our RNA samples.

Please do not use commercial language in the manuscript. Please use generic term instead. Please refer to the commercial language in the Table of materials.

Modified

Same primers as before?

No, the primers are found from the table 1

This detail should be included either in the introduction or in the protocol section (as a note) where the term is introduced.

Added as a note in the protocol section

As we are a methods journal, please revise the Discussion to explicitly cover 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

Please do not make numbered steps in the discussion. Please use paragraph style instead.

Discussion modified

Please do not abbreviate journal titles.

The reference list was made with the jove's own template found in refworks (proquest). Please consider editing the template according to your instructions.

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