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Diagnosis and Management of Atypical *Mycobacterium* spp. Infections in Established Laboratory Zebrafish (*Brachydanio rerio*) Facilities

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Abstract | Two established zebrafish colonies experienced increased mortality and decreased reproductive performance. Initial examination of several fish from one facility revealed hyperemic gills, petechia around the opercula, abdominal distention, and emaciation. Affected fish had congested liver with inflammation and multifocal hepatic necrosis. Large numbers of acid-fast-positive, rod-shaped bacteria were evident in multiple tissues and the blood. *Mycobacterium fortuitum* was subsequently isolated from several fish. Zebrafish from the second facility had skin erosions and ulceration along the flank just caudal to the pectoral fins. Large numbers of acid-fast-positive, rod-shaped bacteria were observed within the necrotic centers of well-demarcated, multifocal granulomas in gonads, liver, and peritoneum from affected fish. *Mycobacterium abscessus* and *M. chelonae* were isolated and identified biochemically. Definitive diagnosis in these outbreaks was obtained by culture on selective media. Because *Mycobacterium* spp. grow extremely slowly and positive confirmation may require 45 to 60 days, *Mycobacterium* species-specific polymerase chain reaction analysis was used to provide a rapid screening assay for *Mycobacterium* spp. as well as for verification of culture results. To our knowledge, this is the first documentation of mycobacterial infection in laboratory-maintained zebrafish and provides guidelines for diagnosis, management, and prevention of atypical mycobacteriosis in laboratory zebrafish colonies.

The zebrafish, *Brachydanio rerio*, is a freshwater species native to the region surrounding the Ganges River of India. Introduced originally as a popular aquarium fish, the zebrafish (also known as the zebra danio or *Danio rerio*) has emerged as an increasingly popular biomedical model for early vertebrate embryonic development, gene function analysis, and mutagenesis studies (1, 2). It is prudent, therefore, to have proper health management of the established colony in place to avoid substantial losses in terms of valuable animals, research time, and money due to failure of the life support systems, improper environmental conditions, or infectious disease.

Atypical *Mycobacterium* species are ubiquitous microorganisms that have been isolated routinely from soil and water in the natural environment. Recently, these atypical species have been gaining worldwide attention as an important pathogen in immune compromised humans as a result of HIV infection or immunosuppressive therapies, such as radiation and chemotherapy (3, 4). Atypical mycobacterial infections of fish have been most commonly caused by *Mycobacterium marinum*, *M. fortuitum*, or *M. chelonae*. However, recent taxonomic studies, using genetic analysis and 16S ribosomal sequencing, have indicated that *M. chelonae* subspecies *abscessus* is a distinct species and has been reclassified as *M. abscessus* (5, 6).

Mycobacteriosis has been identified worldwide in over 150 species of salt and freshwater fish (7), and is the most common chronic disease affecting tropical aquarium fish (8). Members of the freshwater families Anabantidae (bettas and gouramis), Characidae (tetras and piranhas), and Cyprinidae (danios and barbs) appear to be particularly susceptible (8). Large numbers of bacteria are shed from the infected epithelial ulcers and intestines. Ingestion of bacteria through proximity to or by feeding on infected fish results in spread of the infection (8).Vertical transmission of mycobacteria has been documented in Mexican platyfish (viviparous), but not in salmonids or other ovoviviporous fishes (8, 9) such as zebrafish. Due to the long incubation period and chronic, subclinical form of infection, mycobacteria can remain undetected within established research fish colonies for extended periods. Under these conditions, atypical *Mycobacterium* species can pose a continued health risk to fish and a possible zoonotic transmission to unsuspecting laboratory workers. The purpose of this study was to describe two outbreaks of atypical mycobacteria infection in laboratory-maintained zebrafish, methods used to control the outbreaks, and management practices instituted to prevent recurrence.

Clinical Outbreaks

Laboratory A: Zebrafish in this laboratory consisted of wild-type stocks and mutant inbred strains principally used for early vertebrate embryonic development studies. The laboratory had two facilities housed on different floors of the same building, with each facility having independent life support and a common RO (reverse osmosis) water supply. One facility was a small, recirculating, modular-type housing design of 280-L water capacity, and the second was a larger core facility containing numerous independent aquaria for fish housing.

The initial clinical sign of infection in the zebrafish was an overall decrease in reproductive activity of the fish maintained

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Table 1. Differential biochemical characteristics of atypical mycobacteria species infecting fish

Species	Growth class	Nitrate reductase	Iron uptake	Na citrate	Tween hydrolysis	Growth on 5% NaCl	Catalase	Pigment production	Urease	Aryl-sulfatase
M. marinum	Slow	-	-	-	+	-	-	+	+	NA
M. fortuitum	Fast	+	+	-	+/-	+	+	-	NA	+
M. chelonae	Fast	-	-	+	+/-	-	+	-	NA	+
M. abscessus	Fast	-	-	-	+/-	+	+	-	NA	+

within the smaller facility for a period of 2 to 3 weeks. Further investigation yielded a slight increase in colony mortality. Wild-type zebrafish were frequently obtained for outcrossing from a variety of sources with unknown disease status. Visual inspection of the fish contained within the water system revealed normal behavior and activity. However, one abnormal zebrafish was identified with characteristic "dropsy-like" lesions of abdominal distention, scale edema, and flared opercula.

Analysis of the environment and water quality parameters indicated frequent and substantial pH fluctuations between 6.5 and 7.4 and water temperature of approximately 18°C. Further investigation indicated that the water system heater unit had been off for an unspecified period. Environmental parameters were corrected, but reproductive activity of the fish did not improve.

Three weeks later, a second clinically abnormal zebrafish with severe "dropsy-like" lesions was identified and submitted for histologic analysis and bacterial culture. Although the two facilities within this laboratory were on separate floors with independent life support, clinically normal fish from the facility without previous disease also were evaluated since equipment, fish, and technicians frequently traveled between both facilities.

Laboratory B: This laboratory consisted of only wild-type zebrafish maintained for the sole purpose of egg harvest for toxicologic testing and analysis. The animal facility consisted of two commercially available recirculating, modular water systems with independent life support systems and a common RO water supply. Several independent 10-gallon aquaria also were maintained in the same room for quarantine and hospital purposes.

During the preceding three to four weeks, several affected fish had superficial scale erosions, some of which had progressed to deep ulcers along the flanks. These erosions and ulcers were frequently located just caudal to the pectoral fin. A retrospective analysis of colony health records indicated an increase in the number of fatalities in this water system. Affected fish appeared to be limited to a single modular water system containing fish that had survived an acute exposure to supersaturated system water (known as "gas bubble disease" or GBD) due to a faulty aerator. The GBD outbreak developed approximately two months prior to the mycobacteriosis outbreak. Fish with clinically observable disease were not present in the other modular systems or individual aquaria.

Initial environmental analysis revealed basic water parameters (temperature, pH, ammonia, nitrite, and nitrate concentrations) were within normal limits (10). The facility maintained a sound hygiene and management policy regarding animal health that involved daily monitoring of water systems, system maintenance, quarantine screening, and staff training. Prior to this outbreak and the single incident of GBD, the zebrafish had been free of clinical disease for approximately three years.

Materials and Methods

Zebrafish sources: Zebrafish from four laboratories were evaluated in this study. These laboratories maintain zebrafish facilities for use in research of early vertebrate embryogenesis or toxicologic studies. Fish in two of the laboratories, designated A and B, had naturally acquired clinical cases of mycobacteriosis. The other two laboratories, designated C and D, were used as controls for a polymerase chain reaction (PCR)-based detection assay since there had been no clinical evidence or previous history of mycobacteriosis at these two sites.

Culture technique: Zebrafish from laboratories A and B were euthanized by immersion in water containing MS-222 (tricaine methyl sulfonate) administered to effect. Five minutes after cessation of opercula movement, fish were removed from the water and the external surface was disinfected with 70% ethanol. Using a dissecting microscope, a ventral midline incision was made and the abdominal contents were removed aseptically. Kidneys, liver, and spleen were placed in 5 ml of sterile phosphate-buffered saline (PBS) and were submitted for culture (Mycobacteriology Laboratory at the Massachusetts State Laboratory Institute; Jamaica Plain, MA).

Tissue was then processed by use of the BBL™ MycoPrep™ Kit (Becton Dickinson, Sparks, MD) to eliminate normal microbial flora. Processed tissue homogenate was used to inoculate Lowenstein-Jensen and Middlebrook 7H11 agar slants. The LJ and 7H11 cultures were then incubated at 37 and 24°C at an atmosphere of 5 to 10% $C0_2$ and were maintained for a minimum of 60 days. If growth was present within the first seven days, it was considered a rapid or fast grower. If growth was first noted after two weeks, it was considered a slow grower. After sufficient growth was noted, the culture was transferred to 7H9 broth to perform necessary tests for biochemical identification of species (Table 1).

Preparation and extraction of DNA: Tissue for DNA extraction was obtained from selected tissues (liver, spleen, and kidney) of selected zebrafish. The external surface of the fish was subjected to 70% ethanol for disinfection of surface microbial contaminants. Internal viscera were removed under the dissecting microscope by use of sterilized instruments and were placed in 0.5 ml of sterile PBS.

A High Pure PCR Template Preparation Kit (Boehringer Mannheim, Indianapolis, IN) was used for DNA purification. Internal viscera in PBS were homogenized and incubated at 55°C for 24 hours with 40 µl of a protein digesting enzyme (Oncor, Gaithersburg, MD) and 200 µl of tissue lysis buffer. After a 4- to 18-hour incubation, 200 µl of binding buffer was added to the samples and incubation proceeded for 10 minutes at 72°C. Approximately 100 µl of isopropanol was then added to the solution, which was centrifuged at 11,000 ×*g* for 3 minutes to pellet any large particulates. The solution was transferred to the kit-supplied filter tube and centrifuged at 6,000 ×*g* for 1 minute. The samples were washed and centrifuged twice at 6,000 ×*g* for 1 minute, using 500 µl of the wash buffer. Samples received a final centrifugation step for 1 minute at 16,000 ×*g* to remove residual wash solution from the filter. The DNA was eluted with 200 µl of prewarmed (70°C) elution buffer and centrifuged at 6,000 ×*g* for 1 minute, then stored at -20°C until use.

Polymerase chain reaction method: The PCR primers

and conditions were obtained from Integrated DNA Technologies and have been described (7). Briefly, initial PCR amplification of the 924-bp fragment from a highly conserved region of a 16S rRNA gene involved use of external primers T_{39} (5'-GCGAACGGGTGAGTAACACG-3') and T_{13} (5'-TGCCACAG GCCACAAGGGA-3'). The PCR amplification of the nested 300-bp fragment involved use of internal primers $_{pre}T_{43}$ (5'-AAT TGGGCGCAAGCCTGATG-3') and T_{531} (5'-ACCGCTACAC CAGGAAT-3'). Both reactions involved use of a 50-µl reaction mixture consisting of 5.0 µl of buffer, 5.0 µl of bovine serum albumin (BSA), 5.0 µl of dNTPs, 0.5 µl of each primer, 0.5 µl of Taq polymerase, 1.0 µl of Perfect Match® PCR Enhancer (Stratogene, La Jolla, CA), and 31.5 µl of H_20. The initial reaction contained 1.0 µl of extracted sample DNA, and the second reaction with nested primers contained 1.0 µl of amplified template DNA from the initial reaction.

The PCR amplification cycle parameters consisted of a 5-minute denaturation step at 95°C followed by 30 cycles of 1-minute denaturation at 94°C, 1-minute annealing at 50°C, and 1-minute extension at 72°C. A final extension of 5 minutes at 72°C was then performed. The PCR reaction products were evaluated by use of 1% agarose and VisaGel™ Separation Matrix (Stratagene, La Jolla, CA) gels and ethidium bromide staining. A 15-µl sample and 1.0 µl of dye were loaded, and gels were electrophoresed for approximately 3 hours at a constant voltage of 100 V. Amplified DNA profiles were evaluated by immersing gels in ethidium bromide solution for 15 minutes followed by destaining in water for 10 minutes.

Control of clinical outbreak: Bleach is a common disinfectant agent for water/life support systems since it does not persist in the water system, and previous studies have indicated that high concentrations of chlorine-releasing agents are mycobactericidal (11). All exposed equipment that could be replaced was discarded (gravel, filter materials, plastic holding aquaria, buckets). The water system was then sanitized with 1/4 cup of Chlorox™ bleach per gallon of system water (approx. 800 ppm). Using the bleach formulation, the system was operated for three days with aeration, followed by draining and refilling with fresh water (without bleach), then was operated for an additional three days with aeration. The system was then drained and refilled for stocking. All exterior surfaces of associated hardware and aquaria were subjected to the concentrated bleach solution. Water system pipes that could be dismantled were also scrubbed to disrupt the biofilm. Due to the concentrated fumes produced during the procedure, precautions such as closure of the area to all workers, adequate ventilation, and wearing of gloves were implemented. Restocking the system and microbiological analysis of fish were performed several months later to monitor for reinfection of the system.

Results

Necropsy Findings

Laboratory A: The first abnormal fish had hyperemic gills, increased respiratory effort, mildly distended abdomen, and edema of the epithelium, as evidenced by the lifting of scales on the flank and dorsal aspect just caudal to the head and opercula. Excess mucus secretion also was present around the lifted scales. The water containing the specimen had evidence of excess mucus manifested as white, opaque casts varying in size from 1 to 3 mm.

The second clinically affected fish had a severely distended abdomen extending from the base of the opercula groove to the anal pore and erratic swimming behavior in addition to the lesions previously noted (Figure 1). Skin petechiation was noted at the base of the pectoral fins, opercula, anal pore and lateral line (flank). Approximately 120 µl of a translucent, yellow- tinged, non-viscous fluid was aspirated from the body cavity through a ventral midline incision.

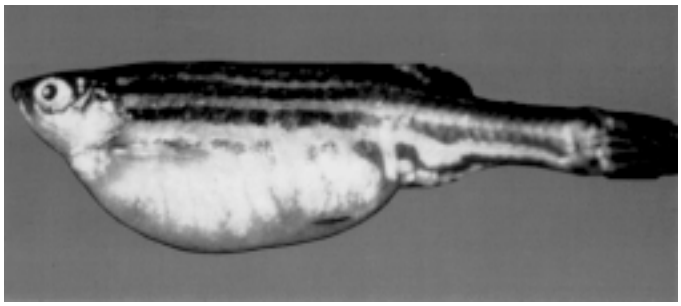

Figure 1. Gross photograph of a confirmed *Mycobacterium fortuitum*-infected zebrafish. Notice "dropsy-like" clinical signs of abdominal distention, scale edema, and petechiae around operculum and pectoral fin.

Microscopic evaluation of the fluid revealed large, rod-shaped bacteria and heterophils suggestive of septic peritonitis. Egg retention and a focal pale-yellow area on the gonad also were present. Mucus scrapings and gill clippings evaluated via low power microscopy did not reveal appreciable findings.

Laboratory B: All fish submitted for necropsy had circular cutaneous foci of erosions or ulcerations approximately 2 to 5 mm in diameter (Figure 2), which were usually localized along the flank of the fish adjacent to the lateral line or just caudal to the operculum. The areas consisted of localized hemorrhage, denuded epithelium, and scale loss. In several affected fish, the area of ulceration extended deep into the subcutaneous tissue and occasionally into the peritoneum. Other affected fish had ulcerative lesions that resolved clinically over a period of 1 to 3 weeks.

Histopathologic Findings

Laboratory A: Multifocal necrosis was present in the liver, spleen, and heart and was associated with variable degrees of inflammation. In the liver, necrotic areas were often accompanied by infiltrates of macrophages, although epithelioid or multinucleate giant cells were not seen. Diffuse hepatic congestion also was evident. In the heart and spleen, necrosis consisting of karyorrhexis or karyopyknosis with little inflammation was noted. Gram staining revealed rod-shaped organisms with staining characteristics similar to those of gram-positive bacteria. Large numbers of organisms were evident in the liver, kidney, peritoneum, epithelium, and heart (bulbous arteriosis) (Figure 3). Ziehl-Neelson staining revealed numerous acid-fast bacilli in macrophages within the liver, in the endothelium of the heart, and extracelluarly in the blood of other organs, including the heart, kidneys, liver, and spleen (Figure 4). The affected fish had peritoneal effusion with abdominal distention and subcutaneous and cutaneous edema. Histologic lesions similar to the first specimen also were identified in the liver, kidneys, and epithelium by use of hematoxylin and eosin (H&E), Gram, and Ziehl-Neelson acid-fast staining of tissue from the second fish.

Laboratory B: Microscopic lesions consisted of granulomatous inflammation with necrosis involving the gonad, peritoneum, liver, and spleen (Figure 5). Foci of inflammation were variably sized (approx. 0.1 to 1.0 mm in diameter) and included well-demarcated granulomas as well as poorly organized aggre-

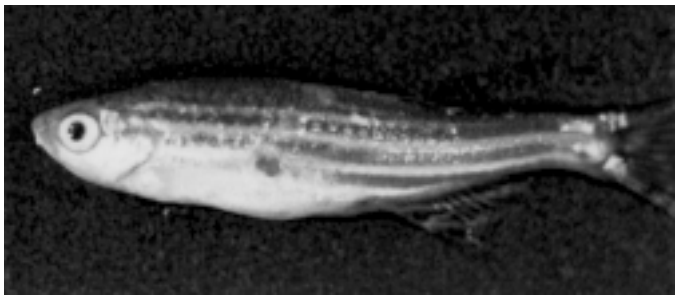

Figure 2. Gross photograph of a confirmed *Mycobacterium chelonae*-infected zebrafish. Notice circular cutaneous ulceration approximately 2 to 5 mm in diameter

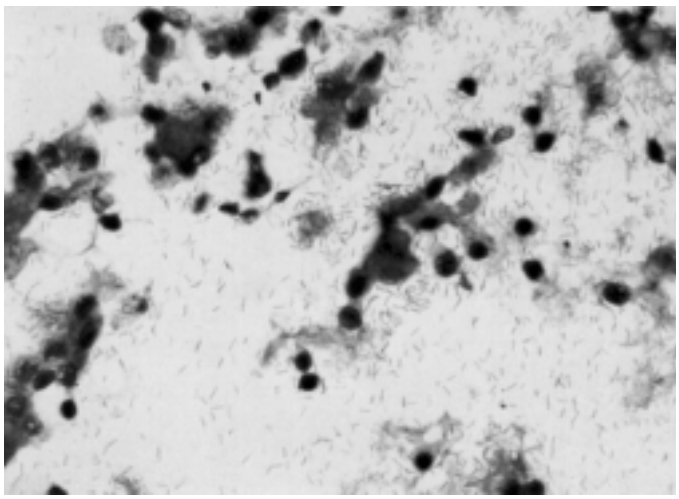

Figure 3. High-power photomicrograph of a *Mycobacterium fortuitum*-infected zebrafish with septicemia. Notice numerous acid-fast bacilli in the lumen of a major vessel. Numerous bacilli are also evident within multiple circulating phagocytic cells.

gates of macrophages. Granulomas were composed of closely spaced collections of macrophages, including numerous epithelioid and foamy macrophages and peripheral circumferential bands of fibrosis. Many granulomas had necrotic centers with coagulated anucleate or karyopyknotic cells, amorphous granular debris, and hypereosinophilic coagulum (Figure 6). A few melanomacrophages were present within the walls of the granulomas. Extracellular brown pigment accumulation and mineralization also were evident in necrotic areas of granulomas. Ziehl-Neelson staining revealed numerous, intracellular and extracellular acid fast-bacilli within most granulomas. However, some granulomas lacked acid-fast bacilli and areas of central necrosis, and others had central necrosis with no discernible bacteria. Also present were unorganized infiltrates of large, epithelioid macrophages in the ovary between or within necrotic eggs, and the peritoneum, liver, and spleen. Multinucleate giant cells were infrequent. Sporadic skeletal muscle vacuolization, necrosis, and mineralization also were seen. The skin of several fish affected with severe peritonitis had foci of ulceration bordered by aggregates of large, epithelioid macrophages that extended into the peritoneum, adjacent subcutis, and skeletal muscle.

Culture

Laboratory A: The viscera of individual zebrafish (No. 1–10) from the small facility where the clinically diseased fish were housed were submitted for culture. All ten fish were culture positive for *M. fortuitum* and seven of ten were positive for acid-fast staining. Two pools (I and II) that each contained the viscera from ten individual fish also were submitted to maximize sampling of the resident population. Both of these pools were culture and acid-fast positive (Table 2).

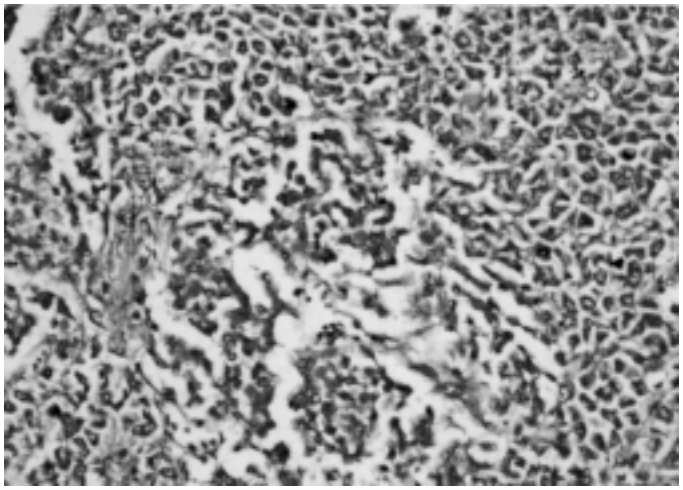

Figure 4. High-power photomicrograph from the liver of a *Mycobacterium fortuitum*-infected zebrafish. Notice typical focus of necrosis with numerous clusters of acid-fast bacilli.

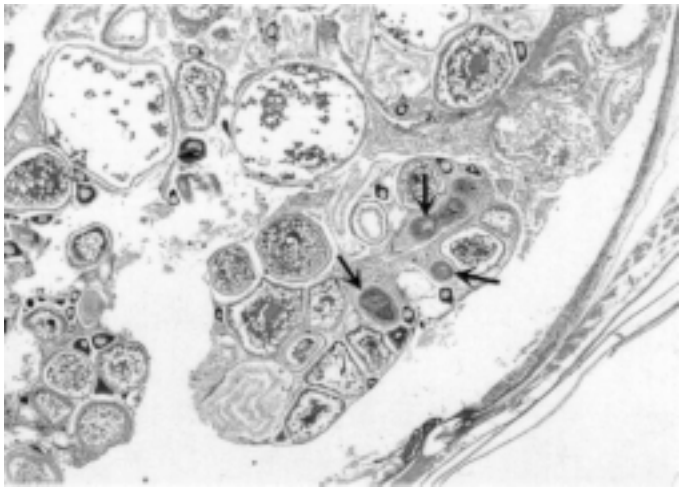

Figure 5. Low-power photomicrograph of the gonad from a confirmed *Mycobacterium chelonae*-infected zebrafish. Notice multifocal areas of granulomatous inflammation (arrows) among follicles in various stages of development.

Four additional pooled samples (pool III – VI) from the core facility with no clinically diseased fish were submitted to assess the possibility of infection from the previous facility. Each pooled sample contained viscera from five individual fish. None of the four pools was positive for acid-fast staining whereas all were culture positive for *M. fortuitum* (Table 3).

Laboratory B: The viscera of five individual zebrafish (No. 11–15) from the system where the clinically diseased fish were housed were submitted for culture. All five fish that were cultured had either active ulcerative skin lesions or recently resolved lesions. Of the five fish submitted, four were culture positive for *Mycobacterium spp.* Three were positive for *M. chelonae* and one was positive for *M. abscessus* (formerly classified as *M. chelonae* subspecies *abscessus*). None of the five individual samples

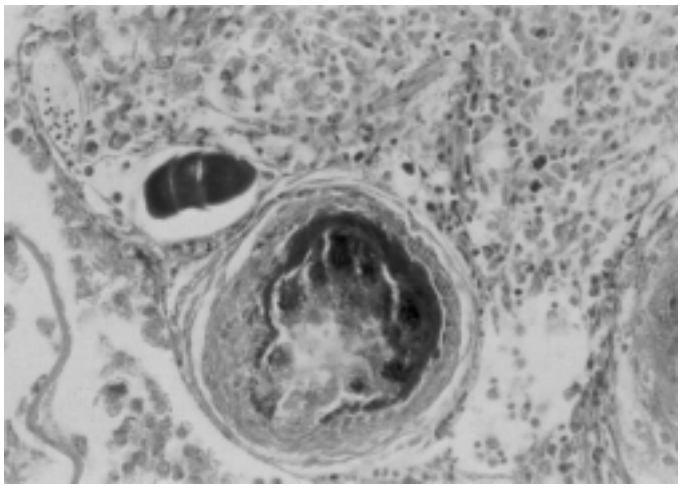

Figure 6. High-power photomicrograph of a granuloma from a confirmed *Mycobacterium chelonae*-infected zebrafish. Notice necrotic center with coagulated anucleate or karyopyknotic cells, amorphous granular debris, and hypereosinophilic coagulum.

Table 2. Culture and acid-fast staining results of individual and pooled samples from the facility in which zebrafish had clinical signs of disease (laboratory A).

Animal I.D.	Acid-fast staining result[1]	Culture result	Days incubated until culture positive[2]
Individual fish samples 1–7	+	+	Mean = 24 Range = 8 – 50
Individual fish samples 8–10	-	+	Mean = 17 Range = 14 – 21
Pooled fish samples I & II[3]	+	+	Mean = 18 Range = 14 – 21

[1]Indicates a +/- result for acid-fast staining bacteria on impression smears.
[2]Indicates number of days the culture had to be incubated until designated positive for *Mycobacterium* spp. growth.
[3]Abdominal viscera of ten zebrafish without clinical signs of disease combined for culture and acid-fast staining.

Table 3. Culture and acid-fast staining results of individual and pooled samples from the facility where zebrafish appeared healthy (laboratory A)

Animal I.D.	Acid-fast staining result[1]	Culture result	Days incubated until culture positive[2]
Pooled fish samples III –VI[3]	-	+	Mean = 26 Range = 8 – 60

[1]Indicates a +/- result for acid-fast staining bacteria on impression smears.
[2]Indicates number of days the culture had to be incubated until designated positive for *Mycobacterium* spp. growth.
[3]Abdominal viscera of five zebrafish without clinical signs of disease combined for culture and acid-fast staining.

Table 4. Culture and acid-fast staining results of individual and pooled samples from the facility where zebrafish had clinical signs of disease (laboratory B)

Animal I.D.	Acid-fast staining result[1]	Culture result	Days incubated until culture positive[2]
Individual fish samples 11 – 14[3]	-	+	Mean = 28 Range = 10 – 60
Individual fish sample 15[3]	-	-	-
Pooled fish sample VII[4]	-	-	-
Pooled fish sample (quarantine) VIII[5]	-	-	-
Pooled fish sample (nursery) IX[6]	-	-	-

[1]Indicates a +/- result for acid-fast staining bacteria on impression smears.
[2]Indicates number of days the culture had to be incubated until designated positive for *Mycobacterium* spp. growth.
[3]Abdominal viscera of zebrafish with either active or resolving skin lesions submitted.
[4]Abdominal viscera of ten zebrafish without gross lesions combined for culture and acid-fast staining.
[5]Abdominal viscera of ten zebrafish without clinical disease from the independent quarantine water system were combined for culture and acid-fast staining.
[6]Ten whole zebrafish (age 1–4 weeks) without clinical disease from independent nursery water system were homogenized for culture and acid-fast staining due to the small size of the individual fish.

were acid-fast positive. A pooled sample (pool VII) containing the viscera from ten individual fish without gross lesions also was submitted to maximize sampling from this system (Table 4). This pool was culture and acid-fast negative.

Pooled samples that each contained the viscera from ten individual fish also were submitted to maximize sampling from the other two water systems (pool VIII = quarantine system; pool IX = nursery system) that did not contain any diseased fish. Both samples were culture and acid-fast negative (Table 4).

Laboratories C and D: None of the zebrafish obtained from these two independent laboratories had any evidence of gross or microscopic disease during routine survey screening of these facilities. Zebrafish from both laboratories were evaluated by use of PCR analysis and H&E staining of histologic sections.

Polymerase chain reaction results: High-quality DNA preparations were repeatedly obtained from the abdominal viscera (kidneys, liver, spleen) of zebrafish and pure cell culture isolates. Initial PCR amplification with the external primers yielded the 924-bp fragment of interest (7). Spurious bands were present in most samples. The secondary PCR with nested internal primers generated a single distinct band at 300 bp (Figure 7).

The DNA extracted from zebrafish housed in two facilities with confirmed *M. fortuitum* and *M. chelonae* infection contained the 924- and 300-bp fragments. Both clinically normal fish obtained from laboratory B previous to the disease outbreak also had the 924- and 300-bp PCR products. The DNA extracted from zebrafish obtained from laboratory A one year after disinfection and restocking of the facility did not have either the 924- or 300-bp marker present. The DNA extracted from the pure culture isolates of the *Mycobacterium* spp. involved in one of the clinical outbreaks (laboratory A) also contained the 924- and 300-bp fragments. The DNA extracted from zebrafish housed in the two control facilities (laboratories C and D) with no previous history of *Mycobacterium spp.* infection did not have either target PCR product.

Preparations of whole genomic DNA extracted from pure culture isolates of other genera of bacteria were evaluated for specificity of the assay. *Escherichia coli*, *Staphylococcus aureus*, *Helicobacter pylori*, *Campylobacter jejuni*, and *Lactobacillus* sp. failed to amplify either the 924- or the 300-bp Mycobacterium spp.-specific PCR products (Figure 7).

Control of clinical outbreak: Twenty-four months after disinfection of the *M. fortuitum*-infected laboratory (laboratory A), zebrafish egg production has remained optimal. Animals in the facility have been free of clinical disease. Routine evaluation of zebrafish in the facility at 3, 6, 15, and 24 months after treatment was performed via acid-fast staining of histologic sections, PCR of DNA extracted from internal viscera, and bacterial culture on selective media. To date, acid-fast bacteria, positive PCR results, or fish culture positive for *Mycobacterium* spp.

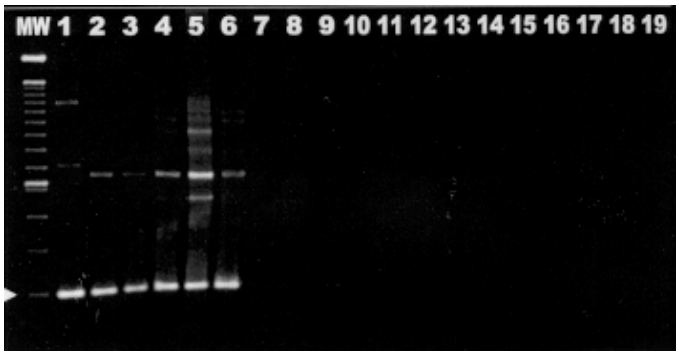

Figure 7. Gel electrophoresis demonstrating 300-bp target sequence, using nested polymerase chain reaction (PCR) analysis (arrow). Lanes 1 and 2 contain extracted tissue DNA from two *Mycobacterium chelonae* infected zebrafish (laboratory B). Lanes 3 and 4 contain extracted tissue DNA from two *Mycobacterium fortuitum* infected zebrafish (laboratory A). Lane 5 contains DNA extracted from a pure bacterial isolate obtained from a *Mycobacterium fortuitum*-infected zebrafish. Lane 6 contains extracted tissue DNA from a clinically normal zebrafish obtained previous to an epizootic with *Mycobacterium chelonae*. Lanes 7 and 8 contain extracted tissue DNA from two clinically normal zebrafish after treatment and repopulation of the water system. Lanes 9–12 contain extracted tissue DNA from four clinically normal zebrafish from two independent laboratories with no previous history of *Mycobacterium* spp. infection (laboratories C and D). Lanes 13–18 contain DNA extracted from the pure cell culture isolates of six bacterial genera unrelated to *Mycobacterium* spp. Lane 19 contains the no template PCR control.

have not been identified.

Approximately twelve months after initial diagnosis of the *M. chelonae/abscessus*-infected fish in laboratory B, the infection has been controlled through quarantine of the affected system and resident fish. Zebrafish with clinical disease are removed to limit the shedding of bacteria directly into the water or exposure of other fish through cannibalism. Subsequent testing (PCR analysis and culture) at 6 and 12 months after diagnosis continued to reveal the presence of *Mycobacterium* spp. Although zebrafish mortality is higher in this water system, compared with that in zebrafish maintained in the other water systems in this facility, egg production for research use has remained constant, although at a level significantly lower than that observed before the outbreak. Routine screening of zebrafish from the other initially uninfected systems in the facility by use of acid-fast staining and PCR analysis of tissue has indicated that the infection appears to be limited to fish housed in the original water system.

Discussion

To our knowledge, this is the first detailed report of atypical mycobacteriosis in laboratory maintained zebrafish. Mycobacteriosis remains the most important infectious disease threat to established fish colonies, resulting in lost research time, valuable animals, and operating capital. Directly preceding both outbreaks described here, the fish were subjected to substantial environmental stress (chronic hypothermia and GBD). In addition to avoiding poor water quality conditions, the avoidance of high stocking densities in laboratory fish colonies significantly reduces environmentally induced stress (10).

The clinical signs of mycobacteriosis in the zebrafish of our study were highly variable. Acute and chronic forms of mycobacterial infection have been characterized in teleosts (12). Histopathologic findings of affected fish were typical of both the acute and chronic forms of mycobacteriosis (13). Chronically diseased fish with mycobacteriosis usually have poor growth rate, chronic wasting, and emaciation. In this study, decreased reproductive rates and slightly increased mortality in both colonies were observed. Acute clinical signs known as "dropsy syndrome," consisting of abdominal distention and scale edema, were present. The edema causes a distinctive elevation or "porcupine-like" effect to the scales. "Dropsy" has been previously described as a common clinical finding in numerous species of fish infected with bacterial pathogens, such as *Mycobacterium* spp. (14). Petechiation, ulceration of skin, and fin erosion are often common clinical findings indicative of bacterial sepsis. Irrespective of clinical signs of disease, affected fish should be immediately removed from the water system to minimize spread of mycobacteria by cannibalism or environmental contamination.

Although bacterial culture currently remains the most specific method for the diagnosis of mycobacteriosis, it does not allow rapid screening that is often necessary in the laboratory research setting. Since culturing for atypical *Mycobacterium* spp. may require up to 90 days for definitive confirmation, most researchers are unwilling to maintain fish in quarantine for that lengthy duration. Also, since the atypical *Mycobacterium* spp. are classified as biosafety level-2 organisms, bacterial culture requires specialized equipment and personnel protection.

The PCR method offers a viable alternative for the rapid diagnosis of atypical mycobacterial infections in the aquatic laboratory setting (7, 15, 16). Direct screening of fish tissue without prolonged DNA extraction procedures can be effectively accomplished within 1 to 2 days (7). Implementation of a PCR-based diagnostic assay provides the quickest and most effective method of accurate and rapid screening for mycobacteriosis in laboratory zebrafish. Our results indicate that PCR-based assays for screening of incoming zebrafish are a valuable diagnostic aid to the detection of clinical and subclinical mycobacteriosis. Furthermore, amplified DNA products yielding the *Mycobacterium* genus-specific 924-bp product can be identified to the species level by subsequent use of restriction enzyme analysis (7). Our study also indicated that, although acid-fast staining of impression smears and histologic examination of tissue were useful diagnostic screening tests, the sensitivity of these techniques was substantially less than that of either bacterial culture or PCR. Similar findings were reported in an infection of a laboratory employee in which an impression smear of purulent discharge from a granulomatous hand lesion was acid-fast negative but culture positive (17).

Since various *Mycobacterium* spp. have been isolated from environmental biofilms that form in water systems, effective treatment can only be accomplished by eradication of infected fish stocks and subsequent disinfection of all exposed surfaces in the facility (18, 19). Once mycobacteriosis is endemic within an established colony, it is difficult to completely eradicate the organism. Various treatment attempts with antibiotics have had limited success in controlling the infection, but unfortunately did not eliminate *Mycobacterium* spp. from affected fish colonies (20–22). In particular, one study suggested that kanamycin was effective in treating clinical mycobacteriosis in guppies (22). However, the infection was reported to only have been completely controlled once culling and disinfection of the infected stocks was performed. Also, although antibiotic treatment was effective in alleviating clinical signs of disease and

the fish were acid-fast negative (via tissue staining) after treatment, attempt was not made to culture the causative organism either before or after antibiotic treatment of the infected fish (22).

Because of the insidious nature of this disease and the inability of antibiotics to effectively eradicate infected fish stocks, strict quarantine and diagnostic screening are the best methods to prevent introduction of *Mycobacterium* spp.-infected zebrafish. Equally important are maintaining optimal animal health and water quality to prevent mycobacteria from infecting fish due to concurrent disease or environmentally induced stress (8).

Atypical mycobacterial infections of humans due to zoonotic transmission of *M. marinum* and *M. fortuitum* have been described. Although these infections are a rare occurrence, frequent, close contact with infected fish, aquaria, or microbiological cultures is a known risk factor (17). These infections are usually self-limiting and restricted to localized areas of granulomatous inflammation of the affected extremity (23). However, appreciable disease in humans, such as persistent cutaneous granulomas, osteomyelitis, tenosynovitis, septic arthritis, and periocular infection, have been reported as a result of trauma with subsequent exposure to infected surfaces or increased susceptibility due to concurrent immunosuppression (24–27). Frequently, resolution of these persistent infections requires lengthy systemic antibiotic treatment regimens and surgical debridement (4, 28). Life-threatening and fatal disease due to *M. marinum* and *M. fortuitum* have also been documented principally in immune compromised humans (29).

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