A Unique *Mycobacterium* Species Isolated from an Epizootic of Striped Bass (*Morone saxatilis*)

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We isolated a *Mycobacterium* sp. resembling *Mycobacterium marinum* and *M. ulcerans* from diseased striped bass *(Morone saxatilis)* during an epizootic of mycobacteriosis in the Chesapeake Bay. This isolate may represent an undescribed *Mycobacterium* species, based on phenotypic characteristics and comparative 16S rRNA gene sequence.

Natural aquatic environments are recognized sources of mycobacteria known to cause disease in both humans and fish. Although *Mycobacterium marinum* is considered the primary causative agent of fish mycobacteriosis, seven *Mycobacterium* species associated with tubercle granulomas in aquarium, cultured, and wild fish populations have been described: *M. abscessus, M. chelonae, M. fortuitum, M. marinum, M. neoaurum, M. scrofulaceum,* and *M. simiae* (1,2). All these species cause disease in humans (3,4). Primary clinical syndromes include skin and soft-tissue infections, cervical lymphadenitis, pulmonary disease, and disseminated infections, the last generally being limited to immunocompromised persons. Human mycobacteriosis following occupational or recreational exposure to the marine environment is frequently associated with trauma such as wounds from handling fish and has been attributed primarily to *M. marinum* (5). Consequently, the discovery of an undescribed *Mycobacterium* species associated with an epizootic of mycobacteriosis in striped bass (*Morone saxatilis*) warrants recognition and additional study.

Mycobacteriosis in fish is a subacute to chronic wasting disease known to affect some 167 freshwater and saltwater species (2). Internal signs of the disease vary according to fish species but typically include granulomas in the spleen, kidney, and liver. External manifestations include scale loss accompanied by hemorrhagic lesions penetrating the musculature in advanced cases. Recently, an ongoing epizootic of mycobacteriosis in striped bass (*Morone saxatilis*) from the Chesapeake Bay was described (Vogelbein W et al., unpub. data). Previous outbreaks of mycobacteriosis in wild striped bass have occurred in Pacific estuaries (6). During the Chesapeake Bay epizootic, we isolated a variety of mycobacteria associated with skin and visceral lesions that included a unique group of slowly growing nonpigmented isolates. We describe one of these isolates, which has specific characteristics similar to those of *M. marinum* and *M. ulcerans*.

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Striped bass (n = 20) we examined included asymptomatic and symptomatic fish with skin ulcerations (Figure) verified histologically to exhibit granulomatous inflammation associated with acid-fast bacilli. All fish were caught in the Chesapeake Bay or one of its tributaries (the James, Potomac, or Rappahannock rivers). Skin and spleen samples from necropsied specimens were processed for routine paraffin histology, sectioned at 5 µm, and stained with hematoxylin and eosin. Selected sections were stained using Ziehl-Neelsen's method for acid-fast bacteria (7). Excised internal tissues (predominately spleen) were homogenized in phosphate buffer using a Ten Broeck tissue grinder and inoculated directly onto culture media or after treatment with one of the following disinfectants (Vogelbein et al., unpub. data): 0.3% Zephiran (Sanofi Winthrop Pharmaceuticals, New York, NY), 2% NaOH, or 2% HCl. Homogenates were inoculated onto Löwenstein-Jensen slants and plates of brain heart infusion agar containing 5% sheep red blood cells and Middlebrook 7H10 agar with albumin-dextrose-catalase enrichment. Initially inoculated media were incubated at 30°C for a minimum of 2 months. Because some isolates exhibited poor growth, a second incubation temperature

Figure. Skin ulcers typical of mycobacteriosis in striped bass (*Morone saxatilis*) from the Chesapeake Bay.

(23°C) was used for primary media inoculated with tissue homogenates.

Purified isolates were characterized phenotypically by traditional methods (8) with incubation at 23°C. Mycolic acids were analyzed by a standardized method for mycobacteria by using reverse-phase high-performance liquid chromatography (HPLC) with UV detection (9,10).

Polymerase chain reaction (PCR) assay and sequence analysis of the 16S rRNA gene were used to characterize one of the slow-growing, nonpigmented mycobacteria, hereafter called isolate M175. This isolate is deposited in the American Type Culture Collection (ATCC), Rockville, MD, as ATCC 700981. The 16S rRNA gene was amplified in 120-µL volumes (11) by using cycle conditions described by van Berkum and Fuhrmann (12). Primers (forward, M16SA, 5'-CGC TGG CGG CGT GCT TA-3' and reverse, M16SB, 5'-ACG GCT ACC TTG TTA C-3') were specifically designed for the amplification of mycobacterial 16S rRNA genes. The PCR buffer (pH 8.5) contained 60 mM Tris-HCl, 15 mM $(NH_4)2SO_4$, and 1.5 mM $MgCl_2$; control reactions without template were included. After purification of PCR products (QIAquick Spin columns, Qiagen Inc., Chatsworth, CA), amplicons were sequenced with a Perkin-Elmer 377 DNA Sequencer in combination with a Dye Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer, Foster City, CA) (11,12).

Granulomatous inflammation was confirmed histologically in spleens of 18 of the 20 fish. Severity of the infection based on the abundance and size of splenic granulomas varied from mild to severe. Skin ulcers were evident in 13 specimens. Granulomatous inflammation was generally associated with acid-fast bacilli in selected stained sections.

Colony development from homogenized tissue was slow, requiring 4 to 6 weeks' incubation at 23°C on the preferred medium, Middlebrook 7H10 agar. Isolate M175 showed little or no growth at 30°C and none at 37°C. Rough nonpigmented colonies were flat with an irregular margin and yielded aggregates of acid-fast nonbranching rods. Isolate M175 was negative for growth on MacConkey agar and Löwenstein-Jensen with 5% NaCl, arylsulfatase, beta-galactosidase, nitrate reductase, semiquantitative catalase, Tween 80 hydrolysis, and Tween opacity. Weak positive reactions for catalase activity after treatment at 68°C and pyrazinamidase after extending incubation to 14 days were observed. Isolate M175 was positive for tellurite reduction, niacin production, and urease. Colonies did not produce pigment after exposure to light for several hours or after prolonged exposure for several days. Based on the aforementioned characteristics, this isolate could not be assigned to an existing species.

The M175 mycolic acid pattern consisted of a single cluster of eight peaks that visually resembled reference patterns (10) for species of the *M. tuberculosis* complex. However, M175 mycolic acid peaks did not superimpose with peaks of *M. tuberculosis* after alignment with the internal size standard. Peak elution times for M175 were suggestive of more polar, shorter, carbon chain-length mycolic acids than those found in *M. tuberculosis* complex species. Comparisons of the M175 pattern with the *Mycobacterium* HPLC mycolic acid database at the Centers for Disease Control and Prevention confirmed a unique pattern suggestive of a new species of mycobacteria.

The sequence of the PCR product of the 16S rRNA gene from *Mycobacterium* isolate M175 was 1,494 nt long. This sequence was deposited in GenBank and was given accession number AY005147. Blast searches of GenBank yielded high sequence similarities of 99.2% to *M. marinum* (13) and *M. ulcerans* (14) and of 98.7% to *M. bovis* (15) and *M. tuberculo-*

Table. Comparison of distinguishing phenotypic features of *Mycobacterium* sp. isolate M175 with genetically (16S rRNA) similar *Mycobacterium* spp. (*M. bovis*, *M. marinum, M. tuberculosis,* and *M. ulcerans*)

Characteristic	*Mycobacterium* sp. (fish isolate M175)	*M. bovis*	*M. marinum*	*M. tuberculosis*	*M. ulcerans*
Optimum growth temperature (°C)	<30	37	30	37	30
Colony morphology	R	R	S	R	S/R
Pigmentation	N	N	P	N	N
Arylsulfatase					
3 days	-	-	V	-	-
14 days	-	-	+	-	+
Niacin	+	-	-/V	+	-/V
Nitrate reduction	-	-	-	+	-
Pyrazinamidase 7 days	-	-	+	+	-
Tween hydrolysis	-	V	+	V	-
Urease	+	+	+	+	-

Source: Data for known species cited from references 17 and 18.
Abbreviations: d = days; N = nonpigmented; P = photochromogenic; R = rough; S = smooth; + = at least 85% strains positive; - = at least 85% negative; V = variable.

sis (16). High sequence similarities between 16S rRNA genes of M175 and other *Mycobacterium* spp. and phenotypic data support the conclusion that M175 belongs within the genus *Mycobacterium*. However, despite the similarities, the 16S gene sequence of M175 differed from *M. ulcerans* by 11 nt (3 insertions and 8 substitutions [one base of the *M. ulcerans* sequence in GenBank is N]) and from *M. marinum* by 10 nt (4 insertions, 6 substitutions [one base of the *M. marinum* sequence in GenBank is N]). Based on sequence differences and contrasting phenotypic characteristics (Table), we conclude that isolate M175 appears to belong to a new, previously undescribed species of *Mycobacterium* (19). Comparative genetic studies of *M. ulcerans* and *M. marinum* based on 16S rRNA sequence analysis have shown very close relationships between these species despite contrasting phenotypic profiles (20-24). The presence of two DNA insertion sequences, IS*2404* and IS*2606*, in *M. ulcerans* but not in *M. marinum* has been used to distinguish the former (22-25).

The public health significance of this unique *Mycobacterium* species is not known. Frequently, mycobacterial disease in fish and cutaneous infections in humans are diagnosed on the basis of clinical presentation and generally attributed to *M. marinum*. Isolation of the causative agent either is not attempted or is unsuccessful, possibly because of loss of viability during specimen decontamination, inappropriate culture conditions, lack of technical experience with mycobacteria, or the prevailing assumption that detection of acid-fast rods is synonymous with a diagnosis of *M. marinum*. Consequently, the extent of environmentally acquired human infections caused by *Mycobacterium* species is not known. Studies to investigate the clinical importance of isolates obtained from persons exposed to marine or estuarine sources would provide data on which to evaluate the public health import of these isolates.

As in the present study, environmental mycobacteria may have lower temperature optima and not grow well on traditional media such as Löwenstein-Jensen. However, a preference for low temperature does not necessarily negate their ability to cause disease in humans, as demonstrated by disseminated infections caused by *M. marinum* and *M. haemophilum* or ulcerative skin disease caused by *M. ulcerans*. An epizootic of mycobacteriosis in striped bass, possibly the most important recreational fish in the Chesapeake Bay, could serve as a reservoir for transmission of mycobacterial infections to humans.

Laboratory challenge studies using striped bass are in progress to evaluate the pathogenicity of isolate M175. Additional research is needed to understand the persistence, distribution, and ecology of these mycobacterial isolates in natural waters, particularly with regard to their transmission to fish. Furthermore, this study also underlines a need to isolate and identify mycobacteria responsible for nontuberculosis infections in humans. This information is essential to determine the extent of human mycobacteriosis associated with occupational and increasingly popular recreational exposure to the natural aquatic environment.

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