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NOTE

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A case of mycobacteriosis associated with *Mycobacterium pseudoshottsii* in aquarium-reared fish in Japan

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ABSTRACT. In 2019, several aquarium-reared fish died at a sea life park in Japan. Necropsy revealed micronodules on the spleen in the dotted gizzard shad (*Konosirus punctatus*). Seven of 16 fish exhibited microscopic multifocal granulomas associated with acid-fast bacilli in the spleen, kidney, liver, alimentary tract, mesentery, gills, and/or heart. Bacterial cultures yielded isolates from the dotted gizzard shad and a Japanese sardine (*Sardinops melanostictus*). Microbiological and molecular biological examinations revealed the isolates as *Mycobacterium pseudoshottsii*. To our knowledge, this is the first isolation of *M. pseudoshottsii* from aquarium-reared fish.

KEYWORDS: aquarium-reared fish, *Mycobacterium pseudoshottsii*, mycolactone-producing mycobacteria, nontuberculous mycobacteria

Mycobacterium pseudoshottsii, a slow-growing, photochromogenic, nontuberculous mycobacterium, is a member of the *Mycobacterium marinum* group (MMG; *M. marinum*, *M. ulcerans*, *M. pseudoshottsii*, *M. shottsii,* and *M. liflandii*), whose members are closely related genetically [7, 19, 21]. MMG species include mycolactone-producing mycobacteria; mycolactone is a cytotoxic macrolide toxin whose gene is encoded on pMUM plasmids [6, 18].

M. pseudoshottsii is reported to have caused infectious diseases in at least ten species of fish. In Chesapeake Bay, the pathogen was initially isolated from wild striped bass (*Morone saxatilis*) in 2005 [19] and from wild white perch (*Morone americana*) in 2007 [22]. Nakanaga *et al*. isolated the bacteria from yellow tail (*Seriola quinqueradiata*), greater amberjack (*Seriola dumerili*), striped jack (*Pseudocaranx dentex*), sevenband grouper (*Epinephelus septemfasciatus*), and yellowtail amberjack (*Seriola lalandi*) farmed in western Japan [15]. Mugetti *et al*. reported *M. pseudoshottsii*-associated mycobacteriosis in red drum (*Sciaenops ocellatus*), European sea bass (*Dicentrarchus labrax*), and gilthead sea bream (*Sparus aurata*) on farms in the Mediterranean Sea [14].

Tokyo Sea Life Park, an aquarium in Japan, rears and exhibits approximately 50 fish from over ten species in a single tank, including the Japanese sardine (*Sardinops melanostictus*), dotted gizzard shad (*Konosirus punctatus*), marbled sole (*Pleuronectes yokohamae*), bluefin searobin (*Chelidonichthys spinosus*), horse mackerel (*Trachurus japonicus*), Japanese butterfish (*Psenopsis anomala*), Japanese conger (*Conger myriaster*), Japanese seabass (*Lateolabrax japonicus*), Japanese whiting (*Sillago japonica*), and white croaker (*Argyrosomus argentatus*). The fish were captured in Tokyo Bay and are housed in an elliptical/cylindric tank with an approximately 9-t capacity, closed-circulation system and water temperature of approximately 20°C. Several fish reared in this tank have died each month since February 2019 from nontuberculous mycobacteria. A field test (AFB-Color staining kit, Merck KGaA, Darmstadt, Germany) conducted on a few dead fish revealed acid-fast bacilli (AFB).

We collected six freshly dead fish and ten live fish from the aforementioned tank. The live fish were euthanized via cervical transection after anesthetization with an overdose of 2-phenoxyethanol in accordance with the American Veterinary Medical Association Guidelines for the Euthanasia of Animals (2013 edition) [12]. The collected fish were frozen at −20°C until necropsy. The fish were thawed and routinely dissected, and their external and internal gross features were observed. Tissues were collected from the major

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organs (i.e., spleen, liver, kidney, heart, gills, and/or alimentary tract), fixed in 10% phosphate-buffered formalin solution, routinely processed and embedded into paraffin blocks for histopathology. The tissue blocks were sectioned and stained with hematoxylin and eosin, Ziehl-Neelsen stain, and a modified Nyka's method as described by Harada (1977) [8, 17].

Gross examination revealed a few white micronodules of approximately 0.5 mm in diameter on the spleens of two dotted gizzard shad (1907-f16, f19), skin ulceration in one fish (1907-f7), and exophthalmos in two fish (1907-f5, f14). Microscopic examination revealed AFB-associated multifocal granulomas in the serosal membrane to muscular layers of the alimentary tract, mesentery, interstitial kidney tissue, and/or parenchyma of the liver and spleen in a Japanese sardine, four dotted gizzard shad, and the marbled sole (Supplementary Table 1). The granulomas often had a necrotic center with AFB, surrounded by epithelioid cells and a thin outermost rim of fibroblasts and connective tissue (Fig. 1). Some fish exhibited granulomas composed of mononuclear macrophages without epithelioid cells and a thin outermost rim.

Mycobacteria were isolated as follows. Tissues of the spleen, liver, and kidney were collected from 16 of the frozen fish. The tissue samples were homogenized with 0.5 mL of phosphate-buffered saline (PBS) (−) and decontaminated with 1 mL of N-acetyl-L-cysteine-sodium citrate (NALC)-NaOH or 0.75 mL of 1N HCl. After decontamination for no more than 15 min, the NALC-NaOH-treated samples were neutralized by adding at least 6 mL of 0.067 M phosphate buffer adjusted to pH 6.8, and the HCl-treated samples were neutralized by adding 0.75 mL of 1N NaOH. The samples were centrifuged at 3,000 × *g* for 20 min, then the supernatant was discarded, and the pellet was resuspended in 1 mL of Middlebrook 7H9 broth base (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) supplemented with 10% BBL Middlebrook Oleic Albumin Dextrose Catalase (OADC) enrichment (Becton, Dickinson and Co.). Aliquots (25 µL of each sample) were inoculated on Middlebrook 7H10 agar supplemented with 10% OADC enrichment and spread on 2% Ogawa egg slant (Kyokuto Pharmaceutical Industrial Co., Ltd., Tokyo, Japan). The media were incubated at 25°C for 2 months and checked daily for the first week, then once weekly thereafter. A single colony was subcultured on Middlebrook 7H10 agar to obtain pure isolates. The isolates were stained with Ziehl-Neelsen on glass slides to check their acid-fast stainability. Two of the three mycobacterial isolates obtained from a dead fish and a live fish (NJB1907-Z4 and NJB1907-f19, respectively; Supplementary Table 1) were subjected to the following examinations.

To classify the two isolates based on the Runyon classification system [20], their pigment production ability was tested on 2% Ogawa egg slants as per Fukano *et al*. (2015) [4]. The characteristics (e.g., arylsulfatase and Tween 80 hydrolysis) of the isolates and type strains (*M. marinum* JCM 17638 and *M. pseudoshottsii* JCM 15466) were tested according to the procedure described by the Japanese Society for Tuberculosis (2016) [24] with a modification of the incubation temperature to 25°C. The arylsulfatase test was conducted on days 3 and 14 [5]. Runyon classification and biochemical examination results indicated that isolates NJB1907-Z4 and NJB1907-f19 showed similar characteristics to those of *M. pseudoshottsii* JCM 15466 (Table 1).

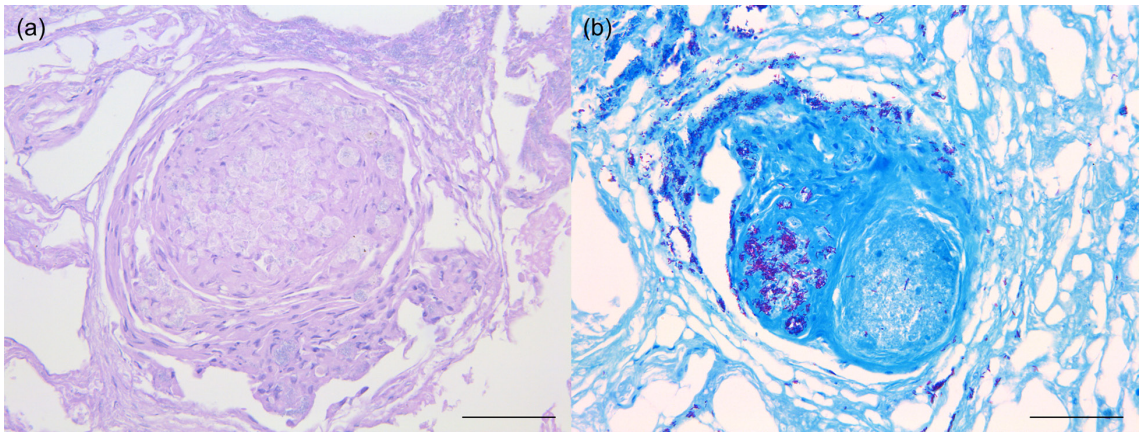

Fig. 1. Epithelioid granulomas in the mesentery of an infected fish (ID 1907-f16). A necrotic area with acid-fast bacilli, surrounded by epithelioid cells and a thin outermost rim of fibroblasts and connective tissue was observed. (**a**) Hematoxylin and eosin stain; (**b**) Ziehl-Neelsen stain. Bars=50 µm.

Table 1. Phenotypic and genomic characteristics of the isolates and type strains

Strain	ZN	Runyon group[a)]	Arylsulfatase reduction (3 days)	Arylsulfatase reduction (14 days)	Tween 80 hydrolysis	IS*2404* /*2606*
NJB1907-Z4	+	I	-	-	-	+/+
NJB1907-f19	+	I	-	-	-	+/+
Mycobacterium pseudoshottsii JCM 15466	+	I	-	-	-	+/+
M. marinum JCM 17638	+	I	-	+	+	−/−

[a)]Classified based on the Runyon classification system [20]. ZN, Ziehl-Neelsen stain.

Genomic DNA was extracted from five strains (*M. marinum* JCM 17638, ATCC BAA535, *M. pseudoshottsii* JCM 15466, NJB1907-Z4, and NJB1907-f19) and the frozen tissues of the spleen, liver, and kidney following the method of Komine *et al*. (2021) [10] and subjected to the following experiments. Molecular phylogenetic analysis was conducted based on a total of 1,997 bp of concatenated sequences of three housekeeping genes: 913 bp of 16S rRNA, 683 bp of RNA polymerase b-subunit (*rpoB*), and 401 bp of 65-kDa heat-shock protein (*hsp65*) following the method of Komine *et al*. (2021) [10]. The obtained sequences for these three genes from NJB1907-Z4 were deposited into the DNA Data Bank of Japan under accession numbers LC699671, LC699669, and LC699670, respectively. Presence of the insertion sequences, IS*2404* and IS*2606*, relating to mycolactone production in the isolates, was confirmed via polymerase chain reaction using the primer sets MU5–MU6 and MU7–MU8 through the cycles as described by Stinear *et al*. (1999) [23].

Isolates NJB1907-Z4 and NJB1907-f19 were closely related to *M. pseudoshottsii* JCM 15466 on the molecular phylogenetic tree using the concatenated DNA sequences (Fig. 2). PCR of the insertion sequences showed that both isolates as well as *M. pseudoshottsii* JCM 15466 were positive for IS*2404* and IS*2606* (Table 1). These results suggested that isolates NJB1907-Z4 and NJB1907-f19 were *M. pseudoshottsii*. *M. pseudoshottsii* has been isolated from several wild and cultured fish. However, to our knowledge, this is the first isolation of *M. pseudoshottsii* from aquarium-reared fish.

Because MMG species are reported to be highly genetically homologous, they are difficult to identify based only on sequencing analysis of the 16S rRNA gene [2]. Therefore, they should be identified using adequate methods as described herein, including biochemical examinations (arylsulfatase and Tween 80 hydrolysis) and molecular biological examinations (phylogenetic analysis based on the housekeeping genes 16S rRNA, *rpoB*, *hsp65* and detection of the insertion sequences IS*2404* and IS*2606*).

M. marinum, a representative MMG species, is a common causative agent of mycobacteriosis in fish and causes a skin infection known as “fish tank granuloma” in humans, thus posing a zoonotic concern [9]. Regarding the effects of *M. marinum* on aquatic resources, human economy and public health, appropriate measures, including sanitation, disinfection of the facility, and eradication of carrier fish, are necessary as primary control strategies to control *M. marinum* infection in fish [1, 3, 13, 16]. However, *M. pseudoshottsii* has never been isolated from humans. Thus, the risk of *M. pseudoshottsii* infection in humans may be lower than that of *M. marinum* infection. In conclusion, the pathogenesis of each clinical case should be clarified, and the causative agent should be identified in detail using adequate methods as described herein to avoid applying the wrong countermeasures to suspected clinical cases associated with MMG.

POTENTIAL CONFLICTS OF INTEREST. The authors have nothing to disclose.

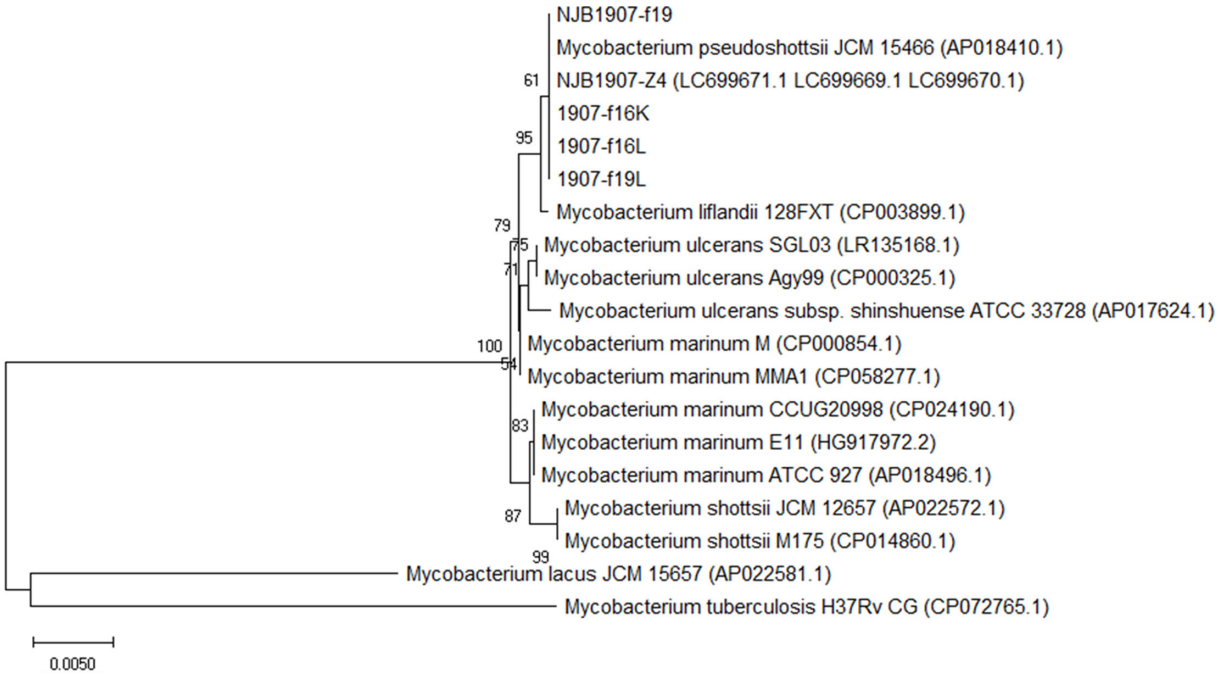

Fig. 2. Molecular phylogenetic tree generated from a concatenated 1,997-bp sequence of the 16S rRNA, *rpoB*, and *hsp65* genes from a kidney (1907-f16K), livers (1907-f16L, f19L) and two isolates (NJB1907-Z4, f19). The tree was constructed using the neighbor-joining method with Kimura’s two-parameter model in Mega X [11]. Bootstrap values are shown at nodes as percentages of 1,000 replicates. GenBank accession numbers are in parentheses.

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