Source or Sink: Examining the Role of Biofilms in Transmission of *Mycobacterium* spp. in Laboratory Zebrafish

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Abstract

Zebrafish health is a primary research concern because diseases can have unintended impacts on experimental endpoints. Ideally, research would be conducted using disease-free fish or fish with known disease status. Mycobacteriosis is a common bacterial disease in wild and captive fishes, including zebrafish. Despite its prevalence, the dynamics of transmission and potential sources of mycobacterial infections in zebrafish are only partially understood. One suspected natural infection source is surface biofilms on tanks and other system components. This study investigates the role that tank biofilms play in mycobacteriosis in laboratory zebrafish by evaluating the establishment of biofilms from bacteria shed from fish, and conversely, the acquisition of infections in fish from surface biofilms. We found that zebrafish infected with *Mycobacterium chelonae* shed bacteria through feces, and bacteria are transmitted to tank biofilms from one to 16 weeks postinfection. We also found that zebrafish acquire *M. chelonae* infections as soon as 2 weeks when introduced to tanks with established *M. chelonae* biofilms. The results from this study highlight the role that tank biofilms play as both a reservoir and source of mycobacterial infections in zebrafish. Results support the inclusion of biofilm surveillance and prevention as part of a disease control program in zebrafish research facilities.

Keywords: zebrafish, biofilm, *Mycobacterium* spp., *Mycobacterium chelonae*, husbandry

Introduction

THE ZEBRAFISH (*Danio rerio*) is a popular aquatic model organism, and recognized as one of the “rising stars” among aquatic animal models of human disease.¹ With increased popularity and investment from the research community, increased value is placed on the maintenance of healthy stocks of both mutant and wild-type lines. The health of laboratory zebrafish is a primary concern, as it is prudent to maintain and conduct research on healthy organisms.

Ideally, research would be conducted on animals free of background infections, and if this is not possible, the disease status of the research subjects should be reported. The impacts of such infections is sometimes obvious when resulting in mortality or declines in reproductive fitness, but equally concerning are subclinical infections that can be a source of uncontrolled experimental variance.² Due to these concerns,

measures to prevent and monitor disease in laboratory zebrafish should be taken.

One common infection in laboratory zebrafish is mycobacteriosis. Mycobacteriosis affects ~40% of zebrafish facilities submitting cases to the Zebrafish International Resource Center's (ZIRC) Diagnostic Services.³ There is no single etiological agent of mycobacteriosis, as it is caused by several species and strains of *Mycobacterium*.^{4,5} The severity of mycobacterial infections in zebrafish is variable and species specific, ranging from high mortality with *Mycobacterium marinum* and *Mycobacterium haemophilum*, to few or no mortalities with *Mycobacterium abscessus* and *Mycobacterium chelonae*.^{6–9} Similarly, morbidity is also variable and includes nonpathognomonic signs, including skin lesions, emaciation, raised scales, swollen abdomen, and irregular or lethargic behavior.^{4,10} Internal signs include granulomas, particularly on hematopoietic organs,^{4,11} and in some cases

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diffuse, chronic inflammation.⁸ Subclinical infections lack any observable signs and may go unnoticed.^{9,11}

Control and management measures for mycobacteriosis in zebrafish facilities highlight the importance of prevention, as established infections are difficult to eliminate.^{9,12} Current preventative measures include quarantine of imported fish and those that appear morbid, disinfection of embryos and fomites, ultraviolet sterilization of water, and sentinel programs.^{9,13} While these measures have, and continue, to effectively limit the establishment of mycobacterial infections in zebrafish facilities, more insight is needed into additional factors contributing to this disease. One factor that has been speculated to be involved in the mycobacterial disease cycle in zebrafish facilities is surface biofilms.

Nontuberculosis mycobacteria normally inhabit a variety of habitats that are shared by both humans and animals, including water distribution systems.¹⁴ Piscine mycobacteria species are able to persist in surface biofilms that form in aquaria.^{5,15} The hydrophobic and oligotrophic characters of mycobacteria allow for survival in environments with low levels of dissolved carbon while readily adhering to surfaces.^{14,16} Thus, mycobacteria are well adapted to survive in "clean" water systems such as aquaria.⁹

Many different species of mycobacteria can be found in biofilms,¹⁷ with several species isolated specifically from aquaria.^{1,5,8,9} In zebrafish facilities, there have been reports of detection of the same species of mycobacteria in both biofilms and fish, but many more species are only found in the biofilms.^{5,8} In addition to bacterial communities within aquatic biofilms, protozoans are an important component of biofilms.¹⁸ Protozoans rapidly colonize aquatic biofilms, occurring in high abundances.¹⁸ These biofilm-dwelling protozoans may support bacterial survival and virulence as the protozoan/bacteria interaction allows for survival, replication, and distribution of pathogenic bacteria.¹⁹

Genetic comparisons between *Mycobacterium* isolates identify identical strains in both fish and biofilms of *M. chelonae* in zebrafish⁵ and *M. marinum* in pompano *Trachinotus carolinus*.²⁰ These results demonstrate a link between fish and biofilms, but not the direction of transmission, that is, whether the fish are the source for biofilms, biofilms the source for the fish, or that both are possible.

Biofilms can be found on all surfaces in the aquatic environment, and it is thought that these biofilms and detritus at the bottom of tanks are a source of mycobacterial infections in zebrafish.⁹ In a study where zebrafish found in the sump tank were examined, they were found to be infected with mycobacteria.⁹ The only source of food for these fish was the surface biofilms and tank detritus. This is consistent with what is known about the feeding habits of zebrafish, as they feed in the water column and on tank surfaces and substrates²¹ as generalist consumers.²² Sentinel zebrafish residing in system sumps have also been observed to hunt for benthic organism and slow zooplankton along the biofilm scaffold.⁹ Larvae (8–10 mm SL) are very active as hunters and grazers, which may result in the incidental consumption of mycobacteria.⁹

A previous study found that zebrafish in larger tanks had a higher prevalence of *M. chelonae* infections compared with fish in smaller tanks.²³ This difference in prevalence was attributed to the different tank cleaning methods used for removing biofilms for the two tank sizes. The zebrafish in the

larger tanks remained in the tanks during cleaning compared with zebrafish in the smaller tanks, which were swapped into new tanks before tanks were cleaned. The fish in the larger tanks were likely feeding on the tank debris that were suspended in the water column during the cleaning while the fish in smaller tanks were not exposed to this. In addition, the oral route of infection has been demonstrated as a natural mode of mycobacterial transmission in zebrafish.²⁴

Conversely, support for biofilms as the sink for mycobacteria includes the natural shedding of bacteria from infected fish,^{9,25} which contributes to biofilms. A potential advantage of this is that biofilms or feces shed from fish can be used as diagnostic samples for screening fish facilities.²⁶ However, the specific timing of mycobacterial shedding and subsequent uptake into tank biofilms from zebrafish has not yet been studied.

The goal of this study is to investigate the route of transmission of mycobacteria between zebrafish and biofilms and to elucidate the role of biofilms as source or sink in these infections. We first investigate the transmission of mycobacteria from zebrafish with established *M. chelonae* infections (both through experimental intraperitoneal [IP] injection infection and also experimental oral ingestion infection) to feces and tank biofilms over a 16-week period. We then investigate the transmission of *M. chelonae* green fluorescence protein (GFP) mutant from established tank biofilms to *casper* (*nacre*^{-/-} *roy*^{-/-}) zebrafish over a 16-week period, using biweekly live fluorescent imaging of zebrafish. We hypothesize that biofilms play both roles and simultaneously are a source of infection at the same time as being a sink for mycobacteria shed from infected zebrafish. Results from this study will contribute toward our understanding of zebrafish mycobacteriosis and contribute to disease control and management improving the overall health of laboratory zebrafish.

Methods

Fish

All fish used in this study were bred and maintained in the zebrafish facility at the SUNY-ESF Center for Integrated Teaching and Research in Aquatic Science. Adult AB wild-type zebrafish ($n=144$; 72 male and 72 female; age=6 months) and *casper* (*nacre*^{w2/w2}; *roy*^{a9/a9}) zebrafish ($n=30$; 15 male and 15 female; age=6 months) lines, originally obtained from the SARL, a specific pathogen-free facility for *P. neurophilia* at Oregon State University (Corvallis, OR), and bred for two generations at SUNY-ESF, were utilized in this study. It should be noted that our facility is only stocked with zebrafish originating from this facility. Animals were housed at a density of 6–10 fish/liter in 1.8 L tanks on a timed, flow-through housing system (Aquaneering, San Diego, CA).

The housing system included ultraviolet disinfection of dechlorinated (carbon filter) municipal tap water as the source, which was maintained at pH 7.6, conductivity of 600–700 $\mu\text{S}/\text{cm}^2$, a temperature of 28.5°C, and ammonia levels ranging from 0 to 0.25 ppm. The zebrafish facility maintained a 14:10 light:dark photoperiod. Fish were fed a commercial feed for zebrafish (Gemma, Skretting) twice daily on weekdays and once daily on weekends during periods where they were not being fed a treatment gelatin feed. Before the experiment, routine cleaning of all equipment (e.g., tanks, lids, baffles, nets, and tubing) consisted of biweekly washing and scrubbing in

warm water with a new soft sponge and bleaching in 1000 ppm chlorine bleach for 30 min, followed by rinsing three times in dechlorinated water and drying. All tanks, lids, and baffles were also autoclaved using a program specified by the tank manufacturer that reaches a temperature of 105–110°C for 15 min. During experiments, this tank cleaning procedure occurred when placement of fish in a new tank, as mentioned in the methods below. All animal work was approved by the SUNY-ESF Institutional Animal Care and Use Committee, protocol #151001.

Transmission to biofilms

Experimental infections. Before the setup of experimental groups, mycobacterial infections or sham injections were established in the fish.

IP Injection: *M. chelonae*-injected wild-type zebrafish ($N=36$ fish) were injected IP with 5.0×10^4 cfu/fish of *M. chelonae* (H1E2) mutant expressing GFP provided by M.L. Kent, and sham-injected fish ($N=36$) injected with sterile saline, following the method described in Watral and Kent.⁶ The concentration of the bacterial inoculum was determined using a nephelometer (Sensititre) and confirmed with colony counts following culture on Middlebrook 7H10 agar plates. After IP injection, fish were allowed to recover in a recovery tank and then fish from each injection type (*M. chelonae* or sham) were randomly divided equally into three new tanks (12 fish/tank) and maintained on the flow-through system for 1 week before the beginning of the experimental sampling. During this week period, fish were fed a commercial feed (Gemma, Skretting) twice daily on weekdays and once daily on weekends.

Oral Ingestion Infection: *M. chelonae*-ingested wild-type zebrafish ($N=36$) were experimentally infected through the ingestion of a gelatin feed containing *M. chelonae* (H1E2) mutant expressing GFP through five consecutive daily feeds containing 1.0×10^4 cfu/feed/fish to reach a total ingestion of 5.0×10^4 cfu/fish. Gelatin feed (Gelly Belly, Florida Aqua Farms) was prepared as described in a previous study.²⁷ Once gelatin feed was set overnight at 4°C, a flame-sterilized metal spreader was used to spread a known concentration of *M. chelonae* culture diluted in sterile water to make a final volume of 1 mL. The inoculated gelatin plate was then dried for ~24 h at 28.5°C in an incubator. Following this, a flame-sterilized razor blade was used to mince the gelatin, and aliquots for each treatment tank were measured by mass and stored at -20°C until feeding.

At feeding, aliquots were emptied into treatment tanks. Sham-ingested wild-type zebrafish ($N=36$) were fed a gelatin feed containing sterile saline for five consecutive daily feeds. After ingestion infection, fish from each ingestion type (*M. chelonae* or sham) were randomly divided equally into three new tanks (12 fish/tank) and maintained on the flow-through system for 1 week before the beginning of the experimental sampling. During this week period, fish were fed a commercial feed (Gemma, Skretting) twice daily on weekdays and once daily on weekends. Tanks were siphoned daily following feeding during this period.

Sample collection and processing. Sample collection started on the second week postinfection. Sampling was repeated weekly for 16 weeks in total. For each sampling event,

zebrafish from each treatment tank were transferred to a new tank and incubated overnight in the new tank on the benchtop (static water). The old tank was also kept on the benchtop. Following overnight incubation, a biofilm swab sample was taken for each old tank as previously described (Whipps *et al.* 2007) and stored in 1% cetylpyridinium chloride (CPC)²⁸ in a sterile 2.0 mL screw cap tube. Collection of feces/detritus from the overnight incubation tank was done using a sterile disposable pipette and stored in 200 μ L 1% CPC in a sterile 2.0 mL screw cap tube. Biofilm and fecal samples were stored in 200 μ L 1% CPC tubes for 2 h at room temperature before sample processing. Following sample collection, fish were returned to their old tank and placed back on the zebrafish system racks unless otherwise noted.

Processing of the biofilm swab samples consisted of swab samples stored in 2.0 mL screw cap tubes containing 200 μ L 1% CPC being vortexed vigorously for 10 s. Next, flame-sterilized forceps were used to remove the swab, which was disposed of in 95% EtOH. To the remaining solution in the screw cap tube, 900 μ L of autoclaved dH₂O was added, followed by vortexing. Tubes were then centrifuged for 10 min at 8000 *g* to form a pellet. The supernatant was removed, 100 μ L of autoclaved dH₂O was added, and tube vortexed. Next, 10 μ L of this solution was streaked onto a Middlebrook (MC) 7H10 agar plate, which was sealed with Parafilm and incubated at 28°C for 5–7 days. Following incubation, resulting colonies were observed under fluorescence to identify those colonies expressing GFP. The number of tank biofilm swabs resulting in the growth of GFP-positive colonies was recorded.

To avoid reidentifying the same *M. chelonae*-positive biofilm samples, the following procedure was followed to identify when biofilm *M. chelonae*-positive tanks needed to be switched out with new tanks. In the case that two or more of three replicate tanks for each treatment were recorded as positive, following the next sampling occurrence (1 week from determining 2/3 or more positive samples), fish incubated overnight for sample collection were placed back on system racks in the new tank instead of returning to their old tank so that future observation of transmission of bacteria to tank biofilms could be detected.

For fecal/detritus samples in 2.0 mL tubes containing 200 μ L 1% CPC, they were first homogenized using a sterile plastic tissue pestle. Next, 900 μ L of autoclaved dH₂O was added, followed by vortexing. The vortexed tubes were centrifuged, pellet resuspended in water, and plated on MB agar plates as the biofilm samples described previously. The number of tank fecal samples resulting in the growth of GFP-positive colonies was recorded.

End analyses. After the 16th week of sampling, all fish were euthanized by submersion in 0.3 g/L MS222 buffered to a pH of 7.5 for a minimum of 10 min following the loss of opercular movement. Once euthanized, fish were dissected to create an ~5 mm by 5 mm window on the lateral side so that internal organs could be observed under FITC fluorescence for signs of GFP expression. Images were taken when GFP expression was observed. Liver and spleen samples were also taken and stored for 1 h in 1% CPC followed by the same homogenization, pellet, and culture procedure described above. Following imaging, euthanized fish were fixed in Davidson's solution for 48 h and then dehydrated to 70% EtOH for histology.

Each fish was sectioned and stained with hematoxylin and eosin (H&E) and Kinyoun's acid fast stain. Following staining, slides were examined for granulomas in H&E sections. For acid fast-stained sections, the presence of granulomas containing acid fast bacilli (AFB) was recorded. The location of granulomas containing AFB was recorded, and the location of AFB not contained in granulomas was also recorded. AFB not within granulomas were considered to be internally located if they were located inside the epithelial boundary (e.g., not in the gut lumen or on the outside of the skin/scales). The prevalence of mycobacterial infections was calculated based on any positive reading of granulomas, granulomas containing AFB, and/or free AFB.

Statistics

There was detection of bacteria in sham-infected fish (both IP injection and oral feed) for a single time point and these groups were excluded from further analyses and figures. For each set of three replicate tanks for each infection type (*M. chelonae* IP injection, *M. chelonae* gelatin feed), a chi-squared analysis with a Monte Carlo simulation method using 20,000 replications was used to compare replicate tanks to determine whether data from replicate tanks can be pooled for further comparisons. No significant differences between replicate groups were identified, and replicate data for each infection-type group were pooled. Fisher's exact test for count data was used to compare prevalence between infection types using the "stats" package.²⁹ Comparisons of prevalence were made between diagnostic methods (*in vivo* GFP expression, liver/spleen culture, and histology) as well as between sexes. The results were organized into bar plots using the "sciplot" package.³⁰

Transmission from biofilms

Experimental biofilm setup. An experimental biofilm was created in three 1.8 L tanks (Aquaneering). Cleaned and autoclaved tanks were filled with zebrafish system water and inoculated with *M. chelonae* (H1E2) mutant expressing GFP that was diluted to 2.7×10^8 cfu/mL, verified by a nephelometer (Sensititre). Following dilution, 3 mL of the diluted bacteria was inoculated into each tank and tank water was stirred using a sterile 25 mL graduated pipette. Tanks were incubated on the benchtop for 4 weeks at room temperature and topped off with zebrafish system water to maintain the water level. Each week, a biofilm swab was taken and processed as previously described. Following 2 consecutive weeks of isolation of GFP colonies from all three tanks, the tanks were emptied, rinsed, filled with fresh zebrafish system water, and placed on the flow-through zebrafish system. Tanks were then checked weekly to confirm the isolation of GFP colonies from the biofilm of all three tanks throughout the experiment.

Tagging zebrafish with individual markers. Adult AB *casper* (*nacre*^{w2/w2}; *roy*^{a9/a9}) zebrafish ($n = 30$; 15 male and 15 female; age = 6 months) were individually tagged so that fish could be individually tracked over the course of the study. Each of the three tanks received 10 *casper* zebrafish (5 male, 5 female) with the following tag designations: male, no tag; male, right-side pink tag; male, right-side orange tag; male,

left-side pink tag; male, left-side orange tag; female, no tag; female, right-side pink tag; female, right-side orange tag; female, left-side pink tag; female, left-side orange tag. Before tagging, fish were anesthetized in 0.15 g/mL MS222 buffered to a pH of 7.5. Following anesthesia, fish were placed in an autoclaved sponge soaked in 0.15 g/L MS222 with a slit cut in the middle of the sponge to stabilize the fish with its dorsal side exposed. For tagging, a manual injection kit for visible implant elastomer (VIE) tags was used (Northwest Marine technology, Inc., Shaw Island, WA, www.nmt.us). Elastomer was prepared according to the manufacturer's instructions (Northwest Marine Technology, Inc., 2011, "Manual elastomer injection systems. Instructions for 10:1 visible implant elastomer," www.nmt.us/products/vie/manual_vie_instructions.pdf). Tags were injected following the procedure described,³¹ with the syringe needle inserted 2 mm further than the desired tag location, followed by elastomer expression and needle retraction until ~1 mm before the injection site. Tags were placed below the dorsal fin base, as a study³¹ found this site to be optimal for tag retention and visibility. Following tag insertion, fish were put into a recovery tank filled with zebrafish water. Following recovery, fish were placed in system tanks and observed for tag retention and tag-related morbidity for 2 weeks before placement in biofilm tanks.

Biweekly live-imaging. Live-imaging occurred immediately before placing fish in the biofilm tanks, and biweekly thereafter. For live-imaging, fish were anesthetized in 0.15 g/L MS222. Following anesthetization, tag location and color were noted and fish were imaged under FITC fluorescence to detect GFP expression. If GFP expression was detected, an image was taken and the location of the GFP expression was noted. Following imaging, fish were placed in a recovery tank containing fresh zebrafish system water. Once, recovered, fish were placed back in their respective biofilm treatment tank. If a fish appeared moribund, it was euthanized in 0.3 g/L MS222, fixed, and stored as previously described.

End analyses. After the 16th week of sampling, all fish were euthanized as above. Once euthanized, four representative fish from each tank (two male, two female) were dissected to create an ~5 mm by 5 mm window on the lateral side so that internal organs could be observed under fluorescence for signs of GFP expression. Images were taken when GFP expression was observed. Liver and spleen samples were also taken and stored in 200 μ L sterile dH₂O followed by the same homogenization, pellet, and culture procedure described above. DNA extraction from the liver/spleen homogenate was performed using the MO BIO Laboratories, Inc. UltraClean[®] Microbial DNA Isolation Kit following the manufacturer's protocol. Next, an *M. chelonae*-specific PCR was performed using primers described by Meritet *et al.*,³² and a conventional thermocycler program followed by an evaluation of PCR products by gel electrophoresis. All euthanized fish were fixed in Davidson's solution for 48 h, then transferred to 70% EtOH, and processed for histology.

Statistics

A chi-squared analysis with a Monte Carlo simulation method using 20,000 replications was used to compare

replicate tanks to determine whether prevalence data from these tanks could be pooled for further comparisons. There was no significant difference between replicate groups, and prevalence data were pooled. Fisher's exact test for count data was used to compare prevalence between infection types using the "stats" package.²⁹ Comparisons of prevalence were made between diagnostic methods (*in vivo* GFP expression, *in vivo* GFP expression with dissection, liver/spleen culture, and liver/spleen PCR) as well as between sexes. The results were organized into bar plots using the "sciplot" package.³⁰

Results

Transmission to biofilms

The detection of GFP-positive colonies in tanks over time shows variable detection in feces and biofilm samples over time between IP-injected and oral feed infection modes (Table 1). Detection for the feces and biofilm samples for all *M. chelonae* IP injection replicate tanks occurred as soon as the first week of sampling (Table 1) and continued to be consistently detected for the first 9 weeks of sampling in both the feces and biofilm with the exception of week 5. By week 10, detection of GFP-positive colonies became more intermittent; however, persisted through the 16 weeks of sampling.

For fish infected with *M. chelonae* through oral feed, detection in colonies only occurred in the biofilm of one *M. chelonae* oral feed infection tank for week 1. By week 3, GFP colonies started to be detected intermittently in two or more of the three replicate tanks for the oral feed infection fish. GFP colonies were detected in feces in two or more tanks starting in week 2 and were detected intermittently through week 16. GFP colonies were not detected in any sham infection tanks with the exception of weeks 7 and 8 where colonies were detected in biofilm samples for oral feed sham infection tanks, for 1 and 2 weeks, respectively.

When comparing the prevalence of *M. chelonae* infections between the two modes of infection (IP injection vs. oral feed), differences depended on the diagnostic method used (Fig. 1, plus sign). We were able to use three different methods to evaluate prevalence of *M. chelonae* infections: *in vivo* GFP screening (Fig. 2A, D), culture of liver and spleen samples, and histology (Fig. 2G–I). There was no detection of bacteria in sham infection fish. Prevalence determination through dissection and *in vivo* GFP screening (gray bars) resulted in no difference between the two different modes of infection when comparing groups of both sexes, only males, and only females ($p=0.57$). When using culture of liver and spleen samples to isolate GFP colonies (black bars), IP-injected groups of both sexes and males had a significantly higher prevalence compared with oral feed-infected groups of both sexes and males ($p=8.15 \times 10^{-3}$). Histology (white bars) resulted in a significantly higher prevalence in all IP-injected compared with all oral feed-infected groups ($p < 2.2 \times 10^{-16}$). No mycobacteriosis or other infectious agents were observed in sham controls fed a gelatin diet lacking mycobacteria.

When comparing the prevalence based on each method within groups of the same infection mode and sex (Fig. 1, asterisk), liver culture resulted in a significantly lower prevalence compared with histology and *in vivo* GFP screening for IP injection-infected groups of both sexes ($p=6.02 \times 10^{-8}$), males ($p=8.46 \times 10^{-4}$), and females ($p=5.94 \times 10^{-5}$). For oral

TABLE 1. TRANSMISSION OF *MYCOBACTERIUM CHELONAE* TO FECES AND TANK BIOFILMS FROM EXPERIMENTALLY INFECTED ZEBRAFISH OVER A PERIOD OF 16 WEEKS BASED ON GREEN FLUORESCENCE PROTEIN COLONY FORMATION IN CULTURE

Infection Mode	Tank	Week															
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
IP Injection	Sham 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Sham 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Sham 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Oral Feed	<i>M. chelonae</i> 1	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	<i>M. chelonae</i> 2	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	<i>M. chelonae</i> 3	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	Sham 1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Sham 2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Sham 3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	<i>M. chelonae</i> 1	-	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	<i>M. chelonae</i> 2	-	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
	<i>M. chelonae</i> 3	-	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X

The presence (X) or absence (-) of GFP colonies is indicated, with the exception of unavailable biofilm samples (N). When two or more tanks for a given treatment resulted in the formation of GFP colonies, fish were placed in new autoclaved tanks (X). GFP, green fluorescence protein.

FIG. 1. Prevalence of *M. chelonae* infections for the two modes of infection [oral Gelly Belly (GB) infection vs. IP injection infection]. Different diagnostic methods include histology (white bars), *in vivo* GFP screening (gray bars), and liver/spleen culture (black bars). Statistically significant differences between diagnostic methods used are indicated (+), as well as those between sexes are also indicated (*). GFP, green fluorescence protein.

feed infection, *in vivo* GFP screening resulted in significantly higher prevalences for groups of all sexes ($p=3.39 \times 10^{-14}$), males ($p=2.57 \times 10^{-9}$), and females ($p=2.57 \times 10^{-9}$).

Transmission to fish

The VIE tags were retained for the entirety of the study and we were able to differentiate individual fish from each tank. We also were able to observe GFP expression *in vivo* through

the skin/scales of the *casper* mutant fish (Fig. 2B, C, E, F). The number of fish with detectable GFP expression increased over the 16 weeks of this study (Table 2). When the fish were first placed in the biofilm tanks, no *in vivo* GFP expression was observed. Beginning at week 2, GFP expression was observed in one female fish in Tank 2. At week 6, GFP expression was observed in two female fish in both Tank 2 and Tank 3. The number of fish with GFP expression gradually increased at each sampling time point, with a higher proportion of females expressing GFP (observed in the ovaries). By the last sampling time point, the tank prevalence of GFP expression ranged from 60% to 100%. Through the dissection of a subset of fish from each tank, additional GFP expression was detected increasing the prevalence (Table 2, X) in Tank 2 and 3 to 70%.

Similar to the previous results for transmission to biofilm, the prevalence of *M. chelonae* infections depended on the method used (Fig. 3, plus sign). *In vivo* GFP expression and *in vivo* GFP expression postmortem after subset dissection resulted in a significantly higher prevalence compared with liver and spleen culture or PCR for both sexes ($p=1.48 \times 10^{-4}$) and females only ($p=6.90 \times 10^{-4}$). There was no difference in prevalence values for males between diagnostic methods ($p=0.19$). There was also no significant difference ($p>0.05$) in prevalence between groups when considering each diagnostic method.

Discussion

In this study, transmission of *M. chelonae* between zebrafish and tank surface biofilms, and vice versa, was demonstrated. Transmission from infected zebrafish to biofilms occurred within 1 week postinfection for fish exposed orally or by IP injection. Observation of GFP *M. chelonae* in feces

FIG. 2. Examples of the different diagnostic methods used in this study. Comparative brightfield and FITC screening was used to identify areas of GFP expression (compare A, D, G to B, E, H). For the transmission from fish to biofilms, study histology was also used to identify acid fast bacteria in granulomas (encircled in C, F, I). SB, swim bladder.

TABLE 2. TRANSMISSION OF *MYCOBACTERIUM CHELONAE* TO ZEBRAFISH FROM TANK BIOFILMS OVER A PERIOD OF 16 WEEKS BASED ON DETECTION OF GREEN FLUORESCENCE PROTEIN EXPRESSION IN ZEBRAFISH DURING LIVE-IMAGING

Tank	Number	Sex	Week									
			0	2	4	6	8	10	12	14	16	
1	Fish 1	M	-	-	-	-	-	-	-	-	-	X
	Fish 2	M	-	-	-	-	-	-	-	-	X	X
	Fish 3	M	-	-	-	-	-	-	-	-	X	X
	Fish 4	M	-	-	-	-	X	X	X	X	X	X
	Fish 5	M	-	-	-	-	-	-	-	X	X	X
	Fish 6	F	-	-	-	-	X	X	X	X	X	X
	Fish 7	F	-	-	-	-	X	X	X	X	X	X
	Fish 8	F	-	-	-	-	-	-	X	X	X	X
	Fish 9	F	-	-	-	-	X	X	X	X	X	X
	Fish 10	F	-	-	-	-	X	X	X	X	X	X
2	Fish 1	M	-	-	-	-	-	-	-	-	-	X
	Fish 2	M	-	-	-	-	-	-	-	-	-	-
	Fish 3	M	-	-	-	-	-	-	-	-	-	-
	Fish 4	M	-	-	-	-	-	-	-	-	-	-
	Fish 5	M	-	-	-	-	-	-	-	-	-	X
	Fish 6	F	-	-	-	-	X	X	X	X	X	X
	Fish 7	F	-	-	-	X	X	X	X	X	X	X
	Fish 8	F	-	-	-	-	-	X	X	X	X	X
	Fish 9	F	-	X	X	X	X	X	X	X	X	X
	Fish 10	F	-	-	-	-	-	X	X	X	X	X
3	Fish 1	F	-	-	-	-	X	X	X	X	X	X
	Fish 2	M	-	-	-	-	-	-	-	-	-	-
	Fish 3	M	-	-	-	-	-	-	-	-	-	-
	Fish 4	M	-	-	-	-	-	-	-	-	-	X
	Fish 5	M	-	-	-	-	-	-	-	-	-	X
	Fish 6	F	-	-	-	-	X	X	X	X	X	X
	Fish 7	F	-	-	-	-	-	X	X	X	X	X
	Fish 8	F	-	-	-	-	-	-	-	-	-	-
	Fish 9	F	-	-	-	X	X	X	X	X	X	X
	Fish 10	F	-	-	-	X	X	X	X	X	X	X

The presence (X) or absence (-) of GFP expression is indicated for each biweekly sampling event.

occurred in week 1 for IP-injected fish, and by week 2 for orally infected fish. Detection of *M. chelonae* in feces and tank biofilms persisted through the end of the study period for both groups; however, as the study progressed, detection became intermittent. This observation of initial consistent transmission of *M. chelonae* to tank biofilms and feces followed by more sporadic transmission is likely due to the amount of bacteria shed by fish during the phases of infection, with a greater amount of shedding following initial infection.

It is possible that early detections in feces and biofilms of tanks containing orally exposed fish are due to bacteria passed through the gut. However, early detections in biofilms were also observed in IP-injected fish, suggesting these would have been shed across the gut. Regardless, we continued to get positive results for 16 weeks, from biofilms and feces, even when tanks were changed weekly, suggesting that shedding occurs regularly once infections are initiated. There were also 2 weeks where GFP-positive colonies were detected in sham oral infection tanks, which we believe to be due to contamination during sample processing because mycobacteria were never detected in any other sham samples.

FIG. 3. Prevalence of *M. chelonae* infections following incubation in *M. chelonae* biofilm tanks based on four diagnostic methods: *in vivo* GFP screening with and without subset dissection, subset liver/spleen culture, and subset liver/spleen PCR. Statistically significant differences between diagnostic methods used are indicated (+).

We expect that the observations from the oral ingestion infection fish simulate what would naturally be observed in zebrafish colonies. Many studies investigating zebrafish mycobacteriosis end at 8 weeks postinfection, and little is known about infections past these experimental endpoints. It is possible that some natural infections are eventually cleared while some shedding occurs²⁴; although a stressful event³³ could exacerbate the situation and result in an outbreak.

We also observed transmission of *M. chelonae* from tank biofilms to uninfected zebrafish, demonstrating that biofilms can be a natural source of infection in zebrafish colonies. Although we cannot entirely determine whether infection occurred through ingestion from zebrafish feeding directly on biofilms or through infection by planktonic mycobacteria detached from biofilms (which could be ingested or infect the fish by another means), the originating source of *M. chelonae* in this study was a tank biofilm. This result is significant because biofilms form readily in aquatic systems and *Mycobacterium* species are commonly found there.^{5,9}

Regular tank cleaning and removal of biofilms from tanks and other equipment in zebrafish systems should be carried out to minimize potential sources of infection. Removal of biofilms within systems can be labor intensive as pieces of equipment need to be scrubbed and disinfected but has previously been successful in controlling established outbreaks.^{9,34} Also, adjustment to tank cleaning measures have previously been demonstrated to be effective for management of asymptomatic *M. chelonae* infections in zebrafish stock.²³ These husbandry considerations include tank size, population density, and changes in biofilm cleaning methods.²³ For example, prevalence of subclinical mycobacteriosis was lower in tanks stocked at a lower density and cleaned by scrubbing with increased water flow, compared with higher density tanks cleaned through siphoning, where fish are more likely to feed on mycobacteria-laden debris.

There are some clues to the natural transmission routes of mycobacteria in laboratory zebrafish and other fishes. Transmission through oral ingestion has historically been suspected as the major source of infection, as major outbreaks in hatchery salmon in the 1950s and 1960s were correlated with unpasteurized fish feed.^{35,36} In zebrafish, Harrieff *et al.*²⁴ initiated mycobacterial infections through ingestion, highlighting the intestinal tract as a route of entry. In a comparison of feeding versus immersion, mosquito larvae infected with *Mycobacterium marinum* were more infective to Japanese medaka (*Oryzias latipes*) compared with immersion challenge at a high dosage.³⁷

Furthermore, passage through another organism, which may be present in a biofilm, may also be important. Peterson *et al.*³⁸ found that in zebrafish, ingestion of the ciliated protozoan *Paramecium caudatum* containing *M. marinum* and *M. chelonae* resulted in a higher prevalence of infection compared with ingestion of culture alone. These results follow previous studies describing an increase in virulence in mycobacteria in tissues (e.g., granulomas) compared with culture.³⁹ Infection through immersion has also been demonstrated in zebrafish embryos.⁴⁰ Biofilms as a source of infection in laboratory zebrafish have been suspected,⁹ but had not yet been experimentally addressed until this study.

As demonstrated in our study, mycobacterial transmission between tank biofilms and zebrafish occurs rapidly, emphasizing the need for zebrafish facilities to incorporate these adjustments into husbandry measures, including both preventative and control measures. The control of mycobacterial biofilms has already been identified as a major challenge in the medical setting, as nosocomial infections due to mycobacterial biofilms have been identified to contaminate water systems and medical equipment, as reviewed in a study.⁴¹ Preventative environmental monitoring, including sampling of biofilms, feces, and detritus, can be useful for surveillance of *Mycobacterium* spp. pathogenic to zebrafish in conjunction with a disease surveillance program,^{42,43} as not all infections result in observable signs of disease.⁶

It is also important to note, as demonstrated in this study, bacterial shedding and detection in the biofilm can be intermittent and this should be considered when interpreting environmental sampling results. Recently, real-time PCR analysis of environmental samples was found to be more sensitive compared with antemortem zebrafish samples for detecting the presence of mycobacteria in zebrafish facilities.²⁶ Subclinical infections with *Mycobacterium* spp. such as *M. chelonae* can go unnoticed and be a potential source of nonprotocol experimental variation. Biofilm monitoring can assist in detecting *Mycobacterium* spp. in systems before morbid fish are observed and allow for identification and control of potential risk factors. It is also important to note that several species of *Mycobacterium* are ubiquitous in water systems and have not been observed to be pathogenic to zebrafish. Thus, simply testing for any mycobacteria in water or biofilm samples will almost certainly yield positive results, but only certain species and strains can establish infections.

In terms of observable morbidity, the fish in this study showed a range of signs of morbidity and different diagnostic methods allowed for a higher degree of detection of *M. chelonae* infections. First, there were no observed mortalities due to any of the experimental *M. chelonae* infections in this study. This result is not surprising as *M. chelonae* has pre-

viously been reported to result in more chronic, low-level infections showing few clinical signs of infection.⁶ When using *in vivo* screening for GFP expression as a diagnostic, there was no difference in prevalence between IP-injected fish and orally infected fish. When liver/spleen culture or histology was used as diagnostics, IP-injected fish had a higher prevalence of infection compared with orally infected fish. Although both methods of exposure resulted in infection, the IP infections are more severe, and therefore more likely to be detected by histology and liver/spleen culture.

Unlike oral exposures, the IP injections allow bacteria to surpass natural barriers (e.g., epithelium of the intestinal tract) and likely spread more easily within the fish and be detected by these traditional diagnostic methods. This also suggests that visual screening for GFP is more sensitive than other diagnostic methods in this experimental system. Results from the fish infected from tank biofilms were similar, as *in vivo* screening for GFP resulted in the highest prevalence value compared with liver/spleen culture and PCR when looking at groups of both sexes or only females. This difference did not occur when only comparing males. This difference between males and females could be related to the ovaries as an important location of granuloma formation. All the female fish infected through tank biofilms had granulomas observed in their ovaries, while males were observed to have GFP expression in their hematopoietic organs, which would influence detectability of bacteria in liver/spleen samples.

Differences in detection were observed between *in vivo* GFP whole-fish screening compared with histological and liver/spleen culture and PCR methods already established for studies investigating mycobacterial infections in zebrafish.^{4,5,11} In terms of sensitivity, the ability to correctly detect positives, the *in vivo* GFP screening had a high sensitivity compared with histology. This method was much less specific than histology, because detection was higher in *in vivo* GFP screening fish than histology, and we have no secondary method of diagnostic validation. This could be attributed to additional potential sources of autofluorescence within the zebrafish (e.g., gallbladder and degenerating eggs) that may result in false positives by an untrained observer, requiring further analyses of bacterial smears or culture.

The usefulness of *in vivo* GFP screening depends on the skill level of the observer, a potential pitfall to this diagnostic method. It is important to note that the evaluation of histological sections may not be optimal for detecting a true negative, as only select midline sections were observed and not the entire fish. Future comparison of this new *in vivo* GFP screening method for detecting mycobacterial infections should be carried out and compared with real-time qPCR detection values to gain a better understanding of the specificity of this method. Importantly, it should be emphasized that this method was useful for the purpose of demonstrating the transmission of *M. chelonae* between tank biofilms and zebrafish, which was confirmed by all diagnostic methods (GFP observation, PCR, and histology).

In this study, we demonstrated that *M. chelonae* can be shed in feces and transmitted to tank biofilms from infected zebrafish, and that zebrafish can naturally acquire *M. chelonae* infections from tank biofilms. In addition, the demonstration of this mode of mycobacterial transmission emphasizes other important disease control measures that

reduce risk of biofilms forming in zebrafish facilities (e.g., quarantine and “eggs-only” policies for fish from an outside facility, quarantine of moribund fish, regular tank cleaning). This transmission of mycobacteria between biofilms and zebrafish is also likely to be true for other *Mycobacterium* and fish species, implying that control and management of mycobacteriosis in laboratory zebrafish may also be applicable to other fishes.

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