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***Mycobacterium marinum* Produces Long-Term Chronic Infections in Medaka: A New Animal Model for Studying Human Tuberculosis¹**

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Abstract

Human infection by *Mycobacterium tuberculosis* is endemic, with approximately 2 billion infected and is the most common cause of adult death due to an infectious agent. Because of the slow growth rate of *M. tuberculosis* and risk to researchers, other species of *Mycobacterium* have been employed as alternative model systems to study human tuberculosis (TB). *Mycobacterium marinum* may be a good surrogate pathogen, conferring TB-like chronic infections in some fish. Medaka (*Oryzias latipes*) has been established for over five decades as a laboratory fish model for: toxicology, genotoxicity, teratogenesis, carcinogenesis, classical genetics and embryology. We are investigating if medaka might also serve as a host for *M. marinum* in order to model human TB. We show that both acute and chronic infections are inducible in a dose dependent manner. Colonization of target organs and systemic granuloma formation has been demonstrated through the use of histology. *M. marinum* expressing green fluorescent protein (Gfp) was used to monitor bacterial colonization of these organs in fresh tissues as well as in intact animals. Moreover, we have employed the See-Through fish line, a variety of medaka devoid of major pigments, to monitor real-time disease progression, in living animals. We have also compared the susceptibility of another prominent fish model, zebrafish (*Danio rerio*), to our medaka-*M. marinum* model. We determined the course of infections in zebrafish is significantly more severe than in medaka. Together, these results indicate that the medaka-*M. marinum* model provides unique advantages for studying chronic mycobacteriosis.

Keywords

Danio rerio; granuloma; medaka; *Mycobacterium marinum*; *Oryzias latipes*; tuberculosis; zebrafish

1. Introduction

Worldwide, an estimated two billion humans are infected with tuberculosis (TB), which remains the most common cause of adult death due to an infectious agent (Dye et. al., 1999). The questionable efficacy of the current BCG vaccine (Ponnighaus et. al., 1992; Fine, 1995)

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and prevalence of latent/chronic TB makes better understanding of the host and pathogen interactions of TB of great medical importance. Difficulties in studying *Mycobacterium tuberculosis* include a slow growth rate (~24 hr generation time), the necessity of working in high containment biosafety level-3 (BSL-3) facilities, and expensive and non-ideal mammalian hosts (Frank and Orme, 2003). Investigators have sought surrogate mycobacterial models for *M. tuberculosis* pathogenesis. Major surrogate model pathogens for *M. tuberculosis* include *Mycobacterium smegmatis* and *Mycobacterium marinum* offering lower risk and greater ease of manipulation in the laboratory setting (Jacobs et. al., 1987; Lee et. al., 1991; Ramakrishnan and Falkow, 1994; Ramakrishnan et. al., 1997).

We would expect the ideal surrogate model pathogen to be closely related to *M. tuberculosis*, causing similar chronic diseases, and to be genetically tractable. *M. marinum* is the mycobacterial species most closely related to the *M. tuberculosis* complex and causes TB-like infections in many poikilothermic hosts, such as frogs and fish (Rogall et. al., 1990; Tønnum et. al., 1998). Diseases caused by *M. marinum* usually result in chronic infections and systemic formation of granulomas, the hallmark lesion of tuberculosis (Ramakrishnan et. al., 1997; Talaat et. al., 1998; Decostere et. al., 2004). The comparatively fast growth rate (~4 hr generation time), ability of investigators to work with lower risk (BSL-2), genetic relatedness, similar pathology to human TB, a genome sequence, and ability to genetically manipulate this mycobacterial species makes *M. marinum* a good model for studying mycobacterial pathogenesis (for review see Cosma et. al., 2003).

The ideal surrogate model host for human TB would be susceptible to infection by *M. tuberculosis* or a closely related bacterium producing a comparable chronic disease, and would also be genetically tractable. Previously described aquatic models for *M. marinum* infection include Goldfish (*Carassius auratus*), zebrafish (*Danio rerio*) and leopard frog (*Rana pipiens*) (Ramakrishnan et. al., 1997; Tallat et. al., 1998; Prouty et. al., 2003). Of these three hosts, zebrafish is not only an excellent laboratory animal but its genetics have become highly sophisticated. The zebrafish model offers numerous mutant lines (Amsterdam et. al., 1999), a completed genome sequence, the ability to make transgenic strains, and a large research community (Woods et. al., 2000). Goldfish and the leopard frog do not currently provide such resources.

Medaka (*Oryzias latipes*) also offers many resources, including classical genetics, an extensive database in toxicology, molecular genotoxicity and carcinogenesis, a genome project, and existing inbred and transgenic lines (Abner, 1994; Ishikawa, 2000; Winn et. al., 2000, 2001; Wittbrodt et. al., 2002; Naruse et. al., 2004; Medaka Home Page, <http://biol1.bio.nagoya-u.ac.jp:8000/>). In addition, medaka has technologies not yet available for zebrafish, which include the See-Through (ST) medaka, which are devoid of most major pigments, allowing organs to be observed in living individuals (Wakamatsu et. al., 2001). Furthermore, medaka has a genome of about half the size of zebrafish; with lower gene redundancies medaka may be especially suited for comparative genomics and targeted mutagenesis (Amores et. al., 1998; Ishikawa, 2000; Taylor et. al., 2003). Because medaka and zebrafish share similar resources while still having unique advantages, together they serve as useful companion models.

Prior to our experimental infections of medaka with *M. marinum*, the only “natural” reported mycobacterial infections were by *Mycobacterium fortuitum* and *Mycobacterium abscessus* (Teska et. al., 1997; Sanders and Swaim, 2001) or unresolved species of *Mycobacterium* (Abner et. al., 1994). Histological studies of medaka from large colonies found high frequencies of granulomas, which have suggested endemic mycobacteriosis (Abner et. al., 1994). The only direct evidence we are aware of, for “natural” *M. marinum* infections in medaka were unpublished reports from facilities housing large medaka colonies (Camus, A., Mississippi

State University, and Hawkins, W., University of Southern Mississippi; personal communication). Although these retrospective studies suggested *Mycobacterium* species can establish chronic infections in medaka, we wished to better evaluate the suitability of this host to study chronic mycobacteriosis with a known strain of *M. marinum* under defined conditions.

Here, we present a new experimental model for tuberculosis using medaka as the host for *M. marinum*. We have demonstrated the ability to produce both acute and chronic infections in a dose dependent manner. A reproducible maximum chronic dose, 50% lethal dose (LD₅₀) and minimum acute dose has been established. Histology has revealed the formation of granulomas, a diagnostic lesion for human TB, in several visceral organs. To assist in tracking the progression of chronic infections, we have used a strain of *M. marinum* expressing green fluorescent protein (Gfp). Fluorescence has assisted us in not only monitoring the colonization of target organs, but also viewing formation of granuloma-like spherical aggregates of bacilli expressing Gfp within infected tissues. We have also begun to use Gfp-expressing bacteria to monitor infections, in situ, in live See Through (ST) medaka. Furthermore, a true chronic state of infection has been established in fish held out to 24 weeks post-infection. To compare our medaka-*M. marinum* model to the established zebrafish-*M. marinum* model, we have infected zebrafish under the same conditions as our medaka infections and have found the maximum chronic dose, LD₅₀, and course of infection to differ considerably.

2. Materials and methods

2.1 Bacterial Strains and Culture

M. marinum strain G13R (1218R/pG13), a strain derived from a fish outbreak isolate, was provided by Lucia Barker (University of Minnesota Medical School, Duluth, Minnesota) and was used in all experiments (Barker et. al., 1999). The pG13 plasmid allows for high constitutive expression of Gfp. All cultures of *M. marinum* were grown in Bacto™ Brain Heart Infusion (BHI) broth, with the addition of Tween 80 (0.2%) and kanamycin (80 µg/mL), at 30°C shaking. Prior to inoculation of fish, 100 mL cultures were collected at an optical density 600 nm of ~1.2, washed twice in phosphate buffered saline (PBS) (pH 7.4), and adjusted to the appropriate infective doses (colony forming units (cfu)/fish). Viable counts for each dose were determined by plating the appropriate serial dilutions onto BHI agar plates supplemented with kanamycin (80 µg/mL) and cycloheximide (100 µg/mL). Plates were incubated at 30°C for 7–12 days before counting colonies.

2.2 Medaka and Zebrafish aquaculture

Medaka (*Oryzias latipes*) (orange-red variety) were originally obtained from Richard Winn (University of Georgia, Athens, GA, USA). Medaka (were then propagated in the laboratory and housed in self-contained 10-gallon aquaria at a maximum density of 50 adult fish per aquarium with recirculating filtration. A photoperiod of 16 h light and 8 h dark was kept in all animal facilities. Twenty percent water changes were done twice monthly. Fish were fed twice daily with bulk flake food (40% protein) and once daily with brine shrimp (*Artemia sp.*).

Upon transfer to the BSL-2 laboratory for inoculation with *M. marinum*, fish were placed in self-contained 40 L aquaria, at 12 fish per aquarium. Filtration was provided by corner filters using activated carbon and polyester filter media. Ten percent water changes were done twice weekly. AmmoLock® and Stress Zyme® (Aquarium Pharmaceuticals, Inc., Chalfont, PA) were added to aquaria for the first three water changes, according to the manufacturer's instructions. This was done to counter any effect of ammonia or nitrite levels in the new aquaria while establishing a biological filter. The temperature was increased to 28°C at a rate of 2°C per day. Fish were allowed to acclimate to the new environment for two weeks prior to inoculation.

Zebrafish (strain AB) were obtained from Monte Westerfield (University of Oregon, Institute of Neuroscience, Eugene, OR, USA). Zebrafish were maintained under identical conditions as medaka. All infections and maintenance of infected animals were held in BSL-2 facilities; they were conducted as prescribed by NIH BMBL guidelines and approved by both the Institutional Biosafety Committee and by the IACUC.

2.3 Medaka inoculation with *M. marinum*

Prior to inoculation, fish were anesthetized with Tricaine Methanesulfonate (MS-222) (0.0175%). Medaka were injected with doses of 10^3 to 10^7 cfu *M. marinum*/fish. Doses were delivered by intraperitoneal injection with 20 μ l of bacteria suspended in PBS, using a 26-gauge needle. Negative-control infections, or “sham-infections” were fish inoculated with sterile PBS concurrently with experimentally infected fish and kept under the same environmental conditions. Survival, gross behavior, and mortality were monitored until at least eight weeks post-infection. The dose-response experiment (shown in Fig. 1) was repeated three times and yielded reproducible results. The results were averaged and used to calculate the LD₅₀ value, using the method of Reed and Muench (1938), and estimate the minimum acute dose and maximum chronic dose.

2.4 Zebrafish inoculation with *M. marinum*

Zebrafish were infected with *M. marinum* doses ranging from 10^4 to 10^6 cfu/fish, as described above for medaka dose-response experiments. As with medaka, PBS was injected in the “sham” control. Survival, gross behavior, and mortality were observed until eight weeks post-infection. The experiment was conducted twice, with highly reproducible dose responses. The results were averaged and used to calculate the LD₅₀ value, using the method of Reed and Muench (1938), and estimate the minimum acute dose and maximum chronic dose.

2.5 Plating for viable counts of Gfp-expressing *M. marinum* in infected organs

To determine viable counts of Gfp-expressing *M. marinum* in infected organs, livers and kidneys were removed from infected medaka at 2-week intervals from 2 to 8 weeks post-infection. Organs were weighed and then homogenized in 0.5 mL PBS. Appropriate serial dilutions were plated onto BHI agar plates supplemented with kanamycin (80 μ g/mL) and cycloheximide (100 μ g/mL). Plates were incubated at 30°C for 7–12 days before counting kanamycin resistant, Gfp-expressing colonies.

2.6 Histopathology

Medaka infected with 10^4 cfu *M. marinum*/fish were collected for histology at 2-week intervals from 2 to 14 weeks post-infection and also at 20 and 24 weeks post-infection. Fish were sacrificed following an overdose of MS-222 (0.1%). A single anterior to posterior incision along the abdomen of the fish was done to allow for penetration of the fixative. Fish were fixed in Bouin’s fixative for 2 days and then transferred to 10% neutral buffered formalin until further processing.

Medaka were processed for paraffin embedding and stained with hematoxylin and eosin stain or Ziehl-Neelsen’s acid-fast stain using standard techniques. Sections were evaluated for inflammation and granuloma formation, as well as the presence of acid-fast bacilli.

Zebrafish infected with 10^4 cfu *M. marinum*/fish were collected at 2 to 8 weeks post-infection and were fixed and prepared for histology as stated above for medaka. Hematoxylin and eosin stains were conducted, but Ziehl-Neelsen’s acid-fast staining was not carried out. Instead, infection was confirmed by observing Gfp expression within fresh organs.

2.7 Monitoring bacterial colonization by fluorescent microscopy

Microscopic inspection of whole fresh organs was performed using: (1) a NikonSMZ800 (Nikon, Tokyo, Japan) Stereoscopic Microscope equipped with X-Cite™ 120 for fluorescence illumination and a filter for detection of Gfp, and (2) an epifluorescence compound microscope fitted with a K2 SBIO accessory unit (Technical Instrument Company, San Francisco, CA), allowing for confocal microscopy; and using 10X SPlan Apochromat Olympus (N.A. = 0.3) and 30X Achromat water immersion LOMO (N.A. = 0.9) objectives. Organs were evaluated for the presence of dispersed bacilli expressing Gfp and aggregates of fluorescing bacteria; infection was confirmed by plating for colony counts of Gfp-expressing *M. marinum* (see above).

Live ST medaka infected with *M. marinum* expressing Gfp were anesthetized with MS-222 (0.01%) prior to observation under the stereoscopic microscope. Fish infected with 10^4 cfu/fish were inspected at 2-week intervals from 2 to 6 weeks post-infection.

3. Results

3.1 *M. marinum* establishes an acute or chronic infection in medaka in a dose-dependent manner

We wished to evaluate the suitability of medaka to study chronic mycobacteriosis with a known strain of *M. marinum* under defined conditions. To initiate these studies, we first established controlled infection conditions based on the goldfish model (Talaat et. al., 1998) in which known doses of an established pathogenic strain of *M. marinum* were administered by intraperitoneal injection. Fish were infected by a range of doses (10^3 – 10^7 cfu/fish) and their survival was monitored over time. The maximum chronic dose was determined to be approximately 10^5 cfu/fish and the LD₅₀ was about 10^6 cfu/fish, while 10^7 cfu/fish caused acute disease with >90% mortality at one week post-infection (Fig. 1). The LD₅₀ value was in turn calculated to be 2.6×10^6 cfu/fish. During the course of chronic infection by $\leq 10^5$ cfu/fish, the animals showed no gross signs of disease (Fig. 2). In contrast to the ulcerative skin lesions documented to occur in zebrafish and striped bass (*Morone saxatilis*) infected with *Mycobacterium* spp., neither inflammatory lesions nor any gross signs of inflammation were observed in medaka, at any time during infection (Fig. 2) (Rhodes et. al., 2001; Van der Sar et. al., 2004). The behavior of infected fish was judged to be normal; they remained active, continued to eat and lay eggs like the unexposed control group. At 8 weeks post-infection the average survival of two experiments for fish injected with 10^6 , 10^5 , 10^4 , 10^3 cfu/fish and the PBS control, was 17%, 83%, 73%, 95%, and 87%, respectively. Further, 3 fish infected with 10^4 cfu/fish were spared and held out to 24 weeks post-infection; they appeared normal and expression of Gfp was observed in various organs of these fish. On various subsequent experiments, numerous fish infected with either 10^4 or 10^5 cfu/fish have been held out to 24 weeks post-infection or longer and were found infected but, again, appeared normal. *M. marinum* expressing Gfp was recovered from the livers and kidneys of all of the experimentally infected medaka sampled. Generally, fish infected with doses of 10^4 or 10^5 cfu/fish had recoveries of 10^3 – 10^5 cfu/mg kidney or liver tissue between 2 and 8 weeks post-infection (data not shown). We conclude that these experimental infections of medaka by *M. marinum* establish a true chronic state of infection.

3.2 Histopathology of *M. marinum* infection in medaka

Control fish at all time points showed no signs of mycobacteriosis, lacking granulomatous lesions and acid-fast bacilli (Fig. 3A,B, and data not shown). Infected fish showed little sign of disease at two weeks post-infection, with only larger-than-normal melano-macrophage centers in the kidney. The first early granulomas were seen at 4 weeks post-infection within the anterior kidney, although the posterior kidney was not visible in the sections that were

examined. At 6 weeks post-infection, a large increase in the number and organization of granulomas was apparent in the kidney, along with the first signs of early granuloma formation in the liver (Fig. 3C,D). When the spleen was present in a section, it was highly infiltrated by inflammatory cells. Granulomas were not observed in the ovaries or testes. As shown in Fig. 3E and F, the number and structural organization of granulomas in the kidney and liver seemed to progress further at 8 weeks post-infection. After this point, granuloma formation in the kidney and liver seems to progress slowly until 24 weeks post-infection, the last time point examined. At 24 weeks post-infection, many highly organized granulomas are found in the kidney and in the liver (Fig. 3G,H). Acid-fast bacilli were observed within granulomas and surrounding tissues at all time points, although they were few in number (data not shown). These observations are consistent with a state of chronic infection. Inoculation with *M. marinum* resulted in the slow, but steady, accumulation of highly organized granulomas in kidneys and livers without severe inflammation.

3.3 Detection of Gfp-expressing *M. marinum* within fresh medaka organs

Consistent with the histology, a steady progression of colonization in kidneys and livers by *M. marinum* was observed through fluorescent microscopy of fresh organs (Fig. 4). At 2 weeks post-infection, few small foci of fluorescing bacteria were observed with an epifluorescent dissection microscope in both organs (Fig. 4A). This is supported by observations under compound epifluorescent microscopy of only small numbers of Gfp-expressing bacilli throughout both organs. At 4 and 6 weeks post-infection, a substantial increase in the number of fluorescing aggregates of bacteria was observed for both the livers and kidneys (Fig. 4B,C). It is also at this time that spherical aggregates of Gfp-expressing bacilli can be observed in livers and kidneys under compound microscopy (Fig. 4D). The spherical shape of the aggregates was confirmed by confocal microscopy and the aggregates are estimated to range in size of about 40–80 μm . We noted that the diameter of the spheres not only matches that of granulomas observed in histological sections, but their appearance also corresponds with the time-course that granulomas appeared post-infection in histological sections. At 24 weeks post-infection, macroscopic inspection of livers and kidneys revealed gross signs of granulomas, which were found to fluoresce intensely under fluorescent microscopy (Fig. 4E,F). When comparing the number of Gfp-expressing bacilli and acid-fast bacilli, greater numbers of Gfp-expressing bacilli were apparent in tissues compared to acid-fast bacilli in histological sections of tissues at the same time point.

3.4 Colonization of See-Through medaka by *M. marinum* expressing Gfp

Early attempts to infect ST medaka with doses of 10^5 cfu *M. marinum*/fish resulted in acute infection. Because it seemed that ST medaka may be more susceptible, doses of 10^4 cfu/fish or lower were used in subsequent infections and resulted in a chronic state of disease. This permitted real-time monitoring of the disease progression in live animals. Infections were observed to spread from the point of injection to the kidneys at 2 to 4 weeks post-infection. Colonization of the liver and spleen was observed at 4 to 6 weeks post-infection (Fig. 5). The timing of colonization of the targeted organs is in agreement with that observed in both histological examination and observation of whole infected organs under fluorescent microscopy. Colonization of regions other than known target organs was apparent in ST medaka. Fluorescence was observed in regions believed to include the swim bladder and peritoneal lining. This was not observed in histological sections probably because tissues from these regions were not adequately represented in sections which were prepared.

3.5 Zebrafish are more susceptible than medaka to *M. marinum* infection

In zebrafish experimentally infected with *M. marinum* under the same conditions as described for medaka, the maximum chronic dose was determined to be approximately 10^4 cfu/fish. The

LD₅₀ was 1.9×10^5 cfu/fish, with an acute response at 10^6 cfu/fish (Fig. 6). By each of these criteria, zebrafish are judged to be about 10-fold more susceptible to *M. marinum* infection than medaka under these experimental conditions.

At a gross level of observation, behavior, such as swimming and eating, were not affected for zebrafish infected with a chronic dose (i.e., 10^4 cfu/fish). However, ulcerative lesions were observed on the abdominal region of all fish infected, even with a chronic dose. The observed lesions were at the site of injection, as well as other regions of the abdomen (Fig. 7A). These lesions progressed in number and severity out to 8 weeks post-infection. Individuals infected with an acute dose showed reddening of the abdomen by 24 hours post-injection and had inflammation in the cranial region prior to death (Fig. 7B). This contrasts with observations of medaka infected with *M. marinum*, which never showed any external signs of inflammatory lesions or inflammation of any type.

All infected zebrafish that were sampled showed Gfp expression in the visceral cavity and organs, while no Gfp expression was seen in negative-control fish (data not shown). Histological examination revealed that, as early as 4 weeks post-infection, large granulomas and progressive inflammation can be observed in the kidney, liver, and visceral cavity. This inflammation was observed to constitute up to one-half the area of livers and kidneys at this time point. Granulomas and inflammation were also observed in the testes and ovaries of zebrafish. The state of inflammation continued to progress from 6 weeks to 8 weeks post-infection, with formation of highly organized granulomas and diffuse inflammation taking place in various regions of the liver, kidney and the visceral cavity (Fig. 7C–F). In one individual sampled at 8 weeks post-infection, highly organized “onion ring” granulomas were observed throughout the viscera of the fish (Fig. 7F). This type of granuloma is histologically similar to some human tuberculosis cases (Nambuya et. al., 1988; Pozos and Ramakrishnan, 2004). In all other individuals sampled, only diffuse inflammatory lesions and large granulomas lacking the “onion ring” morphology were present.

4. Discussion

A major goal of this project was to evaluate the utility of *M. marinum* and medaka to model human tuberculosis. Although we have learned of possible infections of medaka colonies by *M. marinum*, no published accounts, that we are aware of, exist for *M. marinum* infection in medaka. However, mycobacteriosis in medaka and other laboratory fish has been documented in numerous publications (Abner et. al., 1994; Teska et. al., 1997; Sanders and Swaim, 2001; Kent et. al., 2004; Watral and Kent, 2006). Monitoring for the presence of mycobacteria in populations of fish has proven to be difficult, and the potential for low-level background infections is a common risk-factor, especially in large research facilities. Unforeseen mycobacterial infections of animals may complicate and confound research involving infected animals. For this reason, better characterization of mycobacteriosis in fish is of importance as small fish models are more widely used for research purposes.

Three replicates of the dose-response experiment were performed and yielded essentially the same results, indicating that this medaka-*M. marinum* infection model is highly reproducible. The true chronic nature of the infection was demonstrated by infected fish surviving and appearing healthy out to 24 weeks post-infection and by recovery of Gfp-expressing *M. marinum* from all fish sampled. An immunologic manifestation typical of mycobacteriosis was demonstrated through histopathological examination of the entire fish. Slow but progressive granuloma formation in the liver and kidney, as well as inflammation of the spleen, were observed.

The risks of background mycobacterial infections in the medaka population used is reduced due to the precautions taken in our animal husbandry practices. Further, our inspections of bacteria from control fish and routine inspection of our medaka colony during these experiments did not reveal any bacterial colonies matching the morphology of *Mycobacterium* species. Also, granuloma formation was not observed in sampled control fish.

Granulomas were shown to occur in kidney tissue at an earlier time point post-infection compared to liver. Earlier onset of granulomas in kidneys may be due to the role that the anterior kidney plays as the primary hematopoietic organ in teleosts. The anterior kidney of teleost fish is structurally analogous to mammalian bone marrow and contains monocytes, macrophages, neutrophils and lymphocytes (Zapata, 1979; Moore and Hawke, 2004). In medaka, the kidney contains many melano-macrophage centers, while in the liver such structures are unusual. The roles of melano-macrophage centers is thought to include humoral and inflammatory immune responses, storage and destruction of endogenous and exogenous agents, and iron recycling (Wolke, 1992). The fact that such aggregates of macrophage cells exist in kidneys and not in liver tissues prior to infection may account for the early colonization and immune response in kidneys compared to livers. We speculate that *M. marinum* may be trafficked by macrophages to the kidney prior to the liver.

Our ability to use Gfp as a reporter for observing *M. marinum* in fresh tissues proved to be a fast and convenient means to detect the spread to and colonization of host target organs. In situ monitoring of fluorescent bacteria in the See-Through (ST) medaka also permitted us to conveniently document over time, the infectious loads of target organs and where bacteria were sheltered at different stages of disease in the living animal. The colonization sequence and the temporal disease progression in the ST animals was very close to what we observed by other methods. The sensitivity and numbers of bacteria observed in tissues through the use of Gfp was far greater than by acid-fast staining. We presume that the difference in sensitivity likely reflects well documented underrepresentation of stained bacilli embedded in fixed tissues using common acid-fast staining techniques (Nyka, 1967, 1974).

We chose 28°C to conduct our infections because it is within the optimum temperature range for *M. marinum* (28–30°C), a temperature that is also ideal for medaka growth. In order to directly compare the infection process between both medaka and zebrafish, all conditions, including temperature, were the same. Zebrafish under these controlled conditions with known doses of *M. marinum* were significantly more susceptible than medaka. This was demonstrated by a ten-fold lower maximum chronic dose, LD₅₀, and acute dose for zebrafish. Moreover, inflammation of the entire visceral cavity, including most organs, progressed more rapidly in zebrafish and appeared more severe than in medaka. Medaka infections resulted in a number of well organized granulomas, mainly in the liver and kidney. Discrete foci of infection were apparent, and did not result in progressive spread and inflammation of the entire organ. In contrast, zebrafish infections almost always resulted in progressive inflammation occupying almost the entire organ by 8 weeks post-infection. Zebrafish also demonstrated external signs of infection through the appearance of ulcerative lesions in the abdominal region of all infected fish. Acutely-infected zebrafish presented inflammation of the cranial region and reddening of the abdomen. Chronically-infected medaka were very different, never exhibiting abnormal behavior nor presenting any external signs of disease; indeed, they were indistinguishable from the uninfected medaka. Acutely infected medaka ate less than normal and stayed at the bottom of the aquaria, but showed no external lesions nor gross signs of inflammation before succumbing.

When comparing our results in zebrafish with previously reported studies (Prouty et. al., 2003), some differences were noted; in particular, previously reported infections appeared milder. From the published data, we estimate the LD₅₀ at one week post-infection for zebrafish

lies between 10^6 and 10^7 cfu/fish. This was at least ten-fold higher than the LD_{50} of 1.9×10^5 cfu/fish that we determined. Their maximum chronic dose of about 10^5 cfu/fish was also about ten-fold higher. The previously published infections were conducted at room temperature, which was estimated to be at or below 25°C (K. Klose, University of Texas at San Antonio; personal communication), and the lower temperatures may account for the reduced susceptibilities. This explanation seems plausible since 25°C is below the optimal growth temperature for the bacterium. These differences in susceptibilities of zebrafish in the previous study and in our work cannot be explained by differences in *M. marinum* strains, since, in both cases, the same strain was used. Alternatively, the strain of zebrafish used may have been different but was not identified in the previous study (Prouty et. al., 2003). Reduction in virulence by conducting experiments at temperatures below the pathogen's optimum temperature is also consistent with our preliminary infection studies with medaka (data not shown). These observations, in turn, suggest that propagating fish at lower temperatures might be a simple means to reduce disease complications associated with chronic mycobacteriosis in fish colonies.

The research group headed by Ramakrishnan has used *M. marinum* expressing Gfp to pioneer the innate immune response of zebrafish embryos (Davis et. al., 2002; Volkman et. al., 2004). These elegant studies documented the trafficking of *M. marinum* within macrophages of infected embryos in real time (Davis et. al., 2002) and examined the effects of virulence factors on granuloma formation (Volkman et. al., 2004). Monitoring pathogenesis by following these fluorescing bacteria within zebrafish embryos has been facilitated by the embryo's lack of pigment at early stages of development. One disadvantage of this zebrafish system is that during development the embryos and larva progressively develop pigment obscuring the fluorescent bacteria. We anticipate complementing this research on embryos by using See-Through medaka (Wakamatsu et. al., 2001) to monitor real time disease progression, in adult animals with mature immune responses. Our initial infections on ST adults have also demonstrated the feasibility of detecting where bacteria are sheltered at different stages of the disease including outside the targeted organs.

Trucksis and coworkers have used goldfish as a host to identify *M. marinum* virulence factors using signature-tagged mutagenesis (Ruley et. al., 2004). Because, the goldfish model, as well as the leopard frog model, are not highly tractable genetic models, those hosts will not likely be useful in elucidating host factors that play roles in infection. Medaka and zebrafish are not only more convenient to maintain and propagate, but the availability of their excellent classic genetics and molecular genetic technologies offers the promise of a detailed understanding of host-pathogen interactions. We expect that medaka and zebrafish models each offer important, but different advantages in studying mycobacterial infections. Specifically, the zebrafish model may offer better insights into acute mycobacteriosis, while the medaka model may be better suited for studying life-long chronic infections. We anticipate that these highly tractable fish hosts and pathogen models will offer important insights into human tuberculosis.

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Fig 1.

Establishing chronic and acute doses of *M. marinum* in medaka. Survival of medaka infected with differing doses of *M. marinum* by intraperitoneal injection. Fish were injected with 10^3 – 10^7 cfu *M. marinum*/fish. Control fish were injected with PBS. Twelve fish were used per dose. The above graph represents one of three experiments performed, each of which yielded similar results. The LD₅₀ was calculated to be 2.6×10^6 cfu/fish and the maximum chronic dose was approximately 10^5 cfu/fish.

Fig 2.
Gross inspection of medaka post-infection. (A) Negative-control medaka at eight weeks post-injection with PBS showing no gross signs of disease. (B) Medaka injected with 10^4 cfu *M. marinum*/fish at eight weeks post-infection, also absent of gross signs of disease. Scale bars: 1 mm.

Fig. 3.

Time-course of *M. marinum* infections in medaka target organs. Histopathology was conducted on fixed sections of medaka that were experimentally infected with 10^4 cfu *M. marinum*/fish. (A) Control/uninfected kidney. (B) Control/uninfected liver. (C) Kidney at 6 weeks post-infection showing granuloma formation typical at this stage. (D) Liver at 6 weeks post-infection showing the early formation of two granulomas; the first time point at which granulomas are observed in the liver. (E) Highly structured granuloma and two small granulomas in the kidney at 8 weeks post-infection. (F) Infected liver at 8 weeks post-infection, showing progression of granuloma organization. (G) Kidney at 24 weeks post-infection showing typical well-organized granulomas. (H) Liver at 24 weeks post-infection with numerous organized

granulomas. Hematoxylin and eosin stain used in all sections pictured. Scale bars: 100 μm . Granulomas are indicated by arrows. GL, glomerulus; T, renal tubule.

Fig. 4.

Monitoring time-course of *M. marinum* colonization of medaka by fluorescent microscopy. Fresh tissues from medaka infected with 10^4 cfu/fish *M. marinum* expressing Gfp were examined for fluorescence. (A–C) Progressive colonization of medaka kidney tissue by *M. marinum* shown by Gfp expression under low resolution epifluorescence at (A), 2 wks.; (B), 4 wks.; and (C), 6 wks. post-infection. (D) Spherical aggregate of Gfp expressing bacilli in a kidney at 6 weeks post-infection shown under epifluorescence; the spherical shape of aggregates such as this one was confirmed by confocal microscopy. (E) Liver (with attached spleen (S) and gallbladder (G)) from medaka at 24 weeks post-infection shown in brightfield; notice the gross signs of granulomatous sites of infection in the liver and spleen. (F) Same as (E) at a higher magnification under epifluorescence. Granulomatous lesions observed in (E) fluoresce brightly in the liver, but fluorescence in the spleen is not apparent; this is the typical observation. Scale bars: (A–C, E, F), 1 mm; (D), 10 μ m. (D) was imaged with a black and white camera, then color was reapplied with Adobe® Photoshop® to match original fluorescence observed. Arrows indicate positions of representative granulomas. G, gallbladder; S, spleen.

Fig. 5. See-Through medaka infected with 10^4 cfu/fish *M. marinum* expressing Gfp at 6 weeks post-infection. (A) Fluorescent image showing colonization of various organs with *M. marinum* expressing Gfp. (B) The same fish shown in brightfield. Locations of organs are indicated as follows: a, air bladder; g, gills; gu, gut; h, heart; k, kidney; l, liver; t/f, testis/fat. Scale bars: 1 mm.

Fig. 6.

Establishing chronic and acute doses of *M. marinum* in zebrafish. Survival of zebrafish infected with differing doses of *M. marinum* by intraperitoneal injection. Fish were injected with 10^4 – 10^6 cfu *M. marinum*/fish. Control fish were injected with PBS. Twelve fish were used per dose and colors for each dose correspond to Fig. 1. The above graph represents one of two experiments performed, both of which yielded similar results. The LD_{50} was calculated to be 1.9×10^5 cfu/fish and the maximum chronic dose was approximately 10^4 cfu/fish.

Fig. 7.

Zebrafish mycobacteriosis. (A) Typical ulcerative lesion observed on the surface of a zebrafish infected with 10^4 cfu *M. marinum*/fish at 2 weeks post-infection. (B) Zebrafish infected with 10^6 cfu *M. marinum*/fish at moribund, showing reddening of the abdomen and swelling of the cranium. (C) Control kidney showing normal tissue architecture. (D) Kidney at 8 weeks post-infection showing typical inflammation. (E) Liver at 8 weeks post-infection showing typical large area of inflammation with some organization suggestive of granuloma formation. (F) Liver showing numerous “onion peel” granulomas, which were only observed in one individual, and such granulomas were found throughout the viscera. Hematoxylin and eosin stain used for (C–F). Scale bars: (A,B), 1 mm; (C–F), 100 µm.