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Quantitative PCR Assay for *Mycobacterium pseudoshottsii* and *Mycobacterium shottsii* and Application to Environmental Samples and Fishes from the Chesapeake Bay▽

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Striped bass (*Morone saxatilis*) in the Chesapeake Bay are currently experiencing a very high prevalence of mycobacteriosis associated with newly described *Mycobacterium* species, *Mycobacterium pseudoshottsii* and *M. shottsii*. The ecology of these mycobacteria outside the striped bass host is currently unknown. In this work, we developed quantitative real-time PCR assays for *M. pseudoshottsii* and *M. shottsii* and applied these assays to DNA extracts from Chesapeake Bay water and sediment samples, as well as to tissues from two dominant prey of striped bass, Atlantic menhaden (*Brevoortia tyrannus*) and bay anchovy (*Anchoa mitchilli*). *Mycobacterium pseudoshottsii* was found to be ubiquitous in water samples from the main stem of the Chesapeake Bay and was also present in water and sediments from the Rappahannock River, Virginia. *M. pseudoshottsii* was also detected in menhaden and anchovy tissues. In contrast, *M. shottsii* was not detected in water, sediment, or prey fish tissues. In conjunction with its nonpigmented phenotype, which is frequently found in obligately pathogenic mycobacteria of humans, this pattern of occurrence suggests that *M. shottsii* may be an obligate pathogen of striped bass.

Mycobacteriosis is a common disease affecting a large variety of wild and aquacultured fishes worldwide (9). Chronic disease is most commonly observed and is characterized by granulomatous inflammation that may affect all host tissues. External clinical signs include scale loss, dermal ulceration, spinal defects, emaciation, and ascites (5, 6, 16, 25, 31).

Mycobacteriosis in Chesapeake Bay striped bass (*Morone saxatilis*) was first observed in 1997 from histologic findings of acid-fast bacilli in granulomatous lesions (W. Vogelbein, unpublished data). Since the initial finding, surveys have demonstrated a very high prevalence of this disease in Chesapeake Bay striped bass, exceeding 50% in many samples (8, 17). Concomitantly with detection of high prevalence, tag recapture analysis has indicated that natural, nonfishing mortality of Chesapeake Bay striped bass has increased since 1999 (13), and modeling of apparent prevalence data has indicated that some mortality is associated with disease (8). Because the striped bass is an ecologically and economically important finfish along the U.S. Atlantic coast, the high prevalence of this disease creates considerable concern about the continuing health of the resource.

Mycobacteriosis of fishes has traditionally been considered to be caused by *Mycobacterium marinum*, *M. fortuitum*, or *M. chelonae*; however, the recognized diversity of *Mycobacterium* spp. infecting fishes has increased markedly in recent years (9). To date, neither *M. fortuitum* nor *M. chelonae* have been isolated from internal tissues of striped bass in the Chesapeake Bay, and *M. marinum* has been cultured from only a small fraction (3%) of fish (20). Instead, a variety of slow-growing mycobacteria have been isolated, dominated by the recently described species *M. pseudoshottsii* and *M. shottsii* (9, 20–22). The 16S rRNA gene sequences of *M. pseudoshottsii*, *M. shottsii*, *M. marinum*, and *M. ulcerans* are highly similar (≥99.4%), and like *M. ulcerans*, *M. pseudoshottsii* possesses the insertion sequences IS*2404* and IS*2606* and produces mycolactone toxin (19). *M. shottsii* has been reported to be positive for IS*2404* under specific PCR conditions by some authors (22), but not by others (10), and this species is not known to produce mycolactone. IS*2606* has been reported to amplify weakly or not at all in *M. shottsii* (22). *M. pseudoshottsii* and *M. shottsii* differ in pigment production, with the former being a photochromogen and the latter being nonpigmented (22).

In this study, we performed a quantitative real-time PCR-based survey of the presence and density of *M. pseudoshottsii* and *M. shottsii* in water and sediments of Chesapeake Bay, as well as in two dominant prey of striped bass, the Atlantic menhaden (*Brevoortia tyrannus*) and the bay anchovy (*Anchoa mitchilli*) (12, 30). *Mycobacterium pseudoshottsii* was detected by amplification of IS*2404* in a manner similar to that used in previous studies (7, 24). We also amplified and sequenced mycobacterial interspersed repetitive unit (MIRU) loci from menhaden, water, and sediment samples in order to confirm that IS*2404* amplification in these samples was likely to represent the presence of *M. pseudoshottsii* and not another IS*2404*-positive bacterium.

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TABLE 1. Primer/hydrolysis probe sequences used for this study

Target	Primer/probe[a]	Sequence
M. pseudoshottsii IS*2404*	Fwd q2404-1F	5′-GAA ATT CCC TGC GTA CGT GC-3′
	Rev q2404-1R	5′-ACC AGC CAC CGC AAG CTA C-3′
	Probe MYC-MGB1	5′-CCT GCT CAC GCT GC-3′
M. shottsii GSH-derived target	Fwd qMS-1F	5′-GCG CTT TTG GGT TAT GAA TAC G-3′
	Rev qMS-1R	5′-GCT TCT CGG GCT CCT CAT C-3′
	Probe MYC-MGB2	5′-AGT TGA CAA CGA GTC TGG-3′

[a] Fwd, forward; Rev, reverse.

No unique insertion sequences have yet been described for *M. shottsii*, and the high degree of similarity between *M. pseudoshottsii* and *M. shottsii* in genes for which sequences are available (e.g., *hsp60*, *erp*, 16S rRNA, 23S rRNA, internal transcribed spacer [ITS]) makes development of *M. shottsii*-specific assays problematic. We therefore performed genomic subtractive hybridization in a manner similar to that originally described by Akopyants et al. (3) to characterize sequences specific to *M. shottsii* relative to *M. pseudoshottsii.* An *M. shottsii*-specific quantitative PCR (qPCR) assay was developed to target sequences identified in this manner.

MATERIALS AND METHODS

Primer/probe design. The primer/hydrolysis probe set for detection of *M. pseudoshottsii* was designed to target the insertion sequence IS*2404*. Sequence variations exist between *M. pseudoshottsii* and IS*2404*-positive *M. marinum* from Chesapeake Bay striped bass (10), and the primer/hydrolysis probe set used here was designed to specifically target the former.

Subtractive genomic hybridization (GSH). Subtractive genomic hybridization was used to identify unique gene targets in *M. shottsii* for diagnostic purposes. Subtractive hybridization was performed with the Clontech PCR-select bacterial genome subtraction kit and Advantage 2 PCR enzyme system (Clontech, Mountain View, CA) according to the manufacturer's directions. In these experiments, *M. shottsii* (type strain M175) was used as the tester genome and *M. pseudoshottsii* (type strain L15) was used as the driver (reference) genome. AluI was used to digest genomic DNA. Amplified tester-specific products were cloned into Topo TA vector (Invitrogen), and the inserts of the individual clones ($n = 50$) were amplified with M13 primers. Amplified, cloned fragments were resuspended in 0.37 M NaOH-0.02 M EDTA, denatured at 98°C for 10 min, and dot blotted on duplicate positively charged nylon membranes (Boehringer-Mannheim, Mannheim, Germany). Blots were UV cross-linked (1,200 μJ) and probed with AluI-digested DNA from either *M. pseudoshottsii* or *M. shottsii* which had been digoxigenin (DIG) labeled according to the manufacturer's directions (Dig Hi-Prime DNA labeling and detection kit I; Roche Applied Science, Penzberg, Germany). Bound alkaline phosphatase was detected colorimetrically with NBT/BCIP (nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate) according to kit directions. Clones displaying strong hybridization to *M. shottsii* and no hybridization to *M. pseudoshottsii* were selected for further analysis.

Selected clones were sequenced by standard methods on an Applied Biosystems 3130xl automatic capillary sequencer (Applied Biosystems, Carlsbad, CA). Analysis of DNA sequences was performed using either MacVector (Accelrys) or Geneious v4.7 (Biomatters, Auckland, New Zealand) software packages. Homology with known sequences was determined by BLAST searches of the NCBI database. PCR primers were developed to amplify selected clones and tested against a panel of known mycobacteria and other environmentally relevant bacterial (nonmycobacterial) isolates. The bacteria were cultured, and their DNA was extracted according to previously published techniques (10). PCR primers were first tested against DNA from outgroup organisms, including *Enterococcus* sp. (VIMS isolate), *E. coli* (VIMS isolate), *Nocardia otitidiscaviarum* (ATCC 14629), *Rhodococcus rhodochrous* (ATCC 13808), and *Streptococcus parauberis* (VIMS isolate). Primers not showing amplification of these other bacterial DNAs were then tested against DNA from nontarget mycobacteria, including *M. chelonae* (VIMS isolate), *M. fortuitum* (reference culture; U.S. EPA, Cincinnati, OH), *M. gordonae* (VIMS isolate M27), *M. triplex* (ATCC 700071), and unidentified *Mycobacterium* spp. from Chesapeake Bay striped bass (isolates L30, L41, R63, R21, and R15 [see reference 9]). Twenty isolates of *M. pseudoshottsii* and 21 isolates of *M. shottsii* derived from Chesapeake Bay striped bass were then tested with these primers. Six *M. marinum* isolates derived from Chesapeake Bay striped bass (R106, Rp72a, R171, R36, R79, and L46), four *M. marinum* isolates (VIMS) from cobia (*Rachycentron canadum*), and four additional *M. marinum* isolates (ATCC BAA535, as well as strains DL240490, CC240299, and DL045 from the National Centre for Mariculture, Eilat, Israel [see reference 32]) were also tested. DNA from *M. ulcerans* (ATCC 19423) was also used to screen primers. Based on this process of PCR screening, a sequence from clone F5 was selected for the development of *M. shottsii*-specific qPCR primers and probe. Sequence from clone F5 displayed no significant homology with any known sequence by BLAST search, but primers directed against this sequence consistently amplified DNA from *M. shottsii* and no other isolates.

Sample collection and processing. Water samples were collected at locations in the main stem Chesapeake Bay from 39.131 to 36.955 by the Chesapeake Bay Multispecies Monitoring and Assessment Program (ChesMMAP) (see reference 8). Water samples were also collected from several sites within 10 km of the Rappahannock River mouth ($n = 7$) and between km 72 and 73 of the same river ($n = 10$). These locations represent the site of pound nets currently being used in ongoing tag-and-release studies examining the prevalence and impact of disease in striped bass. All water samples were collected between 30 October and 7 December 2007. Water samples (250 ml) were collected at the surface in sterile plastic bottles and kept at 4°C for no more than 1 week until processing. Water (250 ml) was filtered through 0.22-μm nitrocellulose filters (Millipore, Billerica, MA). In some instances (Rappahannock River upriver sites), 5.0-μm prefilters were necessary and were processed as for 0.22-μm filters. Filters were cut into strips and added to microcentrifuge tubes containing ~250 μl of 0.l-mm-diameter glass beads. Tubes were snap frozen in liquid nitrogen and processed in a bead mill at high speed twice for 45 s each time. DNA was extracted from pulverized membranes with a DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, but with the volume of initial proteinase K digestion doubled and elution in a final volume of 100 μl. Duplicate sample extraction blanks were included for every 23 water samples processed. Blanks consisted of 0.22-μm filters wetted with PCR-grade water and processed as described above.

Sediments were collected with a Ponar grab (Wildco, Yulee, FL) from various sites at the mouth of the Rappahannock River, VA (7 December 2007), as well as near river km 72 and 73 (13 November 2007). An ~10-g sample was transferred to a Whirlpak bag (eNASCO, Ft. Atkinson, WI) with a disposable spatula. Sediments were frozen (−20°C) until use. Between 200 to 250 mg of wet sediment was transferred to an extraction tube (PowerSoil kit; MoBio, Carlsbad, CA), and DNA was extracted according to the manufacturer's directions with elution in a 100-μl final volume of Tris-EDTA (TE) buffer. A no-sample extraction blank was included for every 23 sediment samples processed.

Menhaden ($n = 24$; length, 138 to 303 mm; weight, 40 to 392 g) and anchovies ($n = 31$; 51 to 85 mm) were collected by the ChesMMAP survey trawl net during the dates listed for water samples. Menhaden ($n = 22$; 185 to 298 mm; 94 to 411 g) were also collected from commercial pound nets near the mouth of the Rappahannock River (19 November 2007). Samples of liver and spleen were collected using sterile instruments and preserved in 95% ethanol until use. DNA was extracted from weighed menhaden or anchovy tissues with the DNeasy kit, using previously published techniques (10). A no-sample extraction blank was included for every 23 samples processed.

qPCR. All qPCRs were performed on an Applied Biosystems 7500 Fast instrument using standard cycling parameters (initial denaturation at 95°C for 20 s, followed by 40 cycles of 3 s at 95°C and 30 s at 60°C). Primer and probe sequences are given in Table 1. Primers were purified by high-performance liquid chromatography (HPLC) (Invitrogen), and hydrolysis probes were quenched

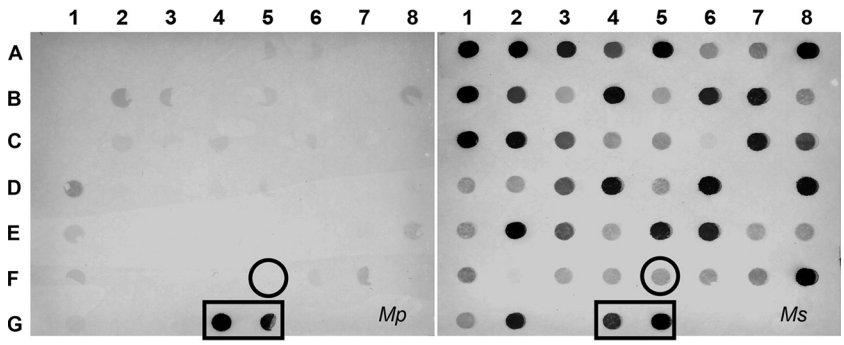

FIG. 1. Dot blot hybridization of tester-specific fragment clones (spots) with AluI-digested, digoxigenin-labeled *M. pseudoshottsii* (*Mp*; left) or *M. shottsii* (*Ms*; right) genomic DNA. Blots are identical with respect to spotted DNA. Spots G4 and G5 (boxed) represent positive controls of *M. pseudoshottsii* and *M. shottsii* AluI-digested genomic DNA (unlabeled), respectively. Clone F5, the sequence of which was used to generate the *M. shottsii* primer/probe set used for assays, is circled.

with minor groove-binding protein (Applied Biosystems). Reaction volumes were 10 μl, containing 1× Fast Universal PCR master mix (Applied Biosystems), 1× Exo IPC mix (Applied Biosystems), 0.9 μM each primer, 0.25 μM hydrolysis probe, 0.1 mg/ml bovine serum albumin (BSA) (Idaho Technology, Inc., Salt Lake City, UT), 1× Exo IPC DNA, and 1 μl DNA sample. The Exo IPC component of the reaction volumes provided an exogenous positive control for detection of reaction inhibition.

Standard curve generation. Standard curves were generated with all reactions. To generate a template for standards, broth cultures of *M. pseudoshottsii* (L15) and *M. shottsii* (M175) were centrifuged and resuspended in Butterfield's buffer with 0.05% Tween 80, and a single-cell suspension was created by repeated passage through a 30-gauge needle. The numbers of cells in suspensions were quantified using Live/Dead staining (BacLight; Invitrogen) with a Petroff-Hauser chamber. The viability of mycobacteria was >95%, and dead bacteria were counted along with the live bacteria. For water standard curves, 1×10^7 *M. shottsii* and 1×10^7 *M. pseudoshottsii* were spotted on triplicate 0.22-μm nitrocellulose membranes, and DNA was extracted as described above. For sediment standard curves, 1×10^7 each of *M. shottsii* and *M. pseudoshottsii* were added to triplicate sediment extraction tubes and DNA was extracted as described above. Standard curves for use with tissues were generated as for sediments, except that DNA was extracted with the DNeasy kit rather than PowerSoil. Neat extracts were serially diluted 10-fold in water, and qPCR was performed on the dilution series.

Assessment of inhibition. Volumes (250 ml) of York River water were filtered through replicate 0.22-μm nitrocellulose filters, and filters were spiked with 20 μl of a suspension containing 10^5 *M. pseudoshottsii* bacteria and 10^6 *M. shottsii* bacteria or 10-fold dilutions thereof. Spiked filters were extracted as described above, and qPCR was performed with a standard curve from a no-residue spiked filter extract and 10-fold dilutions thereof. Sediment inhibition experiments were performed in a similar manner with the MoBio PowerSoil kit as described above, using 200 mg of either of two sediment pools from the Rappahannock River. Each sediment pool contained a mixture of sediments from three sites. The initial density of both *M. pseudoshottsii* and *M. shottsii* was 10^5 bacteria. Quantitative PCR was performed as for water filtration experiments. Assessment of inhibition by background fish DNA was performed in a manner similar to that previously reported (10). Briefly, a pool of striped bass DNA that was PCR negative for *Mycobacterium* spp. was diluted to 1,500 (*M. pseudoshottsii* assay) or 1,100 (*M. shottsii* assay), 750, 375, or 188 ng/μl. These preparations were spiked with *M. pseudoshottsii* or *M. shottsii* bacteria at densities of 2.5×10^4 or 9.7×10^4, respectively, and samples were extracted and amplified as described for tissues above.

Amplification and sequencing of MIRU loci. Mycobacterial interspersed repetitive unit (MIRU) loci are variable tandem repeats that have been successfully used to differentiate *Mycobacterium* strains/types, especially those related to *M. tuberculosis* and *M. marinum*, from different locations and hosts (1, 27, 28). Amplification of MIRU loci from menhaden tissue and *M. pseudoshottsii* culture (L15) was performed at four loci for which primers have been previously published: locus 9 (28), loci 4 and 15 (1), and locus 6 (27). Each reaction was conducted in a 15-μl mixture containing 0.1 mg/ml BSA, 5% (vol/vol) dimethyl sulfoxide (DMSO), 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM (each) deoxynucleoside triphosphates (dNTPs), 1 pmol/μl both forward and reverse primers, 0.3 U of *Taq* polymerase (Invitrogen), and 1 μl DNA template. DNA extracted from *M. pseudoshottsii* (L15) and PCR-grade water were used as the positive and negative controls, respectively. PCR cycling parameters for tissue DNA extracts were as follows: initial denaturation at 93°C for 3 min, followed by 35 cycles of 93°C for 1 min, 58°C for 1 min, 72°C for 1 min, and final extension at 72°C for 7 min. These parameters were identical for amplifications from water and sediment with the exception of annealing temperature, which was varied as described in the following paragraph.

Eight microliters of template was added to MIRU amplification mixtures for water and sediment samples. To reduce nonspecific fragments, a higher annealing temperature (63°C) was used for locus 15. Touchdown PCR cycling, with an initial annealing temperature of 68°C and a decrease in annealing temperature of 1°C every cycle for the initial 10 cycles, was used to amplify loci 4, 6, and 9 for water and sediment DNA extracts. PCR products were electrophoresed on 1.5% agarose gels and visualized under UV light after ethidium bromide staining. PCR products were purified using a PCR product purification kit (Qiagen) and subsequently sequenced bidirectionally using a BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) and the MIRU primers in accordance with the manufacturer's instructions. Each product was sequenced three times in forward and reverse directions. In instances where multiple bands were detected in products amplified from water and sediment extracts, fragments with sizes identical to the amplicon from control *M. pseudoshottsii* (L15) were cloned and sequenced. Fragments with expected sizes were recovered from agarose gels using QIAquick gel extraction kits (Qiagen) and then cloned and transformed into *E. coli* using the TOPO TA cloning kit (Invitrogen) by following the manufacturer's protocol. Bacterial clones were screened for inserts by PCR amplification using M13 vector primers. Products with inserts were purified using ExoSAP (USB, Cleveland, OH) and then sequenced as described above. At least three clones with inserts were sequenced bidirectionally from each sample extract.

Nucleotide sequence accession numbers. A 2,499-bp contig fragment from *M. shottsii* derived from subtractive hybridization and primer walking has been deposited in GenBank under accession number HM149249. A partial sequence for *M. pseudoshottsii* IS*2404* has been deposited under GenBank accession number HM575428.

RESULTS

Subtractive hybridization. Of 50 randomly selected clones from subtractive hybridization using *M. shottsii* as the tester and *M. pseudoshottsii* as the driver, 33 demonstrated no hybridization with DIG-labeled *M. pseudoshottsii* genomic fragments (Fig. 1). Forty-eight clones hybridized with *M. shottsii* genomic fragments. Control spots strongly hybridized in both cases.

After primers were screened for specificity against a panel of known mycobacteria, the hydrolysis primer/probe set for detection of *M. shottsii* in this study was designed to target the sequence of clone F5. DNA walking was used to further characterize sequences in the genome flanking the clone F5 se-

quence, by use of the DNA Walking SpeedUp kit (Seegene, Rockville, MD) according to manufacturer's instructions. A contig of 2,499 bp was assembled, with the sequence of clone F5 occupying positions 1214 to 1604. The sequence for clone C5 was found from positions 1828 to 2270 of the contig. Contig positions 17 to 321 had 90.5% and 90.2% identity with sequences from genomes of *M. gilvum* (GenBank CP000656) and *M. vanbaalenii* (GenBank CP000511), with positions 17 to 136 corresponding to Mflv2877 and Mvan3894 integrases, respectively. The succeeding segment (positions 137 to 321) had significant similarity with sequence upstream of the integrase in *M. gilvum* and *M. vanbaalenii*; however, this region is not annotated in these genomes. Positions 1938 to 2499 returned significant BLASTx matches, with amino acid identities of ~50 to 60%, with lambda phage protein Ea59 protein from numerous unrelated bacteria (e.g., *Pseudomonas syringae* [GenBank accession number YP235422] and *Acidovorax* sp. [GenBank accession number YP985853]. Low (37.1%) amino acid identity was noted for a putative transcriptional regulatory protein from *M. tuberculosis* (GenBank accession number NP216472) corresponding to positions 783 to 1046 of the contig. Primer qMS-1F binds at positions 1383 to 1404 of the contig, while qMS-1R and probe MYC-MGB2 bind at positions 1425 to 1443 and 1406 to 1423, respectively. Lack of nucleotide or translated amino acid similarity in this portion of the contig suggests that this is noncoding sequence, although this region includes one possible open reading frame.

Assay sensitivity. Sensitivities of assays for *M. pseudoshottsii* and *M. shottsii* were determined from standard curves (Fig. 2). From filter extractions, the former produced threshold cycle (C_T) values of ~33 to 35 at 10^{-6} dilution, or 0.1 bacterial genome/reaction, which is equivalent to 0.04 cells/ml in the initial 250-ml environmental water samples. Sediment extractions were somewhat (<10-fold) less quantitatively sensitive, with C_T values of ~32 to 34 at 10^{-5} dilution (500 cells/g). The *M. shottsii* assay was less quantitatively sensitive than the *M. pseudoshottsii* assay, with filter extracts demonstrating C_T values of ~35 to 36 at 10^{-5} dilution (one bacterial genome/reaction; 0.4 cells/ml) and sediment extracts demonstrating C_T values of ~35 to 36 at the 10^{-4} dilution (5,000 cells/g). The sensitivities of tissue extraction assays (omitted from Fig. 2 for clarity) were essentially identical to those of filter extraction assays for both *M. pseudoshottsii* and *M. shottsii*.

Assay specificity. *M. pseudoshottsii* and *M. shottsii* qPCR assays were performed on extracts from pure cultures of bacteria to test assay specificity. All isolates given in Materials and Methods were used to test assay specificity, as were *M. avium*, *M. intracellulare*, and *M. scrofulaceum* (reference cultures; U.S. EPA, Cincinnati, OH). All specificity reactions were performed at least in duplicate. All isolates identified phenotypically as *M. pseudoshottsii* ($n = 20$) and *M. shottsii* ($n = 21$) were amplified by their respective qPCR assays. Additionally, *M. marinum* isolates CC240299, DL240490, and DL045 were positive by qPCR for *M. pseudoshottsii*, as was *M. ulcerans*. No non-*M. shottsii* isolates were positive by the *M. shottsii* qPCR assay. No additional *Mycobacterium* spp. or outgroup organisms were positive by either assay.

Assay inhibition. *M. pseudoshottsii* dilutions in the presence of DNA from 250 ml water filtration residue or 200 mg sediment showed good agreement with standard curves; however,

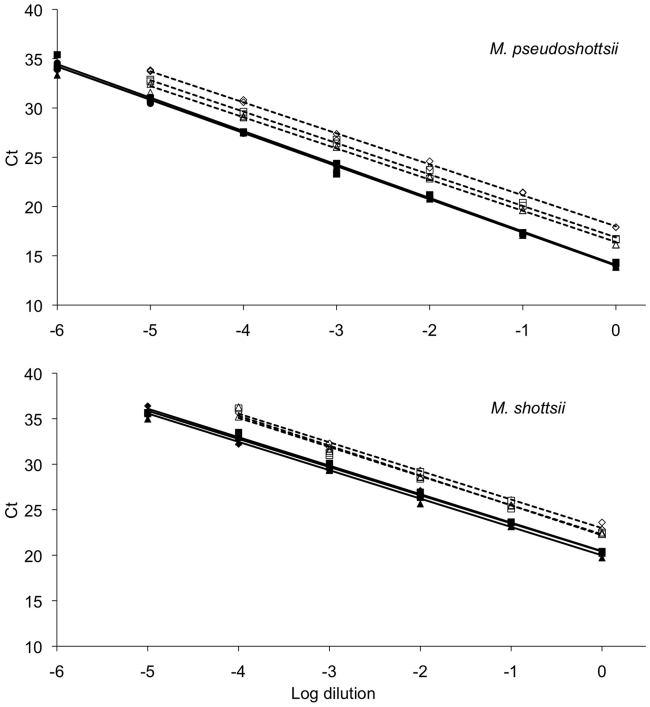

FIG. 2. Standard curves generated from analysis of spiked nitrocellulose filters (solid lines and symbols) and sediment extractions (open symbols, dashed lines). Zero log dilution represents 1×10^5 bacteria/reaction. Dilution series of tissue extracts are not shown for clarity, as C_T values overlapped with those of water extractions. Duplicate C_T values of results for triplicate tissue extractions at the zero dilution for *M. pseudoshottsii* were as follows: (i) 15.70, 15.63; (ii) 15.98, 16.13; (iii) 15.94, 16.16. Duplicate C_T values of results for triplicate tissue extractions at the zero dilution for *M. shottsii* were as follows: (i) 21.29, 21.27; (ii) 21.55, 21.63; (iii) 21.35, 21.38. R^2 values for all standard curves (including those using tissue) were >0.98.

while standard curves demonstrated quantitative sensitivity to the 10^{-6} dilution (0.1 bacterium/reaction), C_T values resulting from amplification of this initial target density in the presence of residue or sediment were generally over 35 cycles and inconsistent between duplicates (Fig. 3A and B). Therefore, the *M. pseudoshottsii* assay was quantitatively sensitive to approximately one bacterium per reaction (=0.4 bacteria/ml; 500 bacteria/g) in the presence of filtration residue or sediment extract, respectively. *M. shottsii* target did not amplify consistently at the 10^{-5} dilution after extraction with the sediment protocol. Therefore, the *M. shottsii* sediment assays were quantitatively sensitive at the level of approximately 10 bacteria/reaction (Fig. 3D). The *M. shottsii* assay was less sensitive in the presence of residue extract, with inconsistent results seen at the 10^{-3} dilution, although amplification was still observed (Fig. 3C). It therefore appears that the *M. shottsii* filtration residue assay is approximately 10- to 100-fold less quantitatively sensitive than the assay for *M. shottsii* in sediment. Amplification of neither *M. pseudoshottsii* nor *M. shottsii* was inhibited by increasing concentrations of background striped bass DNA. Threshold crossing values were within one-half cycle of the standard curve for all background DNA concentrations in the *M. pseudoshottsii* assay and within 0.7 cycle for all concentrations of background DNA for the *M. shottsii* assay.

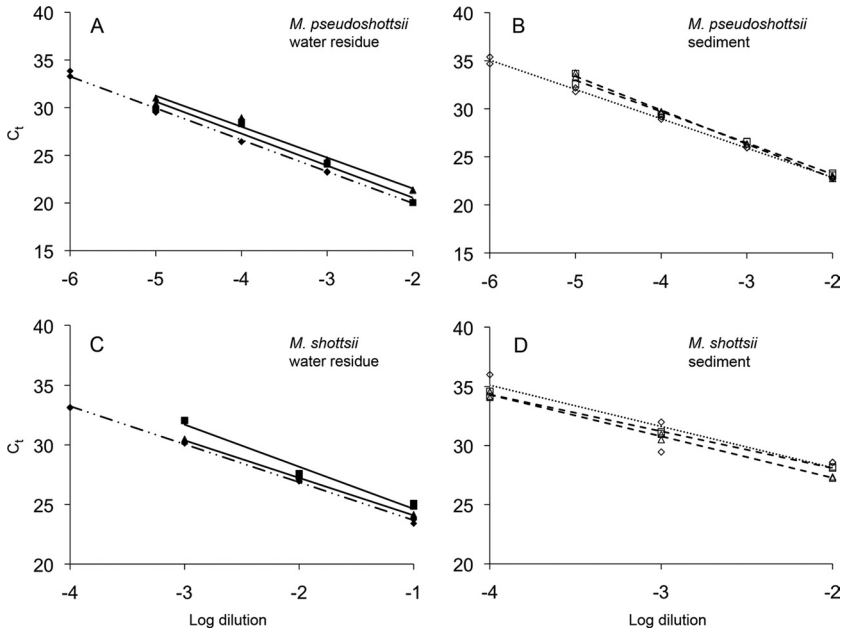

FIG. 3. Inhibition of qPCR assays for *M. pseudoshottsii* and *M. shottsii* by 250 ml water filtration residue or 200 mg sediment at various initial concentrations of target DNA. (A and C) Plots showing inhibition of qPCR detection of 10-fold dilutions of *M. pseudoshottsii* (A) and *M. shottsii* (C) in the presence of water residue (0.22 μm). (B and D) Plots showing inhibition due to sediment (200 mg) for *M. pseudoshottsii* (B) and *M. shottsii* (D). Data points for water are represented by solid symbols; data points for sediments are represented by open symbols. Standard curves (no residue/no sediment) are indicated by broken lines (– · · – · · –) and dotted lines (· · · · · · · · ·) for residue and sediment, respectively. Solid and dashed lines represent amplification in the presence of residue and sediment, respectively. Consistent with Fig. 2, log dilution of −1 represents 10^4 bacteria/reaction. Each reaction is in duplicate, and two dilution series are presented for each target-inhibitor combination. Sediment inhibition curves are each from a separate pool of three Rappahannock River sediment samples. These sediment pools were positive for *M. pseudoshottsii*; however, C_T values exceeded 33 cycles in all cases.

Water samples. *M. pseudoshottsii* DNA (IS*2404*) was found in 100% ($n = 38$) of the water samples from the main stem of the Chesapeake Bay. Densities (extrapolated when below the quantitative limit of the assay) ranged from 0.05 to 5.2 bacteria/ml, with a median density of 0.5 bacteria/ml (Table 2). Sample sites and densities are shown in Fig. 4. Linear regression analysis that included only samples with *M. pseudoshottsii* densities above the quantitative threshold (>0.4 bacteria/ml) revealed a significant increase in bacterial density with increasing latitude [ln bacteria = −46.4 + (1.2 × latitude); $t_{0.05,24} = 6.9$, $P < 0.001$; adjusted $R^2 = 0.65$].

Seven of seven water samples from within 10 km of the Rappahannock River mouth were also positive for *M. pseudoshottsii* (range, 0.08 to 0.65 bacteria/ml), as were 10 of 10 water samples taken between river km 72 and 73 (range, 0.16 to 1.2 bacteria/ml). Upriver water samples required a 5.0-μm prefilter due to high levels of suspended solids. Interestingly, *M. pseudoshottsii* was not detected on these filters, and all

TABLE 2. Summary of qPCR-assessed prevalences and density ranges of *M. pseudoshottsii* in water, sediment, and fish tissue samples

Sample type	Location[a]	No. of samples	Prevalence	Density range (no. of *M. pseudoshottsii* bacteria/g)[b]
Water	Main stem	38	1.0	5.0×10^{-2}–5.2×10^{0}
	RR, km 0–10	7	1.0	8.0×10^{-2}–6.5×10^{-1}
	RR, km 72-73	10	1.0	1.6×10^{-1}–1.2×10^{0}
Sediment	RR, km 0–10	10	1.0	4.3×10^{1}–1.0×10^{4}
	RR, km 72-73	10	0.70	3.8×10^{1}–3.6×10^{2}
Menhaden liver	Main stem	24	0.79	2.0×10^{3}–3.0×10^{7}
Menhaden spleen	Main stem	24	0.88	8.0×10^{2}–2.2×10^{8}
Menhaden liver	RR, km 0–10	22	0.55	1.6×10^{3}–1.6×10^{6}
Menhaden spleen	RR, km 0–10	22	0.59	2.1×10^{3}–2.0×10^{6}
Anchovy liver	Main stem	31	0.16	6.2×10^{2}–9.6×10^{3}

[a] Locations are either in the main stem of the Chesapeake Bay or in the Rappahannock River (RR). For the latter, location is given by river kilometer as measured by the central channel.
[b] *M. pseudoshottsii* densities are given per gram (= ml of water). Densities are extrapolated when below quantitative limits of the respective assays.

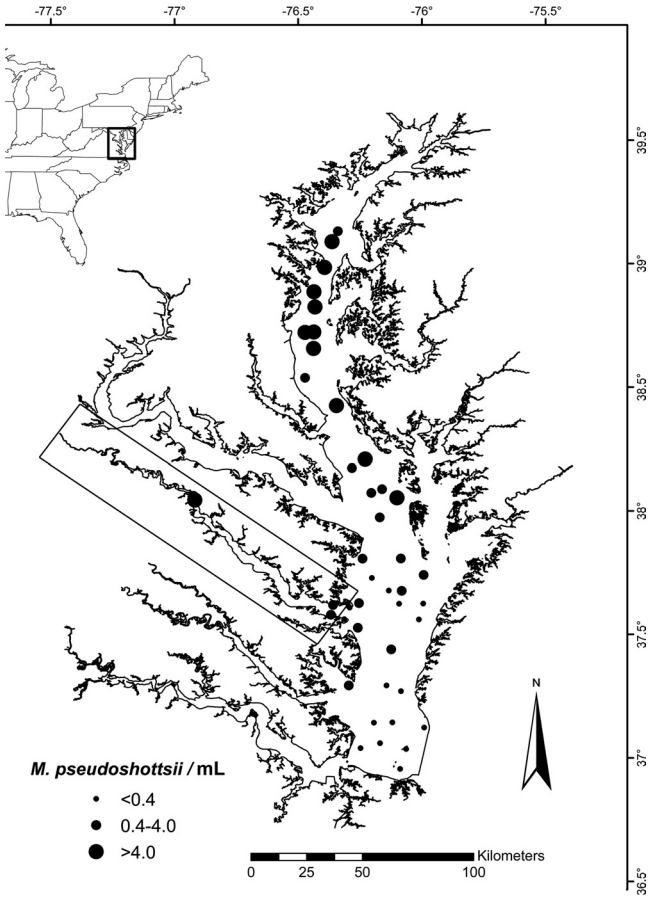

FIG. 4. Densities of *M. pseudoshottsii* bacteria in the main stem of the Chesapeake Bay and the Rappahannock River (boxed) as measured by qPCR. Large circles represent sampling locations (surface water). Small circles (<0.4 *M. pseudoshottsii*/ml) represent positive samples with bacteria below the minimum quantitative threshold of the qPCR assay (see text).

signals were detected from the 0.22-μm residue. *M. shottsii* was not detected in any water sample by qPCR.

Sediment samples. Downriver sediment samples ($n = 10$) were all positive for *M. pseudoshottsii* by qPCR. Extrapolated densities ranged from 4.3×10^1 to 1.0×10^4 bacteria/g. Seven of 10 upriver sediment samples were positive for *M. pseudoshottsii*, with extrapolated densities ranging from 3.8×10^1 to 3.6×10^2 bacteria/g (Table 2). *M. shottsii* was not detected in any sediment sample by qPCR.

Fish samples. *M. pseudoshottsii* was detected in menhaden from the ChesMMAP survey with a prevalence of 79.2% (95% confidence interval [CI], 61.6 to 96.7%) in liver and 87.5% (95% CI, 73.2 to 100.0%) in spleen. Densities ranged from 2.0×10^3 to 3.0×10^7 bacteria/g and 8.0×10^2 to 2.2×10^8 bacteria/g in liver and spleen, respectively. The prevalence of *M. pseudoshottsii* in menhaden collected from pound nets at the Rappahannock River mouth was 54.5% (31.9 to 77.1%) in liver and 59.1% (36.8 to 81.4%) in spleen. Densities ranged from 1.6×10^3 to 1.6×10^6 bacteria/g and 2.1×10^3 to 2.0×10^6 bacteria/g in liver and spleen, respectively (Table 2). Significant relationships were not found between menhaden weights or lengths and the densities of *M. pseudoshottsii* bacteria in either spleen or liver (linear regression; $P > 0.05$). In the case of both ChesMMAP survey menhaden and pound net menhaden, mycobacterial densities in liver and spleen were highly correlated (Pearson coefficient, 0.99 [$n = 21$, $P < 0.01$] and 0.94 [$n = 13$; $P < 0.01$], respectively) in fish for which both organs were positive. In contrast to *M. pseudoshottsii*, *M. shottsii* was not detected by qPCR in any menhaden sample.

In order to independently confirm qPCR findings of *M. pseudoshottsii* in menhaden tissues, ethanol-fixed spleen and liver samples from selected individuals with high densities of *M. pseudoshottsii* bacteria determined by qPCR were processed for routine paraffin histology and stained for acid-fast bacteria via the Ziehl-Neelsen method (18). Acid-fast bacteria were observed in liver and spleen tissues of heavily infected menhaden. Very little pathological change was noted, with the exception of some necrosis in areas of very high densities of acid-fast bacilli. A granulomatous host response to the bacteria was not observed.

Quantitative PCR detected *M. pseudoshottsii* in anchovy liver tissues at a prevalence of 16.1% (3.2 to 29.1%). Densities in positive samples were very low, near the detection limit of the assay, and ranged from 6.7×10^2 to 9.6×10^3 bacteria/g (Table 2). *M. shottsii* was not detected in anchovy tissue.

MIRU analysis. PCR amplified an identically sized single band from DNA extracts of two menhaden livers and cultured *M. pseudoshottsii* (L15) at loci 4, 6, 9, and 15 (Table 3). Direct sequencing of PCR products demonstrated that menhaden amplicons were 100% identical to cultured *M. pseudoshottsii* DNA at all loci. PCR amplification of locus 15 in DNA extracted from water and sediments generated products with 99.8% to 100% identity to cultured *M. pseudoshottsii* DNA. Touchdown amplification of water and sediment DNA extracts for loci 4, 6, and 9 generated products with identical sizes and

TABLE 3. Results of PCR amplification of MIRU loci from two samples each of menhaden, water, and sediment DNA extracts, as well as DNA extracted from pure culture *M. pseudoshottsii* (L15)

Source	Sample or strain	Density (qPCR) (no. of bacteria/g)	PCR results[a] at locus:			
			4[b]	6[c]	9[d]	15[b]
Culture	L15	NA	+	+	+	+
Menhaden	1	3.0×10^7	+	+	+	+
	2	5.4×10^7	+	+	+	+
Water	1	2.9	+[e]	+[e]	+[e]	+
	2	5.2	+[e]	+[e]	+[e]	+
Sediment	1	1.0×10^4	+[e]	+[e,f]	+[e,f]	+[f]
	2	2.4×10^3	+[e]	−	+[e,f]	+[f]

[a] A plus indicates that the size and sequence (when determined; see footnotes below) of the amplified product were identical or nearly identical (similarity ≥ 99.5%) to those of L15. A minus indicates that no fragment with a size similar to that of L15 was amplified or that there was a fragment of similar size to L15 but a BLASTn search of sequenced fragments indicated no significant matches with mycobacteria.
[b] Primers for MIRU loci are as described in reference 1.
[c] Primers for MIRU locus are as described in reference 27.
[d] Primers for MIRU locus are as described in reference 28.
[e] Touchdown PCR cycling was performed (see text). Sequenced products were 99.5 to 100% similar to sample L15.
[f] Multiple fragments were amplified, but only fragments with sizes similar to that of L15 were sequenced. Sequences were 99.8 to 100% similar to L15.

99.5 to 100% sequence identity to *M. pseudoshottsii*. In some cases, multiple bands were generated by PCR, and only the band with a size identical to that of cultured *M. pseudoshottsii* DNA was sequenced (Table 3).

DISCUSSION

The newly described, slow-growing mycobacteria *M. pseudoshottsii* and *M. shottsii* are associated with highly prevalent mycobacteriosis in the economically and ecologically important striped bass in the Chesapeake Bay (10, 20). The reservoirs and modes of transmission of these bacteria to striped bass are unknown, and there is no information regarding the presence and/or density of these bacteria in environmental matrices or in prey species.

In the present study, qPCR for IS*2404* was used to detect *M. pseudoshottsii* in environmental samples. *M. marinum* strain Cyprinum (CC240299) and *M. marinum* strain Eilaticum (DL240490) from Israeli aquaculture and *M. marinum* strain Hellenicum (DL045) from Greek aquaculture were also positive by this primer/probe set, as was *M. ulcerans*. *Mycobacterium ulcerans*, however, is not known to be endemic to North America, nor have *M. marinum* isolates identical to the European strains described above been identified in North America. IS*2404*-positive *M. marinum* isolates from Chesapeake Bay striped bass do not amplify with the primer/probe set described here; therefore, among IS*2404*-positive isolates from the Chesapeake Bay to date, *M. pseudoshottsii* is specifically identified by this assay. There is the potential, however, for undiscovered diversity among *M. marinum* and *M. pseudoshottsii* bacteria in the Chesapeake Bay that may complicate this analysis. We therefore amplified and sequenced MIRU loci from cultured *M. pseudoshottsii* DNA as well as from DNA extracted from menhaden tissues and water and sediment samples. Amplified MIRU loci from infected menhaden tissues demonstrated 100% sequence identity with cultured *M. pseudoshottsii*. MIRU loci amplified from water and sediment DNA extracts demonstrated 99.5% to 100% identity to cultured *M. pseudoshottsii*. These results provide evidence that amplification of IS*2404* in menhaden tissues, water, and sediments is indicative of the presence of *M. pseudoshottsii*. We do recommend, however, further optimization of MIRU PCR at additional loci and application to an expanded range of samples. The importance of this approach as a means of ensuring positive identification of specific *M. ulcerans* strains in various matrices has been demonstrated (7, 15).

Quantitative PCR assays for *M. pseudoshottsii* in the presence of extracted material from both water residues and sediments were found to be quantitative to approximately 1 bacterial genome/reaction, equivalent to 0.4 bacteria/ml or 500 bacteria/g, respectively. Similar assays for *M. shottsii* were found to be less reliably sensitive, with quantitative detection limits of 100 and 10 bacteria/reaction (40 cells/ml and 5,000 cells/g), respectively, and inconsistent quantitative results at the 1,000 bacteria/reaction level for residues. The difference in sensitivity between *M. pseudoshottsii* and *M. shottsii* assays is readily explained by the fact that IS*2404* is likely present in high copy number (~200 copies) in the *M. pseudoshottsii* genome, as it is in *M. ulcerans* (26), whereas the *M. shottsii* target does not appear to be an insertion sequence and is therefore likely present in a low or single copy number.

Problems with PCR inhibition are common when extracts of water or sediment samples are being amplified. Pulverization of nitrocellulose filters by bead milling followed by extraction of DNA with silica column methods was found to be a rapid and convenient method of DNA isolation, but it was also found that addition of bovine serum albumin to PCR mixtures was crucial for minimizing inhibition. This method was found to yield acceptable results for detection of *M. pseudoshottsii* in water samples, but sensitivity problems were apparent for the *M. shottsii* assay. Use of the MoBio PowerSoil extraction kit appeared to effectively reduce PCR inhibition from extracted sediment samples for both *M. pseudoshottsii* and *M. shottsii*, although quantitative sensitivity limits were relatively higher for the latter.

The results of this study demonstrate that *M. pseudoshottsii* is ubiquitously distributed in surface water of the main stem of the Chesapeake Bay, as well as in riverine sediments. Further, *M. pseudoshottsii* is present in Atlantic menhaden and Bay anchovies, two major prey items of the striped bass. This presents the possibility of two major routes of transmission, waterborne and by ingestion, of the pathogen to striped bass. Transmission of pathogenic mycobacteria to finfishes is still poorly understood, although anecdotal information (23) and, more recently, experimental studies (11) have demonstrated that ingestion is a viable potential mode of transmission. Further studies are necessary to explore the contributions to infection of continuous exposure of striped bass to *M. pseudoshottsii* via the water column and ingestion through prey items. Direct fish-to-fish contact through capture in commercial gear (e.g., pound nets) and transmission through handling (e.g., through handling of fishes by fishermen) remain to be evaluated as transmission routes as well. The presence of large numbers of *M. pseudoshottsii* bacteria in menhaden, and the consequent possibility that these fish represent a major reservoir of pathogenic mycobacteria to striped bass, indicates that efforts should be made to confirm transmission via this route. Future research on the infection and disease dynamics of *M. pseudoshottsii* in the striped bass would then need to account for processes at lower trophic levels.

In some cases, Atlantic menhaden were found to be infected with high densities ($>10^7$/g) of *M. pseudoshottsii* bacteria in spleen and liver tissues. Large numbers of acid-fast organisms were confirmed by histology, but interestingly, no host response was observed, and minimal pathology was present. Histological evaluation of menhaden with ulcerative mycosis attributed to *Aphanomyces invadans* consistently reveals severe granulomatous myositis, indicating that menhaden are capable of mounting a typical granulomatous inflammatory response (4, 14, 29). It therefore appears that *M. pseudoshottsii* can infect menhaden but does not cause disease in this host. Alternately, it could be argued that the infections observed in this study represent a commensal state between *M. pseudoshottsii* and menhaden but that this status could be shifted to production of disease in the presence of stressors. Atlantic menhaden is, in its own right, a highly commercially and ecologically important finfish species, and the presence of high levels of a potential pathogen warrants further examination.

An interesting pattern of *M. pseudoshottsii* density in the

main stem of the Chesapeake Bay was noted in this work, with a highly significant relationship of increasing density with increasing latitude over approximately 245 km of the main stem of the Bay. This study was not intended to generate comprehensive analysis of the relationship between *M. pseudoshottsii* density and environmental variables, such as salinity, dissolved oxygen, etc., and geographical location is confounded with several of these variables, especially salinity, which ranged from 25.0 practical salinity units (psu) in the south to 13.1 psu in the north. The fact that patterns of *M. pseudoshottsii* density within the Bay do exist, however, is suggestive that certain hydrologic factors, or even terrestrial factors such as land use, may regulate its density and that this may in turn be related to transmission rates. This is highly speculative, however, and it is likely that host factors, such as stress and condition, may play as much or more of a role in infection and disease than environmental factors.

M. shottsii was not detected in finfishes or environmental matrices in the course of this study. As detailed above, one reason for this may be the relatively lower sensitivity of the qPCR assays for *M. shottsii* than for *M. pseudoshottsii.* Identification of multicopy gene targets specific to *M. shottsii* and development of more sensitive assays would be helpful in resolving this issue. These findings, however, raise the possibility that *M. shottsii* is an obligate pathogen of striped bass. Adaptation of mycobacteria to obligate pathogenicity in specific vertebrate hosts has precedent, most notably with *M. tuberculosis* and *M. leprae*, which have no natural hosts except humans, and armadillos for the latter. The nonpigmented nature of *M. shottsii* is also suggestive, as carotenoid pigments are frequently used by environmental mycobacteria for protection against UV light (e.g., *M. marinum*, *M. pseudoshottsii*), and vertebrate-adapted mycobacteria often lack the capability to produce these pigments (e.g., *M. tuberculosis*, *M. leprae*, *M. ulcerans*), ostensibly because the pigments are no longer necessary. Mycobacteria infecting fishes are generally thought to be facultative environmental pathogens, so the possibility that *M. shottsii* is an obligate pathogen in the manner of significant mycobacteria of humans deserves further examination.

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