Isolation and characterization of mycobacteria from striped bass *Morone saxatilis* from the Chesapeake Bay

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ABSTRACT: Mycobacteriosis in striped bass *Morone saxatilis* of Chesapeake Bay, USA, was first diagnosed in 1997 based on the presence of granulomatous inflammation and acid-fast bacteria in skin and spleen. To confirm histopathology, bacteriological detection and identification of mycobacteria were begun using splenic tissue from fish with and without skin ulcerations. On the basis of initial studies using a variety of selective and nonselective media, decontamination, homogenization and incubation conditions, a simple and quantitative recovery method using aseptic necropsy of splenic tissue was developed. Optimal recovery was obtained by spread-plating homogenates on Middlebrook 7H10 agar with incubation for 3 mo at 23°C. Mycobacteria were recovered from 76% (n = 149/196) of fish examined. Mycobacterial densities exceeded 10^4 colony forming units · g tissue^{-1} in 38% of samples (n = 63/168) that were examined using a quantitative approach. The most frequently recovered mycobacterium, present in 57% (n = 109/192) of characterized samples, was the recently named new species *Mycobacterium shottsii.* Polyinfections of *M. shottsii* and other mycobacteria were observed in 25% of samples (n = 47/192) with densities of *M. shottsii* usually 1 or more orders of magnitude higher than co-isolate(s). Other mycobacteria recovered included isolates that, based on phenotypic traits, resembled *M. interjectum, M. marinum, M. scrofulaceum, M. szulgai* and *M. triplex. M. marinum,* commonly associated with fish mycobacteriosis and human disease, was recovered infrequently (3%, n = 6/192). The presence of multiple mycobacterial types occurring at high densities suggests that a variety of mycobacteria could be causative agents of mycobacteriosis in striped bass from the Chesapeake Bay. Striped bass is the major recreational fish species in the Chesapeake Bay, and the significance of the current epizootic to human health and the potential adverse effects on fish stocks are not known.

KEY WORDS: Mycobacteriosis · Fish disease · Striped bass · *Morone saxatilis* · *Mycobacterium marinum* · *Mycobacterium shottsii*

INTRODUCTION

Striped bass *Morone saxatilis* are the basis of an important commercial and recreational fish resource, particularly along the Atlantic coast of the United States. The Chesapeake Bay states of Maryland and Virginia account for more than half of the total US landings of striped bass and realize economic benefits exceeding \$200 million yr^{-1} (Kirkley et al. 2000). From the mid-1970s to the mid-80s there was a steep decline in striped bass landings, presumably due to overfishing, which resulted in an inter-state fisheries management program which also included restocking efforts (Anonymous 1981). Striped bass populations reboun-

ded during the early to mid-1990s but by the late 1990s concern for the health of these fish returned. An epizootic in Chesapeake Bay striped bass, characterized by skin lesions and granulomatous inflammation with associated acid-fast bacteria, was first described in 1997 (Baya 1998, Vogelbein et al. 1998). Bacterial diseases in striped bass exhibiting skin lesions and scale loss have been attributed to a variety of aetiologies, including *Aeromonas*, *Edwardsiella*, *Flexibacter*, *Pseudomonas*, and *Vibrio* spp. (Toranzo et al. 1983, Mitchell 1984, Sindermann 1988, Plumb 1991, Baya et al. 1997). However, in the current epizootic the presence of nonbranching acid-fast bacteria within granulomas implicated mycobacteria as the causative agent and represented the first time that fish mycobacteriosis had been reported in the Chesapeake Bay or along the Atlantic Coast of the United States.

The mycobacterial species frequently associated with disease in fish are *Mycobacterium abscessus* (formerly *M. chelonae* subsp*. abscessus*), *M. chelonae*, *M. fortuitum* and *M. marinum* (Belas et al. 1995). Less frequently isolated species include *M. neoarum*, *M. poriferae*, *M. scrofulaceum*, and *M. simiae* (Backman et al. 1990, Lansdell et al. 1993, Tortoli et al. 1996).

However, our understanding of pathogenic mycobacteria involved in wild fish mycobacteriosis remains poor for a number of reasons. A presumptive diagnosis of piscine mycobacteriosis is often made based on histological findings, and culture is not attempted or is unsuccessful due to the fastidious nature of the organisms or inappropriate culture conditions. Cultural methods are also sensitive to contamination, and the ease of introducing saprophytic mycobacteria into fish tissues during necropsy complicates identification of pathogenic strains. Decontamination has been employed to remove background microbiota in clinical (Kent & Kubica 1985) and environmental (Dalsgaard et al. 1992, Chinabut 1999) samples but can adversely affect mycobacterial recovery (Brooks et al. 1984, Schulze-Röbbecke et al. 1991).

Mycobacterium marinum was isolated during an outbreak of mycobacteriosis in cultured striped bass (Hedrick et al. 1987) and from wild striped bass collected in San Pablo-San Francisco Bay waters and adjacent Pacific Ocean areas (Lansdell et al. 1993). Histological prevalences ranging from 25 to 68% were reported for epizootics of mycobacteriosis in wild populations of Pacific striped bass (Sakanari et al. 1983). Although cultured diseased striped bass occasionally exhibited external lesions, none were reported for wild populations. Two previously unknown mycobacteria, *M. shottsii* (Rhodes et al. 2001, 2003) and '*M. chesapkeaki*' (Heckert et al. 2001) have been isolated from Chesapeake Bay striped bass during the current epizootic.

This paper describes the types and prevalences of mycobacteria isolated during the current epizootic in striped bass collected from Virginia waters of the Chesapeake Bay. To circumvent the need for decontamination and provide quantitative data, an isolation and enumeration method was developed based on aseptic necropsy of splenic tissue and direct plating on a relatively nonselective medium. Although incubation periods were lengthy, the simple procedure required little specialized equipment or techniques and provided for both enumeration and detection of polyinfections.

MATERIALS AND METHODS

Fish. Striped bass were collected from the Chesapeake Bay and its tributaries ranging from the Potomac River to the Bay entrance. Collection methods included hook and line by recreational fishermen, whereas haul seine, pound nets or trawl surveys were conducted by personnel of the Virginia Institute of Marine Science (VIMS) Department of Fisheries Science.

Necropsy. The majority of samples (n = 44) collected early in the study, from December 1997 through October 1998, were obtained from trawl surveys. These initial samples consisted of diseased fish which had been internally sexed prior to bacteriological analyses, precluding aseptic sampling. Excised splenic tissue was immersed in 70% EtOH for 5 min, drained, and the residual alcohol allowed to evaporate prior to tissue homogenization. Tissue from these samples was decontaminated as described below.

All subsequent samples collected from June 1999 through November 2001 (n = 196) included both healthy appearing fish and fish exhibiting clinical signs, i.e. presence of scale loss, pigmentation and ulceration. Exterior surfaces were disinfected with 70% EtOH prior to necropsy and samples of internal tissue collected aseptically. Spleen and liver were sampled from 18 fish to compare frequency of mycobacterial detection between the 2 sites. Skin scrapings from lesions of selected fish were removed using a scalpel.

Total length, weight and gross clinical signs were recorded for each fish.

Tissue homogenization. Dermal and internal tissue samples were homogenized in 2 to 3 ml Butterfield's buffer (BB) (Anonymous 1995) using a Ten Broeck tissue grinder. Approximately 1 to 2 g of spleen was used to prepare homogenates in the initial group of samples, and was reduced to ca. 0.1 to 0.5 g for subsequent samples. Skin samples were less than 0.1 g. Mycobacterial densities (g tissue^{-1}) were calculated from the weight of tissue used in each homogenate.

Homogenate decontamination. Skin and internal tissue samples requiring decontamination were pro-

cessed using one or more of the following methods. Replicate 1 ml aliquots of homogenate were centrifuged at $4000 \times g$ at 4°C for 30 min and pellets resuspended in 1 ml 0.3% Zephiran™ (Sanofi Winthrop Pharmaceuticals) (Method A), 2% NaOH (Method B) or 2% HCl (Method C). After 15 min exposure to acid or base in Methods B and C, suspensions were centrifuged as above, pellets resuspended in BB containing the pH indicator bromocresol purple, and neutralized by addition of either 1% HCl or NaOH until a blue color persisted (pH 6.8). Neutralized suspensions were centrifuged and resuspended in 0.5 to 1 ml BB. After 2 h exposure to Zephiran™, suspensions were centrifuged, washed twice with BB and resuspended in BB.

Processing of skin lesions was modified in subsequent samples to reduce total exposure time to potential detrimental effects of the selected decontaminant, 2% NaOH, by neutralization prior to centrifugation. Skin scrapings from selected fish with external granulomatous lesions were homogenized in 1 ml BB and decontaminated with a final concentration of 2% NaOH for 15 min. Treated homogenates were neutralized by addition of 0.5% HCl as described above. Suspensions were centrifuged and resuspended in 1 ml BB and spread-plated onto Middlebrook 7H10 agar (MDA) containing 0.5% glycerol and oleic acid, albumin, dextrose and catalase (OADC) or albumin, dextrose and catalase (ADC) enrichment (Difco, Becton Dickson Diagnostic Systems).

Decontamination of pure culture suspensions. Pure cultures of mycobacteria isolated from striped bass were grown at 30°C in Middlebrook 7H9 broth (MDB) containing OADC enrichment (Difco) and 0.05% Tween 80, adjusted to an absorbance of $OD_{590} = 0.1$, and subsequently diluted 50- or 500-fold in phosphate buffer. Fifty µl aliquots were added to duplicate microfuge tubes containing 450 µl 2% HCl, 2% NaOH, 0.3% Zephiran™ or buffer (control). After 45 min exposure to acid or base, treated suspensions were neutralized. Neutralized suspensions and controls were pelleted and washed once in phosphate buffer. Bacterial suspensions exposed to Zephiran™ for 2 h were diluted 2-fold in buffer, pelleted and washed twice. Pellets were resuspended in 500 µl buffer and bacteria enumerated by spread plating the treated suspension and 10-fold dilutions thereof in duplicate to MDA. Plates were incubated at 30°C for 6 wk.

Culturing of mycobacteria. Decontaminated homogenates (0.1 to 0.2 ml) from the initial group of samples (n = 44) were inoculated onto Lowenstein-Jensen (LJ) slants (BBL, Becton Dickson Diagnostic Systems) and Brain Heart Infusion agar (BHI) (Difco) plates containing 5% sheep red blood cells (BA), MDA and MDA modified to contain 0.025% malachite green, 0.04%

cycloheximide and 0.0035% nalidixic acid (MMDA) (Teska et al. 1997). Inoculated media were incubated at 30°C. Agar plates were examined using a stereoscopic microscope at 1 to 2 wk intervals for 2 mo. All morphologically distinct colonies were examined for acid-fastness (Ziehl-Neelsen) and acid-fast colonies streaked to MDA to obtain purified cultures. Growth on LJ slants was streaked to MDA plates and examined for acid fastness.

Tissue homogenates from aseptically sampled internal sites of fish (n = 196) and selected skin homogenates prepared using reduced exposure to NaOH were spread plated on MDA with ADC or OADC. Duplicate spread plates inoculated with homogenate and serial 10-fold dilutions thereof were used to provide a quantitative estimate of mycobacterial density for 168 of the 196 samples. Homogenates were also spread plated onto BHI agar to monitor for contamination by heterotrophic bacteria. Incubation temperature was reduced to 23°C, the incubation period extended to 3 mo and plates were examined as described above. Plates positive for both mycobacteria and non-acid-fast colonies on BHI suggested that either contamination or infection by a nonmycobacteria had occurred. These data were excluded from further analysis.

Phenotypic characterization of isolates. Acid-fast colonies selected from the primary isolation and enumeration plates were purified by streaking. Growth on MDA at 23, 30 and 37°C was compared to determine the preferred growth temperature and pigment production. Purified isolates were characterized using the following biochemical and growth tests (Kent & Kubica 1985, Master 1995): production of arylsulfatase, nitrate reductase, niacin, pyrazinamidase, semi-quantitative and heat-stable catalase (68°C), Tween 80 hydrolysis and urease. Tests were conducted at the preferred growth temperature. Isolates were grouped into major types according to pigment production, niacin and nitrate reactions. Diagnostic keys (Lévy-Frébault & Portaels 1992, Master 1995, Tortoli 2003) were used to assign isolates to an existing species when possible.

Isolates (n = 17) representing the most frequently encountered types were processed for HPLC (Butler et al. 1996) and mycolic acid profiles analyzed as described previously (Butler & Guthertz 2001).

Quantification of mycobacteria. Mycobacterial densities were expressed as colony forming units $(CFU) \cdot g \; tissue^{-1}$ and means calculated from log-transformed data. Mean values preceded by '<' indicated that at least one of the replicate samples was below the detection limit and values preceded by '>' indicated that colonies at the highest dilution plated were too numerous to count. Values below or exceeding the detection level were entered as absolute values based

Table 1. Effects of decontaminants on recovery of mycobacteria from striped bass *Morone saxatilis* homogenates plated on Lowenstein-Jensen medium. Incubation was at 30°C for 2 mo. Values in parentheses indicate number of homogenates positive for mycobacteria/total number of homogenates

Sample type	Percentage positive using		
	2% HCl	2% NaOH	0.3% Zephiran™
Spleen	44 (11/25)	44 (11/25)	20 (5/25)
Skin	50 (6/12)	42 (5/12)	17 (2/12)

Table 2. Effect of decontamination on recovery on Middlebrook 7H10 agar of selected mycobacteria isolated from striped bass *Morone saxatilis.* Data are expressed as colony forming units (CFU; mean of 2 samples). Two isolates of *Mycobacterium scrofulaceum* were examined. ID: indeterminate, <1 CFU 0.1 ml^{-1} plated, equal to <1.0E01 CFU ml^{-1}

Mycobacterium spp.	CFU ml^{-1} following exposure to			
	Buffer (control)	2% HCl	2% NaOH	0.3% Zephiran™
M. interjectum	1.3 E04	ID	<1.5E01	3.5E01
M. marinum	5.5E03	2.6E02	ID	ID
M. peregrinum	2.4E03	ID	ID	ID
M. scrofulaceum 1	3.5E03	1.0E01	ID	ID
M. scrofulaceum 2	1.7E03	1.0E01	ID	ID
M. terrae complex	4.8E03	3.3E01	3.7E02	1.8E01

Table 3. Comparison of different media for recovery of mycobacteria from decontaminated striped bass *Morone saxatilis* homogenates. Incubation was at 30°C for 2 mo. Values in parentheses indicate number of homogenates positive for mycobacteria/total number of homogenates

Sample type	Deconta-minant	Percentage positive on		
		Lowenstein-Jensen	Middle-brook 7H10	Blood agar
Spleen	2% NaOH	39 (14/36)	11 (4/36)	3 (1/36)
	2% HCl	38 (12/32)	12 (4/32)	12 (4/32)
Skin	2% NaOH	47 (8/17)	12 (2/17)	12 (2/17)
	2% HCl	50 (8/16)	19 (3/16)	19 (3/16)

on the lowest level of sensitivity or the highest dilution plated.

Reference cultures. Growth and biochemical testing included reference cultures *Mycobacterium avium* (M1), *M. chelonae* (M3), *M. flavescens* (M4), *M. fortuitum* (M6), *M. gordonae* (M8), *M. kansasii* (M10), *M. marinum* (M11), *M. nonchromogenicum* (M14), *M. phlei* (M15), *M. scrofulaceum* (M17), *M. simiae*

(M19, M20) and *M. terrae* (M21) obtained from the Environmental Protection Agency, Cincinnati, Ohio, and Consolidated Laboratory Services, Commonwealth of Virginia, Richmond, Virginia.

Fish age determination. Fish age was estimated using data provided by the Anadromous Fishes Research Program (VIMS) correlating total length and age based on fish scale assessment for striped bass collected during the study period. Age was determined by linear regression ($y = -2.148 + 0.013x$, p = <0.0001, $r^2 = 0.939$, df = 9406).

Histology. Skin and spleen samples were processed for routine paraffin histology (Prophet et al. 1994) and stained with haematoxylin and eosin (H&E). Selected sections were stained using Ziehl Neelsen's method for acid-fast bacteria. A minimum of 9 random sections of H&E stained splenic tissue for each fish was examined at 40× for the presence and number of granulomas.

Data analysis. All statistical analyses were performed with Statview (SAS Institute).

RESULTS

Initial samples

Homogenates prepared from fish exhibiting skin lesions and plated on multiple media i.e. BA, LJ and MDA, yielded mycobacteria from 58% of spleen (n = 25/43) and 55% of skin lesion (n = 12/22) samples. Mycobacterial detection was significantly affected by the method of decontamination (chi-square, p = 0.0285). Recovery of mycobacteria on LJ (Table 1) following HCL and NaOH treatments was greater than that obtained with Zephiran™, which was less effective in reducing mold contaminants.

Treatment of suspensions of pure cultures with decontamination agents resulted in 1 to 3 log reductions in CFU (Table 2). Observed adverse effects of HCl and NaOH were least on *Mycobacterium marinum* and *M. terrae* complex, respectively.

Detection of mycobacteria from decontaminated samples was significantly affected by culture medium employed (chi-square, p < 0.0001). Recovery for samples incubated at 30°C was highest on LJ medium compared to MDA and BA, which yielded similar percent detection (Table 3). Overgrowth by molds and non-acid-fast bacteria on all 3 media often precluded incubation for the entire 2 mo period.

Randomly selected samples of spleen (n = 22) and skin lesion (n = 14) tissue decontaminated using the aforementioned methods were all negative when plated on MMDA. Individual spleen and skin samples plated in parallel on LJ yielded mycobacteria in 46 (10/22) and 29% (4/14) of samples, respectively. Sixty-seven

Table 4. Growth of mycobacterial reference cultures and mycobacteria isolated from striped bass *Morone saxatilis* on modified Middlebrook 7H10 agar. Broth suspensions of mycobacteria from reference cultures or cultures isolated from decontaminated fish tissue were streaked on Middlebrook 7H10 agar (MDA) modified to contain 0.025% malachite green, 0.04% cycloheximide and 0.0035% nalidixic acid (MMDA) (Teska et al. 1997) and incubated at 30°C for 2 mo. Control = growth of culture on MDA. In the good growth category, individual colonies were easily observed without magnification; in poor growth, individual colonies were only visible with the aid of a stereoscopic microscope. Values in parentheses indicate number positive for mycobacteria/total number

Culture source *Mycobacterium* spp.	No. of isolates	Growth Good	Poor	None
Reference				
M. avium	1		1	
M. chelonae	1	1		
M. flavescens	1			1
M. fortuitum	2	2		
M. gordonae	1		1	
M. intracellulare	1		1	
M. kansasii	1			1
M. marinum	3		3	
M. nonchromogenicum	1		1	
M. phlei	1		1	
M. scrofulaceum	2	2		
M. simiae	2	1	1	
M. terrae	1		1	
Percentage		33 (6/18)	56 (10/18)	11 (2/18)
Fish				
M. flavescens	4		4	
M. gordonae	3		3	
M. interjectum	13	3	9	1
M. marinum	11		9	2
M. peregrinum	1		1	
M. scrofulaceum	12	3	8	1
M. szulgai	2	1	1	
M. terrae complex	20		20	
Unidentified	8	2	5	1
Percentage		12 (9/74)	81 (60/74)	7 (5/74)

(12/18) and 88% (65/74) of reference and fish isolate cultures, respectively, produced no or poor growth when broth suspensions were inoculated onto MMDA (Table 4). Reference cultures of *Mycobacterium chelonae*, *M. fortuitum*, *M. scrofulaceum* and *M. simiae* (1/2) and fish isolates resembling *M. interjectum* (3/13) and *M. scrofulaceum* (3/12) produced good growth on MMDA.

A variety of mycobacteria were recovered using the initial protocol of decontamination and incubation at 30°C for 2 mo. The most frequently recovered splenic isolates from nonaseptically sampled tissues resembled *Mycobacterium scrofulaceum* (22%, 7/32) and *M. terrae* (19%, 6/32).

Aseptically necropsied samples

Because experimental exposure of pure culture suspensions to decontamination chemicals showed adverse effects on mycobacterial recovery, aseptic collection of internal tissue samples was initiated. Due to the finding that mycobacteria were more frequently isolated from splenic tissue (n = 16/18) than liver (n = 2/18), and because aseptic sampling of the spleen was easier than that of the kidney, splenic tissue was chosen as the preferred target site. Mycobacteria were recovered from 76% (149/196) of spleen samples that were removed aseptically from striped bass, homogenized, plated directly to MDA and incubated at 23°C for 3 mo. Sample homogenates that were enumerated yielded mycobacterial densities exceeding 10^4 CFU · g splenic tissue^{-1} in 38% (63/168) of samples. Densities of $\geq 10^{6-7}$ CFU · g tissue^{-1} occurred in 13% (21/168) of samples examined.

Ages of fish sampled were positively skewed (Skewness = 1.2) from the mean age of 5 yr with 9% (17/196) of samples less than 3 yr and 17% (34/196) of samples greater than 7 yr of age. Although there was no correlation (Kendall Tau, p > 0.05) between age and log CFU g^{-1}, mycobacterial densities differed significantly between age groups (Kruskal-Wallis, p < 0.0001). The youngest and oldest fish had both the lowest mean counts and lowest prevalence and, conversely, the 2 middle groups had the highest counts and prevalence (Table 5).

Slowly growing mycobacteria recovered from splenic tissue were grouped using colony pigmentation, a major phenotypic feature, and niacin and nitrate reductase production (Table 6). The dominant mycobacterium recovered, Group A, included isolates that were used to define the new species *Mycobacterium*

Table 5. Splenic densities and prevalence of mycobacteria in striped bass *Morone saxatilis* as a function of age. In the first column, values in parentheses indicate total number of fish in age group. In the last column, values in parentheses indicate number of fish positive for mycobacteria/total number fish examined in age group. SD: standard deviation

Age group (yr)	Mean weight (g)	Mean length (mm)	Culture positive for mycobacteria Mean CFU g^{-1}	%
<3.0 (16)	288	310	150 SD = 34	19 (3/16)
3.0–4.9 (119)	1113	485	6810 SD = 51	90 (107/119)
5.0–6.9 (25)	2137	596	2120 SD = 50	80 (20/25)
≥7.0 (34)	7655	892	180 SD = 44	47 (16/34)

shottsii (Rhodes et al. 2003). *M. shottsii* was isolated alone or in combination with one or more other mycobacteria in 57% (109/192) of the fish examined. Mycobacteria Group B and F were the second most frequently encountered types, each occurring in 12% (24/192) of the fish. Biochemical reactions and mycolic acid profiles of Group B isolates were similar to those of *M. montefiorense*, a moray eel pathogen (*Gymnothorax funebris* and *G. moringa*) (Herbst et al. 2001, Levi et al. 2003), and *M. triplex*, a human pathogen (Floyd et al. 1996). Group F isolates, similar to *M. shottsii* except for chromogenicity and distinguishable from *M. shottsii* on the basis of 3 genetic loci, are described in another publication (Rhodes et al. 2004) All other slowly growing types had a frequency of occurrence of ≤8%. Rapidly growing mycobacterial isolates (data not shown) were present in 4% (8/192) of the fish.

With the exception of Group G isolates, which exhibited phenotypic traits consistent with *Mycobacterium marinum*, all other mycobacteria were more frequently isolated in combination with *M. shottsii* than alone. *M. marinum* was detected in only 3% of samples, and most frequently as a monoinfection.

Mean densities of *Mycobacterium shottsii*, approximately 10^4 CFUg^{-1} in monoinfections and polyinfections (Table 7), were significantly higher than densities of non *M. shottsii* (Wilcoxon, p < 0.0001). *Mycobacterium shottsii* occurred at concentrations ≥10^4 CFU g^{-1} in 33% (56/168) of enumerated samples.

Non-*Mycobacterium shottsii* infections exceeded ≥10^4 CFU g^{-1} in only 5% (9/168) of enumerated samples. *M. marinum* was the only species recovered in 4 of these 9 samples and occurred at densities >10^6 CFU · g spleen^{-1}. One of the *M. marinum* infected fish had splenic granulomas and none presented with external signs. Isolates resembling *M. montefiorense/M. triplex* were present in 3 fish as either the sole species or as the numerically dominant species in a mixed mycobacterial infection. Group D isolates resembling *M. scrofulaceum* on the basis of biochemical tests and HPLC analysis was recovered as the numerically dominant species from 1 fish. An unidentified photochromogenic isolate, Group H, was recovered at elevated densities from 1 sample. Splenic granulomas and petechiae were present in 3 and 2 samples, respectively, of the non *M. marinum* infected fish.

Other mycobacteria recovered, Groups C and E, resembled *Mycobacterium interjectum* and *M. szulgai* on the basis of biochemical and mycolic acid characteristics.

Table 6. Characteristics of slowly growing mycobacteria recovered from spleens of aseptically necropsied striped bass *Morone saxatilis*. Values in parentheses indicate number of splenic homogenates positive for mycobacteria/total number homogenates cultured for mycobacteria and isolates characterized (n = 192). Resulting mycobacterial isolates are characterized using biochemical tests. Sample homogenates were spread plated onto Middlebrook 7H10 agar with oleic acid, albumin, dextrose and catalase (OADC) or albumin, dextrose and catalase (ADC) enrichment and incubated at 23 and 30°C for 3 mo. Niacin: niacin production; NO$_3$ red.: nitrate reductase production; +: at least 85% isolates positive; –: less than 15% positive; v: 15 to 84% positive

Test	Phenotypic group							
	Nonpigmented			Scotochromogenic		Photochromogenic		
	Niacin + NO$_3$ red.– Group A	Niacin – NO$_3$ red.+ Group B	Niacin – NO$_3$ red.– Group C	Niacin – NO$_3$ red.– Group D	Niacin – NO$_3$ red.+ Group E	Niacin + NO$_3$ red.– Group F	Niacin – NO$_3$ red.– Group G	Niacin v NO$_3$ red.+ Group H
Arylsulfatase								
3 d	–	–	–	–	v	–	v	–
14 d	–	v	v	v	+	–	+	+
Catalase								
Semi-quantitative	v	+	+	+	+	–	v	v
Heat-stable (68°C)	–	+	+	+	+	v	v	+
Pyrazinamidase								
4 d	–	–	–	v	v	–	v	–
7 d	–	–	v	v	v	–	+	v
Tween	–	–	–	–	–	–	v	–
Urease	+	v	+	v	v	+	+	+
Percentage occurrence								
Mono-infection	32 (62/192)	2 (4/192)	1 (2/39192)	1 (2/192)	<1 (1/192)	4 (8/192)	2 (4/192)	2 (4/192)
Poly-infection	25 (47/192)	10 (20/192)	5 (9/192)	7 (13/192)	5 (9/192)	8 (16/192)	1 (2/192)	5 (10/192)
Total	57 (109/192)	12 (24/192)	6 (11/192)	8 (15/192)	5 (10/192)	12 (24/192)	3 (6/192)	7 (14/192)

Table 7. Recovery of mycobacteria from aseptically sampled striped bass *Morone saxatilis* splenic tissue using incubation at 23 and 30°C for 3 mo. Values in parentheses indicate number of splenic homogenates positive for mycobacteria/total number of homogenates cultured for mycobacteria and isolates characterized (n = 192). Sample homogenates spread plated onto Middlebrook 7H10 agar with ADC or OADC enrichment. In Mean CFU g^{-1} column, 119 splenic homogenates of 168 splenic samples submitted for enumeration were culture positive. *M. shottsii*: *Mycobacterium shottsii*

Mycobacterium	Percentage			Mean CFU g^{-1}
	Culture	External sign	Splenic granuloma	
Culture positive	**76**	**45**	**54**	
	(145/192)	(86/192)	(104/192)	
Monoinfection				
M. shottsii	32	19	24	23 600
	(62/192)	(37/192)	(46/192)	n = 48
Non-*M. shottsii*	12	6	6	1400
	(23/192)	(11/192)	(11/192)	n = 22
Polyinfection				
M. shottsii and	25	18	21	24 700 (*M. shottsii*),
non-*M. shottsii*	(47/192)	(35/192)	(40/192)	210 (non-*M. shottsii*) n = 39
Non-*M. shottsii*	7	2	4	2400
	(13/192)	(3/192)	(7/192)	n = 10
Culture negative	**24**	**9**	**5**	
	(47/192)	(18/192)	(9/192)	

Skin lesions ranged in severity from small areas of scale loss, often with associated melanotic foci, to infrequently seen large shallow ulcers with hyperpigmented margins. Although external signs and splenic granulomas were more frequent in culture-positive compared with culture-negative fish, many were negative for both parameters (Table 7).

Rapidly growing mycobacteria generally occurred at densities $\leq 10^2$ CFU g^{-1} and were co-isolated with *Mycobacterium shottsii* on 5 of 8 occasions. All isolates except 1 were pigmented and none had biochemical and growth characteristics resembling known rapidly growing fish pathogens, e.g. *M. fortuitum*.

Gram-positive cocci were recovered at $\leq 10^{4-5}$ CFU g^{-1} from aseptically necropsied spleens on 5 occasions. In 3 of these instances *Mycobacterium shottsii* was co-isolated.

Mycobacterium terrae, frequently isolated in non-aseptically collected samples, was never observed when splenic tissue was sampled aseptically.

Skin scrapings

Skin scrapings from 15 fish with skin lesions were decontaminated with NaOH and cultured for mycobacteria. Granulomas were observed histologically in 11 of 15 skin specimens and in all corresponding spleen samples. *Mycobacterium shottsii* was isolated from all 15 skin samples and was the only mycobacterium recovered from 12 samples. Isolates resembling *M. gordonae* and an unidentified

scotochromogen were recovered from 2 and 1 fish, respectively. *M. shottsii* CFUs on plates inoculated with <0.05 g skin scrapings ranged from a few to too numerous to count, whereas non-*M. shottsii* were represented by 1 or 2 colonies in each of the 3 polyinfections. *M. shottsii* was recovered from all corresponding spleen samples at a mean density of 4.0×10^5 CFU g^{-1}.

DISCUSSION

Diagnosis of mycobacteriosis in fish is frequently based on the presence of acid-fast bacilli in histological sections, and culture is not always performed or successful (Dalsgaard et al. 1992, Frerichs 1993). However, pathogenic mycobacteria may be refractory during infection to staining with standard acid-fast techniques, so reliance on histological diagnosis may underestimate disease prevalence (Gauthier et al. 2003). In addition, paramount to identifying an infectious disease is recovery of an aetiological agent. Culturing from diseased tissue allows for isolation, subsequent genotypic and phenotypic characterization of the suspect pathogen(s) and, ultimately, challenge studies to confirm pathogenicity and fulfill Koch's postulates. Determination of mycobacterial densities by plating provides information regarding both intensity of infection and the presence of polyinfections. In the present study of the epizootic of mycobacteriosis in striped bass of the Chesapeake Bay, we isolated and enumerated mycobacteria using a facile method based

on aseptic necropsy. During the course of this study the significance of various aspects of sampling and culture methodology on recovery of the aetiological agent(s) was examined.

During necropsy there is significant potential for introduction of external mycobacterial contaminants whose presence can complicate isolation/identification of the true pathogen. Initial samples examined in this study were received in the laboratory following invasive field procedures that compromised the sterility of internal organs and necessitated chemical decontamination to reduce or eliminate background overgrowth that would prevent detection of slowly growing mycobacteria. Treatment of laboratory suspensions of pure cultures of mycobacteria with NaOH, HCl and Zephiran™ demonstrated marked susceptibility of some mycobacteria to these agents. These findings agree with previous reports of detrimental effects of decontamination on mycobacterial recovery (Brooks et al. 1984, Schulze-Robbecke et al. 1991, Dalsgaard et al. 1992, Yajko et al. 1993, Kamala et al. 1994). Despite the potential loss of culturability from decontamination, treatment of internal tissue samples is still recommended (Chinabut 1999). Our study demonstrates that decontamination can be avoided by careful use of aseptic necropsy to prevent the introduction of external mycobacteria.

Isolation of mycobacteria from skin lesions requires treatment to kill other surface microbiota. Although Bragg et al. (1990) reported better growth of *Mycobacterium fortuitum* from fish homogenates decontaminated with 2% HCl compared with 4% NaOH, we observed no statistical difference in mycobacterial recovery for tissues decontaminated with 2% HCl or 2% NaOH. Modification of our decontamination procedure to reduce treatment time yielded a method that was effective in recovering mycobacteria from skin lesions. Investigators should be aware that mycobacterial susceptibility to disinfection varies according to species, a phenomenon reported by others (Brooks et al. 1984, Dalsgaard et al. 1992) and supported by results for spiked suspensions of fish isolates examined in this study. Varying susceptibilities to decontaminants can result in biased isolation and recovery (Kazda 1983) and reduced enumeration efficiency.

A variety of media for the cultivation of mycobacteria are characterized by various degrees of selectivity and recovery. The selective medium, MMDA, formulated for enumeration of *Mycobacterium abscessus* (Teska et al. 1997), was not effective for recovery of the majority of mycobacteria from striped bass. The ability of MMDA to support growth of various reference strains and field isolates varied within and among species. Although MDA was not as effective as LJ medium for recovery of mycobacteria from decontaminated sam-

ples, i.e. 15 versus 44%, respectively, it provided effective recovery of mycobacteria from samples not subjected to decontamination, i.e. 76% of all striped bass examined. MDA also enhanced recovery of fish isolates compared with BA medium. Additional advantages of direct plating on MDA plates rather than LJ medium included the ability to enumerate colonies and to distinguish different colony types, which facilitated detection of mixed infections. Mixed infections may be difficult to detect on slants due to overgrowth by numerically dominant or faster-growing mycobacteria.

Appropriate incubation temperature and duration are crucial to recovery of environmental mycobacteria. Human diagnostic protocols call for incubation of mycobacterial cultures at 35 or 37°C, with the exception of suspected *Mycobacterium marinum* or *M. ulcerans* infections, when incubation at 25 to 33°C is indicated (Master 1995). For recovery of mycobacteria from fish, we recommend incubation at both 23 and 30°C if possible, and incubation at 23°C if only one temperature is available. In this study, the most frequently recovered mycobacteria, *M. shottsii* and a chromogenic mycobacterium closely resembling *M. shottsii* (Rhodes et al. 2003), grew poorly or not at all at 30°C, and required 2 to 3 mo incubation on primary culture. All other mycobacteria isolated from striped bass grew both at 23 and 30°C, with growth rates enhanced at the latter temperature.

Plating of tissue homogenate on a nonselective medium such as BHI is recommended in order to detect a nonmycobacterial infection or sample contamination. In this study, high densities of nonmycobacteria in splenic tissue occurred infrequently, and when present were characterized by gram-positive cocci. Additional cultural and histological studies are needed to determine whether these cocci are of pathological significance.

Fish mycobacteriosis is a chronic progressive disease that may take several years to progress from a covert state to clinical illness (Austin & Austin 1987). A histological investigation of Chesapeake Bay striped bass showed that disease prevalence in 3 to 5 yr old fish was higher than in younger fish (Cardinal 2001). Our culture data likewise demonstrated that fish less than 3 yr old exhibited a lower frequency of infection, and that prevalence declined from 90% in the 3 to 4.9 age group to 47% in fish 7 yr and older. Kaattari (I. M. Kaattari unpubl. data) observed that more striped bass 6 yr or older were positive by PCR and nested PCR than by culture. It is possible that in older fish, viable mycobacteria are present at very low densities, or PCR is detecting nonviable mycobacterial cells.

Histological evaluation of internal organs from Pacific coast striped bass revealed that, despite a high prevalence of visceral acid-fast granulomas, fish did

not display gross external signs of disease (Sakanari et al. 1983). Similarly, the majority of Chesapeake Bay striped bass with splenic granulomas did not exhibit external lesions (Cardinal 2001). However, in the latter study, dermal lesion prevalence significantly increased with the intensity of splenic infection, i.e. abundance of granulomas, suggesting that external lesions are an expression of late-stage mycobacteriosis. This has also been proposed by Chinabut (1999). In our study, fish displaying external signs of disease typically had splenic mycobacterial densities of $>10^5$ CFU g^{-1}.

Phenotypic characterization revealed a variety of mycobacteria in aseptically processed spleen samples from this study. The most frequently isolated mycobacterium was *Mycobacterium shottsii*, a recently described new species (Rhodes et al. 2001, 2003), present in 57% of striped bass examined. Approximately 25% of fish harboured 1 or more other types of mycobacteria, generally at concentrations 1 or 2 orders of magnitude lower than *M. shottsii*. *M. shottsii* was also the dominant mycobacterium recovered from skin lesions. Frequency of recovery and quantitative data from these wild populations of striped bass suggest *M. shottsii* as an aetiological agent of mycobacteriosis.

In a previous study, *Mycobacterium shottsii* persisted for 45 wk in the spleens of experimentally infected hatchery-reared striped bass, but there was very little granuloma formation and no marked multiplication of mycobacteria (Gauthier et al. 2003). It is possible that environmental parameters of the challenge study, i.e. a flow-through freshwater system maintained at 18 to 23°C with well-fed fish, were not favorable for *M. shottsii* disease development over the time course of the study. The very slow growth characteristic of *M. shottsii* suggests that development of disease in exposed young fingerlings may require a longer period of infection. Environmental stressors, e.g. elevated temperature or reduced food ration, may also be necessary for development of disease.

Mycobacteria resembling *Mycobacterium montefiorense/M. triplex* and a previously unrecognized species related to *M. shottsii* were the next most frequently encountered types in splenic tissue. Granulomatous skin lesions in moray eel were attributed to an *M. triplex*-like organism that did not grow above 30°C (Herbst et al. 2001), a temperature preference shared with many of our isolates and unlike *M. triplex* isolated from humans (Floyd et al. 1996). Eel isolates were subsequently distinguished on the basis of 3 genetic loci from *M. triplex*, and constitute a new species, *M. montefiorense* sp. nov. (Levi et al. 2003). Additional phenotypic and genotypic studies are in progress to determine the relationship of *M. triplex* and *M. montefiorense* to similar mycobacteria from striped bass. The *Mycobacterium* sp. resembling

M. shottsii differed by producing a light-yellow/gold pigment upon prolonged incubation and following light exposure. Based on sequencing results for 3 genetic loci, these isolates are different from *M. shottsii* and are proposed to constitute a new species (M. W. Rhodes unpubl. data).

Mycobacterium marinum, considered to be one of the primary agents of fish mycobacteriosis (Frerichs 1993, Chinabut 1999), was recovered in only 3% (6/192) of spleen samples examined. However, whereas most mycobacteria other than *M. shottsii* occurred as polyinfections (typically with *M. shottsii*), *M. marinum* was present more often as a mono-infection and at elevated densities. Exposure studies (Gauthier et al. 2003) demonstrated that *M. marinum* multiplied rapidly and produced severe pathology. The high growth rate of *M. marinum* may enable it to outcompete slowly growing species.

Mycobacterium marinum is recognized as a causative agent of a variety of infections in the extremities, e.g. skin, soft tissue, tendon, and bones, in healthy individuals with a history of exposure and trauma such as wounds from handling fish, and sometimes results in disseminated infections in the immunocompromised (Falkinham 1996, Anonymous 1997). Other mycobacteria recovered included isolates with phenotypic profiles similar to species known to cause disease in humans: Group B: *M. triplex*; Group C: *M. interjectum;* Group D: *M. scrofulaceum*, and Group E: *M. szulgai.* (Falkinham 1996, Floyd et al. 1996, Horsburgh 1996, Anonymous 1997, Zenone et al. 1999, Hazra et al. 2001). However, assignment of these environmental isolates to existing species is tenuous and requires additional genotypic studies. Mediel et al. (2000) also reported the inability to speciate mycobacteria isolated from fish on the basis of biochemical or restriction fragment length polymorphism analysis patterns. With the development of new molecular tools that have enhanced discriminatory power, there has been an explosion of new species in the *Mycobacterium* taxon (Tortoli 2003). Reliance solely on phenotypic tests and diagnostic keys developed primarily for clinical isolates to classify environmental isolates may be inappropriate.

Neither causative factors of the ongoing mycobacterial epizootic of striped bass in the Chesapeake Bay, nor the impact on fish stocks, are known. Possible transmission mechanisms of fish mycobacteriosis include oral, via contaminated food, surface, via damaged skin and transovarian (Belas et al. 1995). The latter warrants investigation since the striped bass population in the Chesapeake Bay represents a major restocking effort. Although there are no overt signs suggesting mycobacteriosis in other fish populations resident in the Chesapeake Bay, very few samples

have been submitted to culture analysis (Rhodes & Kaattari unpubl. findings). In order to study the ecology of mycobacteria recovered from striped bass, improved methods for application to water samples are needed. Experiments conducted to evaluate methodology for the recovery of mycobacteria from water samples collected during a fish challenge study (Gauthier et al. 2003) showed that sensitivity differences to decontamination chemicals (i.e. NaOH, H_2SO_4, oxalic acid) or selective agents (i.e. cetylpyridinium chloride, primaricin) in culture media resulted in biased recovery and poor detection of slowly growing mycobacteria (M. W. Rhodes unpubl. data). Possible changes in environmental factors affecting mycobacterial populations (i.e. loadings, geographic and seasonal distributions and species composition) and the health status of striped bass have not been identified. Addressing these questions will require not only cultural methods but also development of specific and quantitative molecular tools (Knibb et al. 1993, Belas et al. 1995, Talaat et al. 1999, and Kaattari et al. 2004).

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