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Atypical Piscine Mycobacteriosis in Japanese Medaka (*Oryzias latipes*)

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Japanese medaka, (*Oryzias latipes*), small, freshwater, tropical cyprinodonts, are principally used for toxicologic and carcinogenicity assays, but are finding more applications in developmental genetic and biological research. An increase in mortality began in brood stock of adult medaka that had been shipped and housed separately by sex. Initially, mortality averaged one fish daily and began in females two weeks after they were received. Cohabitation began eight weeks after arrival. After four to six weeks of cohabitation in different spawning aquaria, mortality was observed in males. Clinical signs of disease included loss of scale luster and color, with subsequent blanching of dorsal flank musculature, small raised nodules on various external surfaces, emaciation, fraying of fin tips, and equilibrium disturbances. Histologic examination of affected adults revealed multi-organ granulomatous inflammation with intracellular acid-fast bacilli. Specimens from 46 juvenile medaka that were spawned from affected adults, were submitted for culture and histologic evaluation. Of 18 fish, two had lesions similar to those of adults. The organism isolated from the remaining fish was identified as *Mycobacterium fortuitum*. Due to atypical rapid progression of disease, spread of *M. fortuitum* to progeny, and poor prognosis, the entire colony was euthanized.

Piscine mycobacteriosis is a common disease of freshwater and marine fishes in temperate and tropical waters, and several *Mycobacterium* species have been implicated, including *M. fortuitum*, *M. marinum*, *and M. chelonae* (1-5). *Mycobacterium abscessus*, formerly *M. chelonae* subspecies *abscessus*, has been isolated from Japanese medaka; however, infected fish did not manifest clinical signs of disease, did not suffer appreciable mortality, but developed granulomas with few acid-fast bacteria (6). In fish, *M. fortuitum* causes a generalized granulomatous response, compared with the more focal discrete granulomas caused by either *M. marinum* or *M. chelonae* (7).

We describe an atypical presentation of piscine mycobacteriosis in Japanese medaka, (*Oryzias latipes*), in which use of various diagnostic, histologic, and bacteriologic cultural techniques were employed. Medaka are used principally for toxicologic and carcinogenicity assays, but are increasingly used in genetics and developmental biology (8). They are small, freshwater, tropical cyprinodonts that reach a length of 3.5 cm as adults and have an average life span of three years. Medaka are also oviparous and are easy to propagate and maintain under artificial culture conditions (6).

Case Report

Clinical findings. Excess mortality occurred in a brood stock of 120 adult medaka. Two weeks after arrival, females that had been shipped and housed separately from males began to die. Mortality among the females averaged one fish per day for three months (range: 1 to 3/d). Spawning cohabitation began eight weeks after arrival. Following four to six weeks of cohabitation, mortality was observed among males in several spawning aquaria. Clinical signs of disease included protracted loss of scale luster and color, with subsequent development of focal to diffuse blanching of the caudal peduncle and dorsal flank musculature. Multifocal, white, round, raised nodules developed and were located caudal to the base of the dorsal fin, on the caudal peduncle, at the base of the pectoral fins, and on the ventral surface of the mandible. As the condition progressed, equilibrium disturbances, emaciation, fraying of fin tips, and in one instance, trailing clear fecal casts, were observed. Feeding activity remained normal until equilibrium disturbances were observed. Treatment of affected tanks with 1 ml of malachite green/formalin solution/3.8 L for 18 h (7.2 mg of malachite green dissolved in 37% formalin) resulted in only temporary abatement of some clinical signs. Medaka were euthanized by administration of an overdose of either benzocaine (ethyl aminobenzoate) or tricaine methanesulfonate (MS-222, Finquel, Argent, Redmond, Wash.) when they had decreased feeding or activity behavior, with or without equilibrium disturbances. Five months after arrival, only six of the original 120 adult medaka were alive, exhibiting various stages of disease, and were euthanized. During this outbreak, spawned progeny from affected adults (except the first clutch) had been moved into a recirculating system following egg disinfection, incubation, and larval rearing procedures. During the four months after the initial adult mortality, only one fatality occurred among approximately 25 generations spawned.

Housing and husbandry. A total of 120 five-month-old Japanese medaka (*Oryzias latipes*) were purchased from a commercial vendor (Aquatic Research Organisms, Hampton, NH). Medaka were maintained under the guidelines provided by the *Guide for the Care and Use of Laboratory Animals* and the United States Public Health Service Policy on the Humane Care and Use of Laboratory Animals. Their experimental use was approved by the Institutional Animal Care and Use Committee of the University of Washington.

Medaka (90 females and 30 males) were housed separately as groups in glass aquaria of 120- and 40-L capacity, respectively. Eight weeks after arrival, individual male and female medaka were subsequently transferred into either four 20- or one 40-L

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capacity glass aquaria for spawning. Five to seven females and one to two males were grouped to provide a spawning density of fewer than 1 fish/3.8 L. When eggs were clustered beneath the ventral coelom of the female, the fish were netted and the eggs removed by disposable plastic pipettes to plastic petri dishes for incubation. Eggs were disinfected with 100 mg of povidine-iodine solution/L for 15 min without agitation (9) and were incubated in a hatching solution, containing 0.5 mg of methylene blue (fungal growth inhibitor)/L, for 16 days at 30°C. Larval fish were transferred to 0.5 Liter tanks in a 40 Liter larval rearing system upon hatching. When they were 1 cm long, fish were transferred to 10-L tanks in a 320-L juvenile rearing system. These procedures were performed on all except the first clutch of eggs. As a result, the first clutch of eggs was spawned and reared to juveniles in a glass aquarium without proper disinfection and were moved directly into the larval recirculating system.

All 20-, 40-, and 120-L aquaria were powered by Hagen Aquaclear (Rolf C. Hagen Inc., Montreal, Canada) filters that contained a sponge, activated carbon, and calcium carbonate. Temperature was maintained between 24 and 26°C (Table 1) by use of Visi-Therm water heaters (Aquarium Systems, Mentor, Ohio). The 320-L system involved use of an in-line Ocean Clear (Red Sea Fish pHarm Ltd., Houston, Texas) 50-μm polystrand bio-bead canister filter followed by an Ocean Clear 25-μm cartridge canister filter and an ultraviolet light sterilization unit (Model No. CA-40, Rainbow Lifegard Aquarium Products, El Monte, Calif.) configured to produce 200,000 $\mu W/s/cm^2$. However, this component was not operational until two months after juvenile medaka progeny had been transferred into this system.

Fish were fed salmon starter (No. 1 crumble, Moore-Clark, Vancouver, British Columbia, Canada) and live *Artemia salina nauplii* (cysts from Argent, Redmond, Wash.) twice daily and were maintained on a 14:10-h light:dark cycle. A third of the water volume from each aquarium was replaced weekly. All equipment was washed and disinfected by use of a commercial bleach solution diluted to 10% (200 mg of free chlorine/L) with minimal contact time of one hour (10). Disinfected equipment was rinsed with tap water before air-drying.

Diagnostic laboratory findings. Differential diagnosis for this case included: helminths, particularly digenetic trematodes; the systemic protist-like agent, *Ichthyophonus hoferi*; bacterial pathogens, *Nocardia* and *Flavobacterium* spp.; and microsporidiosis, particularly *Pleistophora hyphessobryconis* and *Glugea anomala*. One month after mortality began, nodules on adult medaka were examined, via dissection microscopy, for presence of encysted stages of helminths. Their absence ruled out the presence of digenetic trematodes.

Histologic examination. After three months, one dead fish, one moribund fish, one fish with early clinical signs of disease, and one fish that appeared normal were submitted for histologic evaluation. All fish had severe to moderate multifocal granulomatous myositis, splenitis, nephritis, peritonitis, hepatitis, myocarditis, enteritis, oophoritis, branchitis, dermatitis, perineuritis, encephalitis, and perivasculitis, with lack of a fibroplastic reaction, that was characterized by the presence of large numbers of intracellular rod-shaped bacteria principally within the cytoplasm of macrophages (Fig. 1). Gram staining revealed gram-variable reaction for intracellular rod-shaped bacteria, which ruled out *Ichthyophonus hoferi*. Use of Kinyoun's modification of the Ziehl-Neelsen acid-fast stain revealed a positive, red, acid-fast reaction for the intracellular rod-shaped bacteria, which ruled out *Flavobacterium* sp., *Pleistophora hyphessobryconis*, and *Glugea anomala*.

Bacteriologic examination. To determine whether the progeny spawned were similarly infected, six juvenile medaka were randomly sampled from various clutches contained in a 320-L recirculating system and were examined by use of similar procedures. One medaka had severe multifocal granulomatous myositis characterized by large numbers of intracellular acid-fast, rod-shaped bacteria observed principally within the cytoplasm of macrophages. Forty live juvenile medaka from various clutches were sent to Washington Animal Disease Diagnostic Laboratory (Washington State University, Pullman, Wash.) for evaluation. Seventeen fish were evaluated histologically and 23 were pooled and homogenized for bacterial isolation. Of the 17 juvenile medaka evaluated histologically, one fish had moderate, multifocal granulomatous myositis, nephritis, myocarditis, enteritis, hepatitis, oophoritis, and encephalitis, with intracellular, acid-fast bacilli consistent with mycobacterial species. Bacterial culture revealed the bacterium, *M. fortuitum*, which ruled out *Nocardia* sp. Due to the severe disease in adults and the presence of *M. fortuitum* in spawned progeny, the entire colony was depopulated and all systems were thoroughly cleaned and disinfected. To the authors' knowledge, this is the first report of disease induced by *M. fortuitum* in medaka.

Discussion

The first isolation of *M. fortuitum* from a lesion (human abscess) was reported in 1938 (11). The initial recovery of *M. fortuitum* from a fish species, the neon tetra *Hyphessobrycon innesi*, occurred in 1953 (12). In 1963, *M. fortuitum* was reported as the

Table 1. Water quality data from systems before and during increased mortality period of Japanese medaka (*Oryzias latipes*)

Water source	Temperature (°C)	pH	Nitrite (mg of NO_2^-/L)	Ammonia (mg of NH_3/L)	Hardness (mg of $CaCO_3$/L)	Conductivity (μs)
Normal ranges	25-28	7 to 8	0.0	<1.0	150 to>300	500 to 1000
Vendor[a]	27	7.3	0.0	0.0	200	ND[b]
De-chlorinated tap water	25.5	7.5	0.0	0.0	51	53
Glass aquaria[c]	24.8 ±0.12[d]	7.6 ±0.03	ND	0.17 ±0.09	ND	1370 ±111.4
Larval/ juvenile systems[c]	25.4 ±0.13	7.4 ±0.03	ND	0.65 ±0.16	ND	1193 ±70.9

[a]Mean values supplied by vendor.
[b]Not done.
[c]Values are means of water sample data taken daily over a four-month period.
[d]Values are ± standard error of the mean of water sample data taken daily over a four-month period.

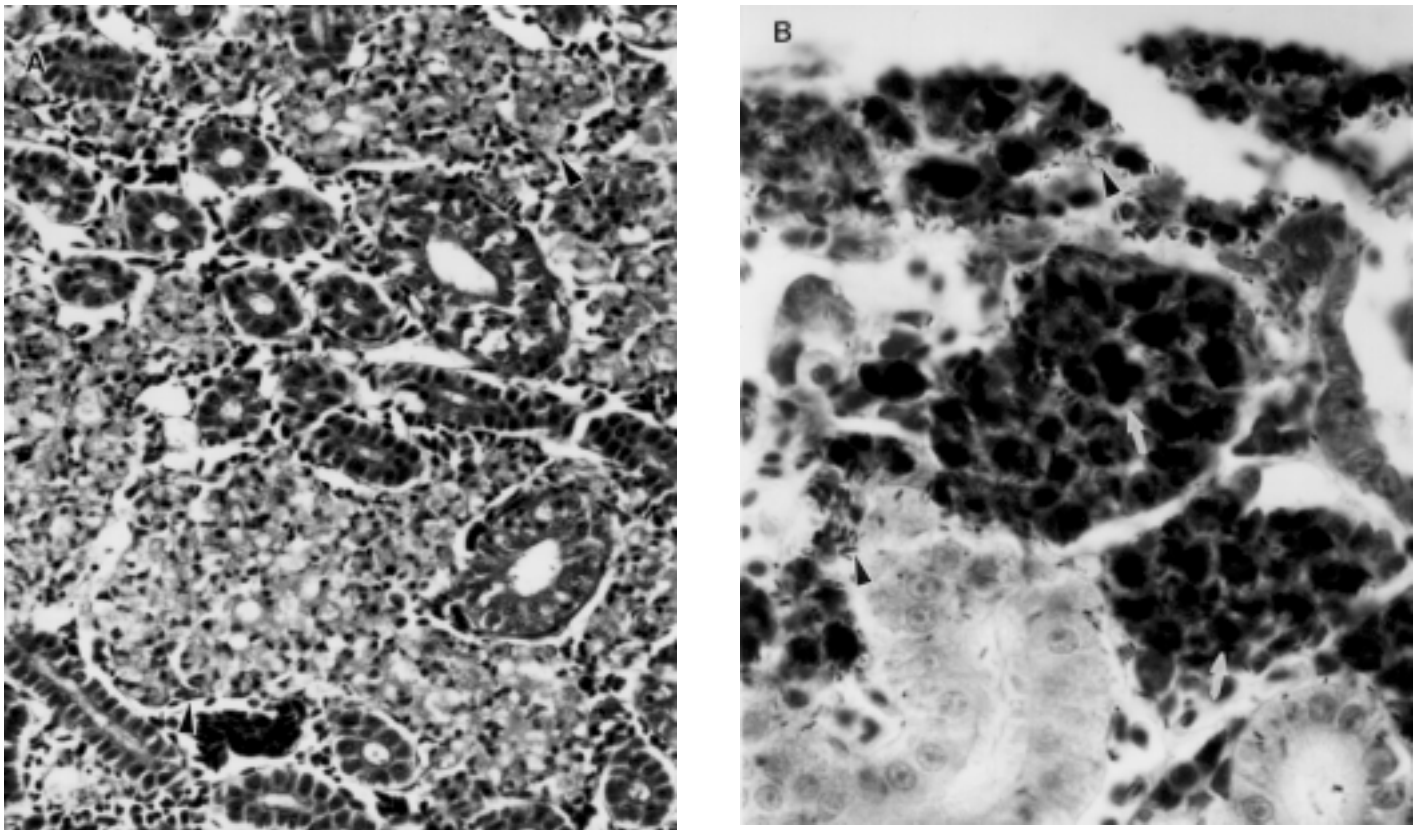

Figure 1. Photomicrographs of sections of kidney from Japanese medaka. (A) Notice numerous histiocytes (arrowheads) crowded within the renal interstitium, and lack of a fibroplastic response by the host to isolate histiocytic aggregates. H&E stain; magnification x 370. (B) Notice multiple histiocytes (arrows) within the renal interstitium filled with densely packed acid-fast-positive, rod-shaped bacteria, and presence of individual bacteria (arrowheads) near histiocytic margins. Kinyoun's modification of the Ziehl-Neelsen stain; magnification x 925.

causative agent of spontaneous tuberculosis in fish and other cold-blooded vertebrates (1). *Mycobacterium fortuitum* is a weakly staining, gram-positive, aerobic, non-motile, non-sporing, acid-fast rod that is saprophytic and found in soil and diverse aquatic environments (13, 14). It is one of the more rapidly growing mycobacterial species, capable of growth between 22 and 40°C, and is known to readily and rapidly form biofilms (15). This species belongs to the Runyon Group IV of atypical mycobacteria that also includes *M. chelonae,* and is usually referred to as the *M. fortuitum / M. chelonae* complex (16). *Mycobacterium fortuitum* is a pathogen, although usually opportunistic, in humans and animals. In addition to fish, it has been isolated from a wide range of vertebrate hosts, including mammals, amphibians, reptiles, birds, and marsupials (17-22).

We have presented evidence that *M. fortuitum* is pathogenic to medaka. The presentation of mycobacteriosis is atypical due to the severity and rapid progression of disease observed in adults. The original source of infection was undetermined, although sub-clinical (latent) infection or natural infection by ingestion of biofilm or feed may have occurred. *Mycobacterium fortuitum* can be transmitted through enclosed recirculation systems. The method of transmission is of concern because the spawned progeny had a low prevalence of infection without induction of clinical signs of disease. Knowledge regarding the pathogenesis of this agent in this host is rudimentary. Mycobacteriosis may have been associated with poor immune response related to environmental shock/stress, increased genetic susceptibility, or exposure to a virulent strain of *M. fortuitum*.

Potential primary sources of *M. fortuitum* infection of adult medaka include: arrival with a sub-clinical (latent) infection; ingestion of bacteria existing in the biofilm within the glass aquaria; or consumption of infected feed. When the vendor was notified, neither the vendor nor any of its recent clients had observed excess mortality or disease outbreaks of any kind. The pasteurization process used during commercial fish feed production should be sufficient to kill *M. fortuitum* and other bacterial pathogens (23). However, samples of the water and feed were not submitted for microbiological culture.

Transmission of *M. fortuitum* may have occurred by a vertical or horizontal mechanism, including transovarial transmission, cross-contamination from improperly disinfected nets, improper spawning tank or egg disinfection techniques, or bacterial colonization of glass tank biofilm or biological filtration media of recirculating systems (15, 24). *Mycobacterium fortuitum* transmission has been achieved experimentally by intraperitoneal inoculation and ingestion of contaminated feed or infected fish carcasses (1). Because infective bacteria can be shed from ulcerated skin lesions, intestines, and decomposing fish carcasses, oral

ingestion is the most likely portal of entry (1, 3, 10, 24). Epidermal trauma, although less common, can become a more important portal of entry for mycobacterial infections under conditions of high stocking density, poor water quality, and poor nutrition (5, 25).

Transovarial transmission of mycobacteriosis has been documented for the viviparous Mexican platyfish (*Xiphophorus maculates*) (26), but not for salmonids and other oviparous fish (27, 28). However, a report from Australia regarding eggs from the oviparous salmon, indicates that mycobacteria may be introduced by eggs and transmitted to a first generation of progeny (29). Transovarial infection studies have yet to be done in the oviparous Japanese medaka (*Oryzias latipes*).

In this case report, the disease presented atypically, especially in the adults, without formation of either caseous necrosis or the classic epitheliod focal to miliary granulomas (2, 7). The lesions seen in medaka are similar to a non-granulomatous-type reaction without the prominent fibroplasia frequently seen in the mammalian histiod response (30). This presentation may be related to the host's ability to respond to the pathogen, virulence of this particular strain of *M. fortuitum*, duration of infection, or a combination of these factors.

Progeny medaka infected with *M. fortuitum* failed to manifest clinical signs of disease similar to those in adults, but had pathologic lesions of similar severity. Although the number of infected fish was small among the juvenile medaka evaluated histologically, the organism was isolated from pooled samples of fish homogenates. This may lead to speculation that *M. fortuitum* may have been transmitted transovarily. However, it is more probable that external colonization of the eggs and subsequent infection of progeny upon hatching may have been the mechanism of *M. fortuitum* introduction into the 320-L juvenile system. Had this occurred with the first clutch, it would have negated the effect of external egg disinfection with an adequate concentration and contact time of the povidine-iodine solution in subsequent clutches. Additionally, the two-month delay in the installation of the in-line (parallel) ultraviolet light sterilization unit provided ample time for *M. fortuitum* to proliferate within the system (15, 31, 32).

The water quality data (Table 1) provided by the vendor, in comparison with the in-house water quality during housing in aquaria, indicated the only appreciable deficiency to be the hardness, 200 mg of $CaCO_3$/L at the vendor versus 51 mg of $CaCO_3$/L at our facility. Although captive-bred fish are less susceptible to stress (environmental shock/delayed mortality syndrome) than are wild-caught fish, the change in water hardness may have induced osmoregulatory shock/stress (10, 33), resulting in clinical manifestations of a sub-clinical (latent) infection of *M. fortuitum* or enhanced susceptibility to a primary infection.

Since *M. fortuitum* is usually resistant to common human antitubercular drugs and little information on the bioavailability in fish of any of the antimycobacterial drugs is currently known, treatment was not recommended (3, 10, 14, 16). However in this instance, the temporary abatement of some clinical signs of disease observed after treatment with malachite green/formalin is related to the antimycobacterial properties of formalin (17, 34, 35). The option of continual treatment with malachite green/formalin was not explored due to previously observed recrudescence.

Due to the chronic nature of disease, difficulty in treatment, and the fact that *M. fortuitum* is a rapid producer of biofilm in aquatic environments (15), disinfection, quarantine, and detection of infected individuals are the best methods of control (10). Ultraviolet irradiation (90,000μW/s/cm^2), 70% ethyl alcohol (without organic matter), and chlorine-releasing agents (100 mg of free chlorine/L) rapidly kill *M. fortuitum* (31, 32, 34-37). Use of 200 mg of free chlorine/L to disinfect equipment is more than adequate to kill *M. fortuitum* if most of the organic material is removed mechanically. Commonly used 10% iodophor disinfectants do not reliably kill mycobacteria, but further aqueous dilutions of the iodophor disinfectant from 1:2 to 1:100 (vol:vol) are more rapidly bactericidal than are undiluted preparations (38, 39). This peculiarity has been thought to be associated with the amount of free iodine available for bactericidal activity (39). It is not known whether these disinfectants penetrate the biofilm produced by *M. fortuitum*; however, without use of a disinfectant with the means of adequately penetrating the biofilm, thorough decontamination of eggs may be hindered (15).

Mycobacterium fortuitum has been documented to cause focal to diffuse epidermal or dermal granulomas in humans and, as such, should be considered a zoonotic agent along with *M. marinum* and *M. chelonae* (14, 16). To minimize exposure to *M. fortuitum*, use of protective gloves during routine handling of fish or during equipment cleaning, in addition to frequent hand washing with soap and water, is recommended.

This case report highlights the necessity of using appropriate quarantine and disinfection protocols with regard to laboratory fish species. In addition, incorporation of a practical method of detecting infective organisms during the quarantine period is recommended. Use of sterilized or reverse osmosis water for rinsing chemically disinfected equipment prior to air-drying or autoclaving of equipment after thorough washing is recommended. Incorporation of additional in-line, successively smaller diameter filters (10, 5, or 1 μm) prior to the ultraviolet sterilization unit also is recommended. Finally, dilutions of a 10% iodophor disinfectant with gentle agitation should be considered for disinfection of eggs.

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