

**Mycobacteriosis in Striped Bass of the Chesapeake Bay:
Expansion of Studies Emphasizing Cultural and Rapid Molecular
Diagnostic Methods to Evaluate Disease Prevalence**

A Final Report

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EXECUTIVE SUMMARY

Researchers in Virginia and Maryland have recently documented an epizootic of mycobacteriosis in striped bass, *Morone saxatilis*, from the Chesapeake Bay. Utilizing histological techniques, prior research at the Virginia Institute of Marine Science (VIMS) confirmed the presence of acid-fast bacilli within granulomas, implicating mycobacterial infections in skin and internal tissues of a large number of wild striped bass (Vogelbein et al., 2001; Cardinal, 2001). Histological studies relied upon fixation of fish tissues in a preservative, followed by specialized stains which allowed for direct detection of the mycobacteria or the typical lesions associated with mycobacteria, called granulomas. In addition, microbiological studies consisting of isolation and phenotypic characterization of the *Mycobacterium* spp. were undertaken for a subsample of these fish (Rhodes et al., 2000). Microbiological methods initially employed traditional processing techniques, including tissue decontamination to eliminate any rapidly growing “contaminant” bacteria. Selective media and incubation at temperatures of 30°C or higher were used. Such routinely used methods, however, were subsequently determined to have a detrimental effect on recovery of mycobacteria from striped bass. For example, one of the common disinfectants for decontamination, Zephiran^T, not only destroyed nonmycobacteria, but killed over 99% of the mycobacteria in pure suspensions. In addition, the predominant mycobacterial isolate from wild striped bass, *M. shottsii*, (Rhodes et al., 2001a; b; c; 2002) seldom would grow at 30°C, so incubation at a lower temperature, 23°C, was essential. Even at their optimal low incubation temperature, many *Mycobacterium* spp. grow very slowly, requiring weeks to months for initial colony detection. Molecular techniques began to be utilized in an effort to quickly detect mycobacteria in fish (Kaattari et al., 1999; 2000; 2001). Amongst the molecular techniques available, polymerase chain reactions (PCR) and a further amplification technique called nested PCR were utilized. The PCR and nested PCR techniques use oligonucleotide primers that detect and amplify a portion of the *Mycobacterium* genus-specific gene, the 16S rRNA gene. This gene is highly conserved amongst all *Mycobacterium* spp. and thus represented a reliable type of DNA to serve as an indication of mycobacterial infections in striped bass tissues.

Surveys of wild striped bass from the Potomac, Rappahannock, and York Rivers (N = 1899) were initially conducted during spring, 1998 through fall, 1999 (Vogelbein et al., 2001; Cardinal, 2001). Histological examination of skin and spleen samples from these fish was analysed. Prevalence of splenic infection by mycobacteria appeared to be much higher than for dermal (skin) infections. Splenic infections ranged from 31.5% in the Rappahannock River in summer, 1999, to 62.7% in the York River in fall, 1999. There seemed to be no significant variance in prevalence spatially (from site to site).

INTRODUCTION

During 1997-99, the Aquatic Animal Disease Diagnostic Laboratory (AADDL) at the Virginia Institute of Marine Science (VIMS) investigated and documented an epizootic of disease in wild striped bass, *Morone saxatilis*, from many portions of the lower Chesapeake Bay. Some of these fish exhibited an ulcerative dermatitis initially suspected of being caused by *Pfiesteria piscicida*, a dinoflagellate. Skin ulcers ranged from pinpoint, pigmented spots to large, shallow hemorrhagic (bloody) wounds. This disease was determined to be due not to *Pfiesteria*, but to a group of bacteria called *Mycobacterium* spp. This disease syndrome is referred to as mycobacteriosis. Further investigations by VIMS researchers and collaborators at the Centers for Disease Control (CDC) identified a new species, *M. shottsii*, as the most frequently isolated mycobacteria from striped bass during this epizootic (Rhodes et al., 2001c; Rhodes et al., 2002). During 2001-2002, the Virginia Saltwater Recreational Fishing Development Board funded a proposal to compare rates of detection of this disease by three methods, histology, quantitative bacteriology, and the molecular technique of polymerase chain reaction (PCR) and nested PCR. This report summarizes the results of our yearlong investigation of this disease.

The striped bass is important both for recreational and commercial finfish fisheries in the Chesapeake Bay and, indeed, along the entire Atlantic coast of the United States. During the mid 1970's to 1980's, a significant decline in striped bass landings (Field, 1997) led to the development of an interstate fisheries management plan by the Atlantic States Marine Fisheries Commission (ASMFC, 1981). Then in 1984, federal legislation was enacted that outlined punitive measures for those states which failed to comply with the ASMFC plan. By 1995, however, ASMFC announced that Bay stocks had returned to healthy levels and thus coastal states could expand their recreational and commercial fisheries (ASMFC, 1995).

Natural populations of striped bass exist along the Atlantic coast from near the United States' border with Canada down to Florida. Striped bass from the Chesapeake Bay are the major component of the Atlantic coastal stock, one of the four major stocks of striped bass in the United States (Austin, 1980). The young striped bass remain in the Bay until they are two to six years old (Austin, 1980; Kohlenstein, 1981). Then a difference in migration based on gender occurs, with female fish migrating to sea, but male fish generally remaining in the Bay. The migrating adults can range from Virginia to Nova Scotia during spring until fall. Then the fish begin to migrate southward to warmer water. Many fish overwinter in the deeper parts of the Chesapeake Bay (Kohlenstein, 1981) and spawning begins near April when the water temperature reaches 8°C (Austin, 1980; Almy, 1999).

Mycobacteriosis in wild striped bass from the Chesapeake Bay has recently been documented (Cardinal, 2001; Rhodes et al., 2001c; Vogelbein et al., 2001). *Mycobacterium* spp., the causative agents of mycobacteriosis, are Gram positive, acid-fast, nonmotile, non-spore forming, rod-shaped bacteria (ASM Press, 1995). The rod shape of such bacteria may vary, ranging from straight to curved, 0.2-0.6 x 1.0-10.0 um, and may exhibit a branched or filamentous growth. The unique waxy nature of their cell walls results in acid-fastness, a property which is characteristic of mycobacteria. With mild heating, the red-colored carbolfuchsin stain of the Ziehl-Neelsen protocol penetrates their cell wall, and subsequent decolorization with acidified alcohol will not remove the carbolfuchsin. A blue counterstain, methylene blue, is generally applied, so that the acid-fast mycobacteria stand out as bright red rods within a background colored blue (Prophet et al., 1994). Thus, the mycobacteria are said to be "acid-fast". To reliably detect acid-fastness for the *Mycobacterium* spp., it is preferable to stain cultured living cells of mycobacteria. When the mycobacteria are within tissues, the

acid-fast characteristic of mycobacteria may not be detected, depending on the staining methodology (Colorni et al., 1998; Daoust et al., 1989; Gauthier et al., 2002). Although there are other nonmycobacterial organisms with various degrees of acid fastness: *Rhodococcus* spp., *Nocardia* spp., *Legionella micdadei*, the cysts of *Cryptosporidium* spp., *Isospora* spp. and other microsporidia, mycobacteria can be “identified by traits such as rate of growth, colonial morphology, pigmentation, and for differential purposes, biochemical profiles” (ASM, 1995).

The genus *Mycobacterium* causes disease in over 160 species of saltwater and freshwater fish (Chinabut, 1999). Wild stocks of fish have been reported to have mycobacteriosis, including cod, *Gadus morhua*, mountain whitefish, *Prosopium williamsonii*, striped bass, *Morone saxatilis*, North-East Atlantic mackerel, *Scomber scombrus*, and yellow perch, *Perca flavescens*, with the prevalence of mycobacteriosis ranging from 8% to 100% (Dalsgaard et al., 1992; Abernethy & Lund, 1978; Sakanari et al., 1983; MacKenzie, 1988; Daoust et al., 1989). There appears to be no bias towards the sex of the fish in the prevalence of mycobacteriosis, but the severity of the infection is apparently related to age (Abernethy and Lund, 1978; MacKenzie, 1988).

Piscine mycobacteriosis is considered a slowly developing chronic disease, which may take two or more years for the number of organisms to grow to readily detectable numbers (Ashburner, 1977). Most species of fish may manifest few or no external signs of disease. In advanced stages, emaciation, exophthalmia, lordosis, hemorrhagic and dermal ulcerative lesions or loss of scales may be observed. Affected fish may be lethargic, floating impassively, with loss of appetite.

The specific *Mycobacterium* species reported for fish include *M. chelonae*, “*M. chesapeaki*,” *M. fortuitum*, *M. marinum*, *M. neoaurum*, *M. poriferae*, *M. scrofulaceum*, *M. shottsii*, and *M. simiae* (Backman et al., 1990; Bruno et al., 1998; Chinabut, 1999; Heckert et al., 2001; Landsdell et al., 1993; Rhodes et al., 2001c, 2002). Many of these *Mycobacterium* spp. are considered ubiquitous in the environment, being able to survive in water or sediment (Falkinham et al., 1980; Brooks et al., 1984), as well as in various fish species. Such “environmental” mycobacteria can survive in water which is nutrient-poor (Bolan et al., 1985), contains chlorine (Collins et al., 1984), or varies over a wide range of pH and temperature conditions (George et al., 1980; Beurey et al., 1981). It is speculated that the water thus serves as a natural habitat and may serve as a means of transmission. Even though environmental mycobacteria can survive outside of an animal host saprophytically, they can also be pathogenic for a wide variety of marine animals and humans (Collins et al., 1984; Falkinham, 1996; Dailloux et al., 1999).

The literature for mycobacteriosis in striped bass includes aquacultured fish on the Pacific coast (Hedrick et al., 1987), experimentally infected striped bass fish (Wolf & Smith, 1999), and wild striped bass (Landsdell et al., 1993). Very recent reports of mycobacteriosis in wild striped bass in the Chesapeake Bay have been published (Heckert et al., 2001; Rhodes et al., 2001, 2002). Research for the project described in this report was unique in that all spleen samples (N=118) were aseptically collected, and three concurrent techniques for detection of mycobacteriosis were employed: histology, quantitative bacteriology, and PCR/nested PCR (Kaattari et al., 2002a & b).

GOALS:

This study had three basic goals. First, we wanted to determine the incidence of mycobacteriosis in wild striped bass of the Chesapeake Bay, utilizing aseptically collected spleen tissues. Both externally asymptomatic and symptomatic striped bass would be included in such studies because the literature often reports that initial mycobacterial infections in fish are asymptomatic. But such asymptomatic fish are nonetheless a potential source of infection for additional fish and furthermore, aid in the documentation of the actual incidence of mycobacteriosis in striped bass. Spleens were selected to evaluate internal infection because spleens often are reported as the most consistently affected visceral organ (Colorni et al., 1993). In addition, this organ is readily harvested aseptically and normally has no bacteria. Thus, if *Mycobacterium* spp. are detected in a fish spleen, such bacteria represent a “true infection”, and are not likely due to incidental contamination by mycobacteria originally in the water and/or sediment (or other part of environment).

The second goal was to enhance communication between the recreational fishermen and researchers by collecting fish, whenever possible, at striped bass tournaments throughout the state. Our research team was successful in arranging for active collaborative assistance at several tournaments: Reedville (6/13/01), Colonial Beach (10/06/01), Lynnhaven in Virginia Beach, sponsored by the American Striper Association (ASA) (11/17/01), and Deltaville, sponsored by the Coastal Conservation Association (CCA) (12/01/01). In addition, several individual fishermen brought striped bass directly to VIMS for our research. A second benefit of such interaction was that in contrast to hiring commercial fishermen to harvest the fish, this method of collection was very economical, allowing us to be successful with a modest budget. To increase the number of striped bass to be studied and also to allow for collection of a greater diversity of sizes, condition, and age of fish, we also conducted several haul seine net surveys of the York River.

The third and primary goal was to conduct a concurrent comparison of three methods of detection of mycobacteriosis: standard histological examination, quantitative bacteriological assay, and molecular polymerase chain reaction (PCR) and Nested PCR assays. Histological examination would quickly confirm the presence of granulomatous lesions, the type of pathology associated with mycobacterial disease. Quantitative bacteriology would allow determination of the severity of the disease (e.g. the density or concentration of mycobacteria per gram of tissue). By culturing the mycobacteria, tests could also be conducted that allowed characterization of the specific *Mycobacterium* spp. involved. Finally, since molecular PCR/nested PCR tests are generally purported to be very sensitive, rapid methods of detecting mycobacteriosis, we wanted to validate the accuracy and reliability of this method for detection of mycobacteriosis in wild striped bass.

METHODS:

Fish Collection and Initial Processing

Striped bass were predominantly collected at tournaments (N= 49, Fig. 1) and from haul seine net surveys (N= 65, Fig. 2) during June, 2001, to December, 2001. Fish were immediately put on ice and transported to VIMS, or in the case of one tournament (Reedville, 6/13/01), the fish were processed on-site, using VIMS' mobile laboratory vehicle. In four instances, individual recreational fishermen directly brought striped bass on ice (either freshly caught or frozen) to VIMS for analysis.

The Virginia Marine Resource Commission (VMRC) issued special permits allowing each tournament volunteer to collect up to five extra striped bass, including sub-legal sized fish. Such permits were only used at the Colonial Beach tournament, where nine captains signed up after a short presentation by Ilsa Kaattari at the Rules Meeting. Unfortunately, the weather did not cooperate on the following day, and thus the number of striped bass caught was very low (N = 6). For both the ASA tournament in Lynnhaven and the CCA tournament in Deltaville, the organizers did not require use of the permits. For fish collected at the CCA tournament, we coordinated our research with Troy Thompson of VMRC, who studies otoliths and scales in order to age the striped bass.

Each fish (and its associated tissues) was assigned a unique, sequential identification number. Various physical measurements were taken: weight, total length, fork length, and standard length. This data was written on a standard form which included a pictorial depiction of both exterior sides of the fish where sketches of any lesions or pathology could be recorded.

The striped bass showing any exterior lesions potentially associated with mycobacteriosis from tournaments or seine surveys were individually wrapped in newspaper and put in a plastic bag before being stored on ice. The photograph shown in Fig. 3 shows the "typical" shallow ulcers in striped bass skin associated with mycobacteriosis. Fish without obvious skin lesions were stored directly on ice.

Aseptic necropsies of the fish were conducted within a laminar flow hood (Fig. 4), using Biosafety Level Two conditions. Spleen pathology was observed and recorded, as well as a basic description of the exterior of each fish.

Small portions (approximately 0.5 – 1.0 g) of spleen from each fish were aseptically collected, and then a weighed subsample (0.1 – 0.5 g) was added to 2 ml sterile Butterfield buffer (BB) (Anon, 1995) for further processing. A second, similar-sized subsample of spleen was thinly sliced and placed in individual containers of 10% formalin buffer fixative for histological processing.

Histopathology Methods

For histological evaluation, spleen tissues were fixed in 10% buffered formalin for 48 hours. The tissues were then transferred to tissue cassettes, placed in a gentle running water bath for approximately 3 hr, then processed through a series of increasing concentrations of ethanol to dehydrate the tissue (e.g. 50% ethanol for 1 hr, 50% ethanol for 1 hr, 70% ethanol for 1 hr), and stored in 70% ethanol until ready for processing and embedding. Just prior to processing, cassetted tissues were transferred to 95% ethanol for 15-30 min and placed in a tissue processor (Shandon Hypercenter) for further dehydration, clearing, and paraffin infiltration. The tissues were embedded in TissuePrep paraffin and sectioned at 5 um on an Olympus or American Optical rotary microtome and mounted on glass microscopic slides. The slides were stained with routine Harris hematoxylin and eosin (H&E) (Prophet et al.,

1994), dehydrated, and mounted in Preservaslide. Tissue sections were then examined at 40X magnification for the presence and number of granulomatous lesions, the type of lesion commonly associated with mycobacterial infections (Fig. 5). A minimum of 9 sections of stained splenic tissue for each fish was examined, and all were observed for the presence and number of granulomatous lesions.

Quantitative Bacteriological Methods

Portions of spleen from each fish were aseptically collected and weighed as described above. The splenic tissue in BB was homogenized in a Ten Broeck tissue grinder and two equal portions of this homogenate were stored in sterile 1.5 ml microcentrifuge tubes. One tube was frozen at -20°C for future molecular testing and the second further serially diluted and spread-plated onto duplicate plates of Middlebrook 7H10 agar with ADC or OADC enrichment (MDA) for quantitative analysis (Fig. 6). Homogenates were also spread plated onto Brain Heart Infusion (BHI) agar, a non-selective medium, to monitor for contamination by heterotrophic bacteria.

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 skin homogenates were neutralized by addition of 0.5% HCl in the presence of the pH indicator, bromocresol purple, until a blue color persisted (pH 6.8). Plates were incubated at 23°C for three months. Culture plates were stored in plastic, Ziploc^T bags to prevent desiccation of the media during incubation.

All morphologically distinct colonies (see Fig 7) were examined for acid-fastness (Fig 8)(Ziehl Neelsen) and acid-fast colonies were streaked to MDA to obtain purified cultures. Purified isolates were characterized as mycobacteria using published methods (Kent & Kubica, 1985; Isenburg, 1992.)

Mycobacterial densities are expressed as colony forming units (CFU) per gram of tissue. Means of these densities were calculated from log transformed data.

Reference *Mycobacterium* spp. and *M. shottsii*

Reference *Mycobacterium* spp. (*M. chelonae*, *M. flavescens*, *M. fortuitum*, *M. gordonae*, *M. kansasii*, *M. marinum*, *M. scrofulaceum*, *M. simiae*, and *M. terrae*) were obtained from the Environmental Protection Agency, Cincinnati, OH and from the Consolidated Laboratory Services, Commonwealth of Virginia, Richmond, VA.

For further description of phenotypic and genetic analyses of *M. shottsii*, the predominant mycobacterial isolated from striped bass, two recent publications (Rhodes et al., 2001c; Rhodes et al., 2002) are appended to this report.

Molecular Methods: PCR and Nested PCR

The purpose of a polymerase chain reaction (PCR) assay is basically to detect and amplify a specific gene within the whole genome (DNA content) of an organism. The gene most frequently targeted for detection of *Mycobacterium* spp. is the 16S rRNA gene, otherwise known as the ribosomal small subunit gene (Kirschner et al., 1993, 1996; Turenne et al., 2001). The sequence of this gene is comprised of approximately 1500 basepairs (bp) of nucleotides. Within this gene is a conserved region which all *Mycobacterium* spp. share, a DNA segment comprised of 924-940 bp located at the 5' end of the gene (reading from left to right, standard for all genes) (Talaat et al., 1997).

Prior to this project, investigators at VIMS had confirmed that the primers designed by Talaat et al. (1997) were genus-specific ("positive") for twelve different *Mycobacterium* spp.

either discovered previously in striped bass or reference cultures for species reported in literature as causative agents of fish mycobacteriosis. These same PCR primers were negative when testing preparations of genomic DNA of closely related genera of bacteria, such as *Nocardia* spp. Essentially, even crudely extracted genomic DNA extracted from pure cultures of mycobacteria when added as template DNA in a PCR reaction, would be “positive”. That is, within only a few hours, the specific-sized (924-940 bp) DNA product was successfully amplified and detected.

It required additional work to detect the presence of mycobacteria within fish splenic tissue. Instead of being the sole source of genomic DNA (as with pure cultures of mycobacteria), the mycobacterial DNA was only a very small fraction of the total DNA present. Most of the genomic DNA used as template in the original PCR reaction was DNA of the fish tissue itself. In addition, many tissues naturally contain potent PCR inhibitors, such as enzymatic proteins that degrade DNA or a variety of other proteins or lipids that interfere with the amplification process of PCR. To decrease the activity or presence of such natural tissue inhibitors, it was necessary to purify the genomic DNA before setting up the PCR reaction. Using a larger amount of DNA as template was also required. Furthermore, an additional amplification step, called nested PCR, was necessary in order to detect the presence of mycobacterial DNA within fish tissue. The primers for nested PCR were originally designed by Talaat et al. (1997) and these primers amplified an internal 300 bp segment of DNA (located within the larger, 924-940 bp DNA segment). The source of DNA template for nested PCR reactions was a 3 ul portion of the whole 100 ul completed, original PCR reaction mixture. By conducting an additional amplification, sufficient mycobacterial DNA product was produced to become detectable.

Specific PCR Conditions/Reagents Utilizing Pure Mycobacterial Cultures

Genomic DNA was extracted from pure mycobacterial cultures according to the procedure described by Reischl et al., 1994. Briefly, one-two bacterial colonies were washed once with phosphate buffered saline (PBS, Sigma Chemical Co., St. Louis, MO) and resuspended in 500 ul extraction buffer (EB) (1% Triton X-100, 0.5% Tween 20, 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). In screw-capped microcentrifuge tubes, the turbid suspension was subjected to five cycles of 3-min freezing in liquid nitrogen and 1-min heating in a boiling water bath. To ensure killing of the mycobacteria, a final 10-min boiling step was added (Buck, 1992). After that treatment, the rigid, lipopolysaccharide-rich mycobacterial cell was disrupted, and following a short centrifugation step, the released genomic DNA from the supernatant could be reliably used for PCR amplification.

A 924-940 base pair fragment of the 16S rRNA gene from *Mycobacterium* spp. was amplified using two published primers (Talaat et al., 1997). The upstream (T39) and downstream (T13) primers each consisted of 20 nucleotides as follows: 5'-GCG AAC GGG TGA GTA ACA CG and 5'-TGC ACA CAG GCC ACA AGG GA, respectively (GibcoBRL, Gaithersburg, MD). PCR amplification was conducted in 100 ul reaction mixture containing 200 uM (each) of dATP, dCTP, dGTP, and dTTP, 10 ul of 10X reaction buffer (RB) (100 mM Tris-HCl, pH 8.3, 0.5 M KCl; 15 mM MgCl₂, 0.01% gelatin), 10-100 ng of each genomic DNA template or 1-5 ul of supernatants of frozen-boiled mycobacterial cultures, 0.6 uM of each primer, and 2.5 U of *Taq* polymerase. All PCR reagents were obtained from Sigma-Aldrich, St. Louis, MO. The reaction mixture was placed in an MJ Research PTC-200 thermocycler (MJ Research, Inc., Watertown, MA) and the following amplification conditions were used: 1 cycle of 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min, extension at 72° C for 1 min; and a final extension at 72°C for 10 min. Twenty

ul of the amplified PCR products were analyzed by electrophoresis on a 1.0 % agarose gel and visualized by ethidium bromide stain.

A no-template DNA negative control was included with each set of PCR assays and during early investigations, DNA from *E. coli* and *Nocardia otitidiscaviarum* (ATCC 14629) were used as additional negative controls.

Specific PCR and Nested PCR Conditions/Reagents for Striped Bass Splenic Tissue

Genomic DNA was prepared from one of the aliquots of the splenic tissue homogenate as described in the quantitative bacteriology methods section. Approximately 500 ul of the thawed homogenate was spun briefly and the tissue pellet was resuspended in 500 ul EB as defined previously. In screw-capped microcentrifuge tubes, the turbid suspension was subjected to five cycles of freezing and boiling as described previously. A final 10-min boiling step was included. At this point, it was necessary to conduct additional purification of the genomic DNA and although several kit methods were as effective, a simple, non-kit, standard method, employing phenol/chloroform/isoamyl alcohol extraction, followed by ethanol precipitation (Ausubel et al., 1992), was most frequently employed. The genomic DNA content was measured, using fluorometric DynaQuant (Hoefer, San Francisco, CA) equipment. Approximately 500-1000 ng of purified DNA preparations was utilized as template DNA in the PCR reaction. PCR conditions as described above for purified mycobacterial culture DNA were followed. Since the splenic tissue samples were negative at this point, a positive *Mycobacterium* spp. template control (as well as a no-template DNA control) was usually included in each set of PCR reactions.

After the PCR reaction utilizing Talaat primers, T39 and T13, was complete, 3-5 ul of the original 100 ul PCR reaction mixtures were used as a source of template DNA for the second amplification, nested PCR. Nested PCR primers were previously designed and designated by Talaat et al., 1997, as upstream (preT43 = 5'-AAT GGG CGC AAG CCT GAT G) and downstream (T531 = 5'-ACC GCT ACA CCA GGA AT.) Nested PCR amplification was conducted in 100 ul reaction mixture containing 200 ul (each) of dATP, dCTP, dGTP, and dTTP, 10 ul of 10X RB, 3-5 ul of the first PCR reaction, 0.6 uM of each nested PCR primer, and 2.5 U of *Taq* polymerase. In order to further enhance the nested PCR reaction, it was occasionally found useful to add 10 ul of sterile DMSO to the reaction mixture. The amplification cycle consisted of 5 min denaturation at 95°C followed by 40 cycles of denaturation at 94°C for 1 min, annealing at 53°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Twenty ul of the amplified PCR products were analyzed by electrophoresis on a 1.0% agarose gel and visualized by ethidium bromide stain. A positive nested PCR reaction would reveal a 300 base pair product.

Additional, Confirmatory Molecular Tests

During early experiments with amplified products of PCR or nested PCR reactions, the gene sequences of such products were characterized by both their appropriate length and sequence. Followed purification of the PCR product (Concert Rapid PCR Purification System, GibcoBRL, Grand Island, NY), cloning utilizing a TA Cloning Kit or TOPO Cloning Kit ((Invitrogen, Carlsbad, CA), and plasmid preparation (Concert Rapid Plasmid Miniprep System (GibcoBRL, Grand Island, NY), the product was sequenced on a Long Read IR 4200 (LiCor, Lincoln, NE), using Thermo Sequenase DYEnamic Direct cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech Inc, Piscataway, NJ). The DNA sequences were

analyzed using the GenBank Data Base and MacVector 6.5 software (Oxford Molecular, Madison, WI).

Fish Age Determination and Statistical Analysis

Age determination of fish was estimated using data provided by the Anadromous Fishes Research Program (VIMS) correlating total length of fish and age based on fish scale assessment of striped bass collected during the period of June, 1999, through December, 2001. Regression analysis of length and age was performed using simple linear regression and the regression ANOVA table.

Statistical tests (paired sign test, regression analysis, Mann-Whitney U test, and Spearman rank correlation) were performed using Statview software (Abacus Concepts, Statview, Berkeley, CA)

RESULTS:

Histopathology

Examination of fixed, H&E stained sections of striped bass splenic tissues revealed the presence and number of granulomatous lesions in the samples. It was deemed unnecessary to also conduct Ziehl-Neelsen stains for AFB because mycobacteria in tissues are not always acid-fast (Colorni et al., 1998; Daoust et al., 1989; Gauthier et al., 2002). Table 1 summarizes the number of granulomas per spleen sample and frequency of occurrence. Of fish that were positive for granulomas, the majority, ~75%, had two or more granulomas per splenic sample. Seven percent of all infected (as detected by histological examination) fish had greater than 100 granulomas per splenic sample, and these fish also generally had a correlating high density of infection as shown by quantitative bacteriology results. The highest number of histologically-detectable granulomas in one fish spleen sample was 261, and not unsurprisingly, this particular fish even had macroscopically visible granulomatous lesions in its spleen (Fig 9). Overall, 52% of all samples (N = 61/118) were histologically positive for the presence of granulomas.

Quantitative Bacteriology

Our aseptic method of collection and processing of splenic tissue was quite successful in that only one spleen sample (N = 119) had a high density of “contaminant” heterotrophic bacteria. This sample was thus omitted from the data analysis since the source of any mycobacteria in the sample could have been the environment. By conducting serial dilutions of known amounts of splenic tissue, the level of infection for each fish was determined. Table 2 presents the frequency distribution of mycobacterial densities in culture-positive tests. Approximately 75% of all infected fish had greater than/or equal to 1,000 (10^3) mycobacteria g^{-1} tissue. Nearly 19% had greater than 1,000,000 (10^6) mycobacteria g^{-1} .

Partial characterization, utilizing phenotypic characteristics, revealed that the predominant *Mycobacterium* spp. from striped bass was *M. shottsii*. *M. shottsii* was cultured from 77% of the infected fish, with approximately half of such fish having only *M. shottsii* and the other half having *M. shottsii* as a co-infection with other *Mycobacterium* spp. Even in co-infections, *M. shottsii* was numerically dominant, being present at more than tenfold higher CFU/g than the other *Mycobacterium* spp. Several phenotypic characteristics readily aid identification of *M. shottsii*: its slow growth, even at its optimal temperature of 23°C, its nonchromogenicity (no pigment produced during light or dark conditions), and its phenotypic

characteristics of being niacin positive, nitrate negative, tween negative, urease positive, and pyrazinamidase negative.

From the remaining 23% of infected striped bass without *M. shottsii*, isolated mycobacteria exhibited biochemical reactions and growth characteristics resembling those of *M. scrofulaceum*, *M. simiae*, and *M. interjectum*.

Overall, 69% (N = 81/118) of the fish collected for this VMRC-funded project were culturally positive for mycobacteriosis.

Molecular Results: PCR and Nested PCR

When working with genomic DNA extracted from mycobacterial cultures, the PCR primers reliably and specifically amplified a 924-940 segment of the 16S rRNA gene. Figure 10 shows PCR results for the twelve different *Mycobacterium* spp. isolated at VIMS from either wild striped bass or from reference strains. Included in this figure are two types of negative controls: a reaction with all PCR reagents except that no template DNA is added and a second reaction with template DNA from *Nocardia otitidiscaviarum*, a closely related, but non-*Mycobacterium* spp., bacterium. These PCR results confirmed that the Talaat-designed primers (1997) facilitated sensitive, specific amplification of a *Mycobacterium*-genus specific gene.

In order to directly detect *Mycobacterium* spp. within splenic tissue of wild striped bass, an additional amplification PCR reaction, called nested PCR, was required. In addition, extraction of original template DNA from the fish tissue required purification steps to reduce the tissue's natural PCR inhibitors. The original PCR reaction also needed a large amount of extracted DNA added as template. Conducting both a PCR and nested PCR reaction for each fish splenic sample did not add more than about a day to the time required for detection. Virtually all of the DNA extracted from fish tissues would be fish DNA. Both the quantity of spleen tissue homogenized for PCR/nested PCR testing and the degree of infection (and thus amount of mycobacterial DNA) in the fish spleens varied significantly. Furthermore, there were many other variables where the quality and quantity of DNA could be affected prior to initiation of the PCR reaction.

An estimation method for assessing the sensitivity of the molecular approach was to examine the results for samples of this study having low densities of culturable mycobacteria. Sixty-four percent of samples (N = 14/22) with $\leq 10^3$ CFU g⁻¹ were positive by PCR. Statistical analysis of these results determined there was a significant difference between detection rates for quantitative culturing and PCR/nested PCR (p = .0078) for this subset of samples. Thus, quantitative bacteriology was more sensitive than PCR/nested PCR for detecting mycobacteriosis in low-density infections of splenic tissue.

When PCR reactions were set up with DNA extracted from splenic tissue, the 924-940 bp positive product was not produced. But when 3 ul of this original PCR completed reaction was used as template in the next amplification, nested PCR, a positive product of 300 bp could be detected. This 300 bp product represents an internal portion of the 924-940 bp product that was too low to be detected in the original PCR reaction (Fig. 11).

Overall, 75% (N = 88/118) of the fish were PCR/nested PCR positive for mycobacteriosis.

Comparison of Methods of Detection

Table 3 shows the locations within the Chesapeake Bay (Virginia's mid Bay to lower Bay) of wild striped bass included in this project. In addition, the percentage of fish positive for mycobacteriosis by each of the three methods of detection are shown. This data revealed

an interesting exception to the overall similarity between quantitative bacteriology and molecular PCR/nested PCR methods in detecting mycobacteriosis: at two out of four tournaments (Colonial Beach and Deltaville), the PCR/nested PCR method detected higher rates of mycobacteriosis.

To further investigate this potential difference, Table 4 compares the method of fish collection, hook and line vs. net of haul seine, to positive mycobacteriosis percentages for each method of detection. The fish caught by haul seine net were generally smaller and younger than those caught by hook and line. The mean age of fish collected by haul seine net versus hook and line was 4.0 yrs \pm 1.1 (N = 65) and 7.4 yrs. \pm 3.1 (N = 53) respectively. Statistical analysis (paired sign test) of the data in Table 4 revealed that the molecular method, PCR/nested PCR, detected a significantly higher number of fish positive for mycobacteriosis than did the culture method ($p = .0118$) for fish caught by hook and line. In addition, there was an even greater difference between histology and PCR/nested PCR ($p < .0001$). In contrast, there was no significant difference between histology and quantitative bacteriology for the hook and line-caught fish ($p = .0923$). For net-caught fish, there was a significant difference between bacteriology and histology ($p = .0023$), between PCR/nested PCR and histology ($p = .0386$), but no difference between quantitative bacteriology and PCR/nested PCR ($p = .3323$).

Table 5 shows a two-way comparison of detection methods, contrasting both positive and negative cases/percentages of mycobacteriosis for each possible pair of methods. Examination of this table shows, for example, that 66 fish were both positive for nested PCR and quantitative culture, whereas 22 fish were positive by nested PCR, but negative by quantitative culture. To complete the story of this specific two-way comparison, 15 fish were negative by nested PCR, but positive by quantitative bacteriology and 15 fish were negative by both methods. Table 6 also shows two-way comparisons between histology and quantitative bacteriology and between histology and nested PCR. Statistical analysis (nonparametric paired sign test) revealed no significant difference between quantitative bacteriology (culture) and nested PCR for the entire sample (N = 118), but very significant differences between quantitative bacteriology and histology ($p = .0003$) and also between nested PCR and histology ($p = <.0001$).

The prevalence of splenic mycobacteriosis based on the various age categories of fish, the mean of weight, length, and log CFU g-1, and the positive percentage for each method of detection is described in Table 6. Despite some slight trends, the only unequivocal conclusion is that fish that were 4 - 4.9 years of age had the highest rate of mycobacteriosis for all methods of detection, particularly for the histology method. 74% of striped bass in this age category were positive by histology, 95% were positive by quantitative bacteriology, and 86% were positive for nested PCR. In the youngest category (0 - 2.9 years of age), only 6% were positive by histology, in contrast to 44% being positive by nested PCR.

Taking essentially the same data (combining the two youngest age categories into one), Figure 12 shows histograms of the positive percentages of mycobacteriosis in each age category for each method of detection. The conclusions stated above for Table 6 are pictorially more obvious.

DISCUSSION:

The histological method of mycobacteriosis detection, based on observing granulomas in splenic tissue, was the least sensitive of the three methods. This finding, along with previous reports describing difficulties in detection of acid-fast bacilli in tissue indicates that histological approaches underestimate the occurrence of mycobacteriosis. Development of granulomas requires a variable length of time depending upon both the specific *Mycobacterium* spp. involved and the degree of immune reactivity of the host. Since the spleen has been reported as a common visceral organ infected by mycobacteria and it is a significant component of the host's immune system, it was reasonable to histologically examine this tissue. Mycobacteria can be present without the presence of granulomas in splenic tissue either because it is too early in the infection or because such granulomas in older, chronic infections have resolved (due to healing). In either of these cases, however, mycobacteria could be detected by culture and/or molecular methods. Thus, there undoubtedly can be false negative results for the histological method. On the other hand, reliance on granuloma detection as evidence for mycobacteriosis can also lead to false positive results, since granulomas can be caused by organisms that are not mycobacteria, i.e. due to parasitic worms. The main advantage of histological examinations is the speed of potential detection, requiring a minimum of three-four days from harvest of fish tissue to histological examination. In addition, our research documented that when fish splenic tissues did have detectable granulomas, those same fish had 100-fold higher densities of mycobacterial culture densities than did fish without granulomas. Thus, detection of granulomas in splenic tissue may be associated with more severe, active mycobacterial infections in striped bass. Overall, 52% of all samples in this study (N = 61/118) were histologically positive for the presence of granulomas. The rate for this project is very similar to the overall 48% positive rate for a previously conducted, larger VMRC-sponsored project (N = 1899) (Vogelbein et al., 2001).

Quantitative cultures of mycobacteria from splenic tissue facilitated determination of the concentration of infection, characterization of cultural isolates, and validation of the molecular PCR/nested PCR method of detection. Overall, 69% of samples (N = 118) was positive for mycobacteriosis, utilizing quantitative bacteriology. Using nonparametric, statistical analyses, there was no difference between the detection methods of quantitative bacteriology and PCR/nested PCR for the whole sample (N = 118). Both of these methods, however, were more sensitive and detected more cases of mycobacteriosis than did the classical method of histology.

In the past two years, VIMS investigators have actually examined a total of 196 aseptically-collected spleens from wild striped bass. Seventy-five percent of such fish were culturally positive for mycobacteriosis (Kaattari et al., 2002; Rhodes et al., 2002). This rate matches the rate detected by PCR/nested PCR for the subset of samples in this project (N = 118).

The predominant isolate from this project was *M. shottsii*. Three other mycobacteria were isolated occasionally and phenotypically resembled *M. scrofulaceum*, *M. simiae*, and *M. interjectum*. The first two types have been reported in the literature on fish mycobacteriosis previously, but fish being infected with bacteria resembling *M. interjectum* is a new observation. The significance of this observation is unknown. *M. interjectum* is a potential human pathogen (Lumb et al., 1997; Emler et al., 1994; Springer et al., 1993). Mycobacteria resembling *M. scrofulaceum*, *M. simiae*, and *M. interjectum* from striped bass were also occasionally present as co-infections with *M. shottsii*. It was interesting that we did not isolate two of the three *Mycobacterium* spp. most frequently cited as major causes of fish

mycobacteriosis, *M. chelonae* and *M. fortuitum*. In addition, we only rarely isolated the third major reported agent of fish mycobacteriosis, *M. marinum*, from striped bass.

It may be speculated that quantitative bacteriology could slightly underestimate the true prevalence of mycobacteriosis in wild striped bass. Evidence of underestimation was shown by the significantly lower rates of detection for quantitative bacteriology, when compared to detection by PCR/nested PCR, when examining the larger, older striped bass caught by hook and line. Perhaps these bigger, older fish have survived because of their partial or complete resolution of the active mycobacterial infection. The mycobacteria in such fish may have been rendered nonviable, and thus would not be culturable. But it is probable that sufficient mycobacterial DNA might still be present to allow the PCR/nested PCR method to detect evidence of mycobacteriosis. Another potential cause of underestimation for quantitative bacteriology would be not providing an essential growth factor or condition for the *Mycobacterium* spp. For example, until our research team learned that a low incubation temperature, 23°C, was essential for detection of the predominant *M. shottsii*, we would have failed to detect this important *Mycobacterium* spp. The quite lengthy period, two-three months, required for *M. shottsii* to grow at 23°C, certainly is not conducive for rapid diagnoses of fish mycobacteriosis. Standard methods employed for studying *Mycobacterium* spp. that infect humans or other mammals (Levy-Frebault et al., 1992) cannot be relied upon to detect mycobacteriosis in fish. *Mycobacterium* spp. that affect fish are generally slow growing and exhibit diversity in their culturable and phenotypic traits. Diverse intra-specific phenotypes are being reported for one of the major agents of fish mycobacteriosis, *M. marinum* (Chemlal et al., 2002; Ucko et al., 2002). Traditional biochemical tests and diagnostic keys are insufficient to speciate many of the so-called “environmental” mycobacteria. Thus the need for an molecular alternative exists, PCR and nested PCR.

The specific PCR protocol for this study amplified a genus-specific 924-940 bp segment of the 16S rRNA gene. This gene segment includes a well-known hypervariable section of the 16S rRNA gene (Kox et al., 1995; Turenne et al., 2001) useful for speciating the mycobacteria. When the predominant isolate from striped bass known now as *M. shottsii* was initially being characterized, such genetic sequencing revealed that *M. shottsii* was equally homologous and most closely related to *M. marinum* and *M. ulcerans*. Later, the nearly complete 16S rRNA gene sequence of *M. shottsii* (1494 bp, GenBank AY005147) was determined. Phylogenetic analyses utilizing the entire gene maintained the same genetic relationship between *M. shottsii*, *M. marinum*, and *M. ulcerans* (Rhodes et al., 2002). *M. shottsii* was determined to be most closely homologous (99.2%) with both *M. marinum* and *M. ulcerans* (Rhodes et al., 2002) and *M. shottsii* was 98.7% homologous with *M. tuberculosis* and other *Mycobacterium* spp. in the *M. tuberculosis* complex (Fig. 12). The *M. tuberculosis* clade includes those mycobacteria in the *M. tuberculosis* complex plus *M. marinum*, *M. ulcerans*, and *M. shottsii*.

When the 300 bp product of PCR/nested PCR was sequenced, a well conserved segment of the 16S rRNA gene was revealed. This small segment, when compared to others in GenBank, was useful in confirming both the type of gene and the genus, *Mycobacterium*, but it was insufficient for speciation purposes.

PCR/nested PCR methods detected that 75% of the wild striped bass sampled for our project were positive for mycobacteriosis. This molecular method appears to have been validated by the similar rates of mycobacteriosis as detected by quantitative bacteriology. The gene being investigated, the 16S rRNA gene, is almost universally accepted as a well-conserved, acceptable target for mycobacterial detection and identification. But the possibility exists, particularly for an amplification of another amplification (nested PCR following PCR)

that errors might occur. To detect false positives, negative controls (no template) were employed in each set of PCR/nested PCR reactions. Are false negative results a possibility as well? Yes, particularly since this specific set of samples employed the spleen, an organ rich in blood, which is known to have many potent, natural inhibitors of PCR reactions (Chakravorty & Tyagi, 2001). Certainly there were examples shown in Table 5 (Two-Way Comparison of Detection Methods) where quantitative bacteriology was positive and yet PCR/nested PCR was negative. Despite such reservations, it appears that this project has shown that PCR/nested PCR is indeed a rapid, specific, and sensitive method for detecting mycobacteriosis in wild striped bass. An important detriment for molecular methods of detection, of course, is their high cost and reliance upon specialized equipment.

We can only speculate on the significance of such a high rate of infection in wild striped bass. It appears that the fish are being infected while less than 3 years, but the timepoint of initial infection is unknown. Mycobacterial infection in wild striped bass is a chronic disease that does not seem to inhibit overall growth and reproductive development of the fishes. Sexually mature striped bass were frequently found positive for mycobacteriosis, thus the possibility exists for vertical transmission (infection through infected eggs). Another potential source of infection is the water itself, which may transiently harbor mycobacteria. A likely source is infected fish that either shed mycobacteria indirectly into the environment or are directly preyed upon by striped bass. Mycobacteriosis is known to infect many species of fish, but since striped bass may be more susceptible to infection by mycobacteriosis (Wolf & Smith, 1999), they may be “sentinel” fish expressing a higher rate of infection than other fish of the Chesapeake Bay.

Much more research remains to be conducted. What is the source of mycobacteriosis in wild striped bass? Has this disease progressed beyond the Chesapeake Bay? Is this disease only occurring in striped bass in estuarine water? Does the disease occur in the freshwater contingent of striped bass? Are humans at risk for disease?

It has been rewarding to discover that the predominant mycobacterial agent in wild striped bass is a new mycobacterial species, *M. shottsii*, belonging to the *M. tuberculosis* clade. *M. shottsii* and striped bass may thus serve as a productive, useful model for a human disease of worldwide importance, tuberculosis. Actually, collaborative challenge studies, studying the pathogenicity of *M. shottsii*, are already being conducted at the National Fish Health Research Laboratory in West Virginia. Plans for a world-class, fish challenge facility at VIMS have also just been approved by voters of Virginia, so our team looks forward to being able to conduct such research at our own site.

SUMMARY:

Our project compared three methods of detection for mycobacteriosis in aseptically collected spleens of striped bass from the Chesapeake Bay. For the whole dataset (N = 118), quantitative bacteriology detected similar rates of infection as did PCR/nested PCR. Both of these methods were more sensitive than the histological method of detection. A significant difference between these two methods was determined to exist between fish caught by hook and line (N = 53) from those caught in a haul seine net (N = 65). PCR/nested PCR may have been detecting mycobacteria that were no longer viable in the older, larger fish caught by hook and line.

All locations sampled in Virginian parts of the Chesapeake Bay had similar rates of mycobacteriosis. Since our dataset was collected during a short period, from June, 2001, to December, 2001, we can make no conclusions as to seasonal trends in infection rates. PCR/nested PCR detected that 44% of striped bass three years of age or younger were already positive for mycobacteriosis, but the number of fish sampled in this age category was small (N = 16). The peak of positive detection for all three methods occurred in fish aged 4 to 4.9 years of age; a similar peak was observed in a previous histological examination of 1899 striped bass (Vogelbein et al., 2001).

Overall, 75% of fish sampled for this project examined were infected with mycobacteria, with the predominant isolate being *M. shottsii*, a new species belonging to the *M. tuberculosis* clade. The molecular method of detection, PCR/nested PCR, thus appears to have been successfully validated as a useful, rapid, and sensitive method of detection for mycobacteriosis in wild striped bass.

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APPENDIX

Table 1. Histology: Number of granulomas/spleen sample and frequency of occurrence for infected striped bass

| | <u>1</u> | <u>2-10</u> | <u>11-100</u> | <u>>100</u> |
|---------------------|----------|-------------|---------------|----------------|
| # Granulomas | | | | |
| # Fish | 13 | 25 | 19 | 4* |
| % Fish | 21% | 41% | 31% | 7% |

*Highest # of granulomas per spleen sample = 261

Although a minimum of 9 sections was examined for each spleen sample, a standardized method for histological examination was not performed, so these results should be considered a “relative distribution”.

Table 2. Bacteriology: Mycobacterial densities (colony forming units = CFU/gram of spleen) and frequency distribution for infected striped bass

| CFU g⁻¹ | % of infected fish |
|----------------------------------|---------------------------|
| 10 ¹ -10 ² | 8.6 |
| 10 ² -10 ³ | 17.3 |
| 10 ³ -10 ⁴ | 22.2 |
| 10 ⁴ -10 ⁵ | 14.8 |
| 10 ⁵ -10 ⁶ | 18.5 |
| >10 ⁶ | 18.5 |
| Total | 100 |

Table 3. Occurrence of mycobacteria in splenic tissue of striped bass (*Morone saxatilis*) collected from the Chesapeake Bay

| <u>Location^a</u> | <u>No. Samples</u> | <u>% of Fish Positive for Mycobacteriosis</u> | | |
|-----------------------------|--------------------|---|---------------------|-------------------|
| | | <u>Histology</u> | <u>Bacteriology</u> | <u>Nested PCR</u> |
| Mid Bay | | | | |
| Colonial Beach Tournament | 7 | 43 | 43 | 71 |
| Great Wicomico River | 1 | 0 | 0 | 0 |
| Reedville | 1 | 100 | 100 | 100 |
| Reedville Tournament | 15 | 53 | 67 | 73 |
| Total | 24 | 50 | 58 | 71 |
| Lower Bay | | | | |
| Deltaville Tournament | 21 | 33 | 43 | 86 |
| Lynnhaven Tournament | 6 | 33 | 83 | 83 |
| Piankatank River | 1 | 100 | 100 | 100 |
| Virginia Beach | 1 | 100 | 100 | 100 |
| York River | 65 | 58 | 78 | 71 |
| Total | 94 | 58 | 71 | 76 |
| Total | 118 ^b | 52 | 69 | 75 |

^aFish collected from specified rivers, sites, or fishing tournaments

^bActually 119 fish were collected, but one fish (MR 30) was excluded from this study due to unacceptable level of “contaminant” bacteria

**Table 4. Occurrence of mycobacteria in splenic tissue by type of capture:
hook and line or net of haul seine***

| <u>Source</u> | <u>Histology</u> | <u>Bacteriology</u> | <u>Nested PCR</u> |
|----------------------|--------------------------|----------------------------|--------------------------|
| Hook and line | 23/53 = 43% ^a | 30/53 = 57% ^a | 42/53 = 79% ^b |
| Net of haul seine | 38/65 = 58% ^a | 51/65 = 78% ^b | 46/65 = 71% ^b |

* Results which are significantly different ($p < 0.05$) between the three methods of detection are indicated by a different superscript.

Table 5. Two-way comparison of detection methods for all striped bass (N = 118)

| | Culture + (%) | Culture – (%) | Totals (%) |
|-------------------------|----------------------|----------------------|-------------------|
| Nested PCR + (%) | 66* (56) | 22 (19) | 88 (75) |
| Nested PCR – (%) | 15 (13) | 15 (13) | 30 (~25) |

| | Culture + (%) | Culture – (%) | Totals (%) |
|------------------------|----------------------|----------------------|-------------------|
| Histology + (%) | 56 (48) | 5 (4) | 61 (52) |
| Histology – (%) | 25 (21) | 32 (27) | 57 (48) |
| Totals (%) | 81 (69) | 37 (31) | 118 (100) |

| | Nested PCR + (%) | Nested PCR – (%) | Totals (%) |
|------------------------|-------------------------|-------------------------|-------------------|
| Histology + (%) | 57 (48) | 4 (3) | 61 (~52)** |
| Histology – (%) | 31 (26) | 26 (22) | 57 (48) |
| Totals (%) | 88 (~75)** | 30 (25) | 118 (100) |

* Number of samples

**Due to rounding off of %s, such totals are approximate.

Statistics: Nonparametric Paired Sign Test Results

| | |
|--------------------------|-------------------|
| Culture Vs. Nested PCR | P-Value = 0.3240 |
| Culture Vs. Histology | P-Value = 0.0003 |
| Nested PCR Vs. Histology | P-Value = <0.0001 |

Table 6. Prevalence of splenic mycobacteriosis in various age groups of striped bass

| <u>Age (yr)</u> | <u># Fish</u> | <u>Weight (g)</u> | <u>Length (mm)</u> | <u>Mean</u> | <u>Percent Positive</u> | | |
|-----------------|---------------|-------------------|--------------------|------------------|-------------------------|----------------|-----------------------|
| | | | | <u>Log CFU/g</u> | <u>Histology</u> | <u>Culture</u> | <u>PCR/Nested PCR</u> |
| 0-2.9 | 16 | 288 | 310 | 2.2 | 6 | 19 | 44 |
| 3-3.9 | 12 | 762 | 439 | 3.8 | 67 | 92 | 67 |
| 4-4.9 | 43 | 1242 | 504 | 4.3 | 74 | 95 | 86 |
| 5-5.9 | 13 | 1964 | 587 | 3.2 | 54 | 77 | 77 |
| ≥ 6 | 34 | 7415 | 882 | 2.3 | 38 | 47 | 76 |

Fish Collection Methods



Fig 1. Striped bass from the CCA Deltaville Tournament being placed in cart for transport to VIMS researchers' vehicle. A total of 49 wild striped bass were obtained from four tournaments and an additional 4 striped bass were collected from individual fishermen.



Fig. 2. This photograph shows a typical haul seine net similar to the one used to collect 65 wild striped bass from the York River.



Fig. 3. This wild striped bass shows the typical shallow, hemorrhagic dermal lesions associated with mycobacterial infection. Lesions may also be subtle, with only darkly pigmented, pinpoint spots. Most wild striped bass examined in our study had no exterior lesions.



Fig. 4. Large wild striped bass (minus tail to fit into VIMS mobile lab's laminar flow hood) being prepared for aseptic collection of its spleen. This fish was collected at the Reedville Tournament, the only tournament at which the mobile laboratory was utilized. Our hood at VIMS was large enough for two fish to be simultaneously necropsied, if required.

A.



B.

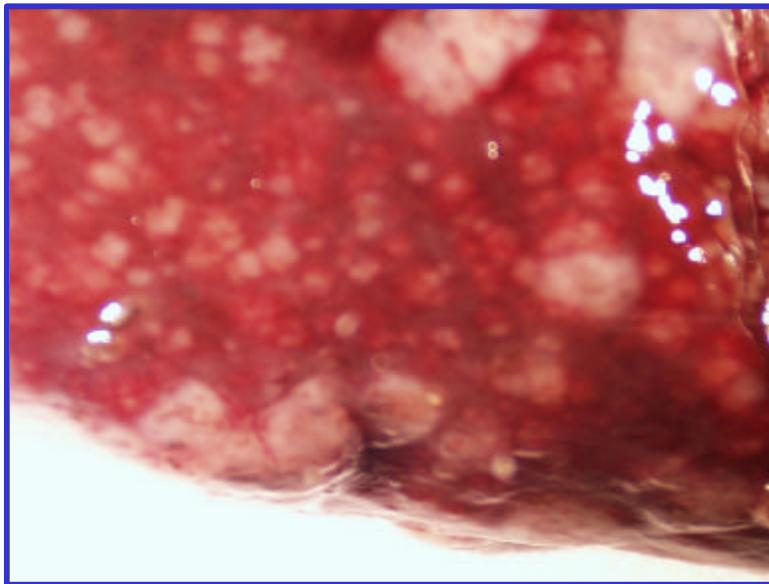


Fig. 5. A. Top photograph shows a section of fish spleen stained by H&E stain, with five, mature granulomatous lesions.
B. The bottom photograph shows a close-up portion of a fish spleen with numerous, macroscopically visible granulomatous lesions due to mycobacteriosis.

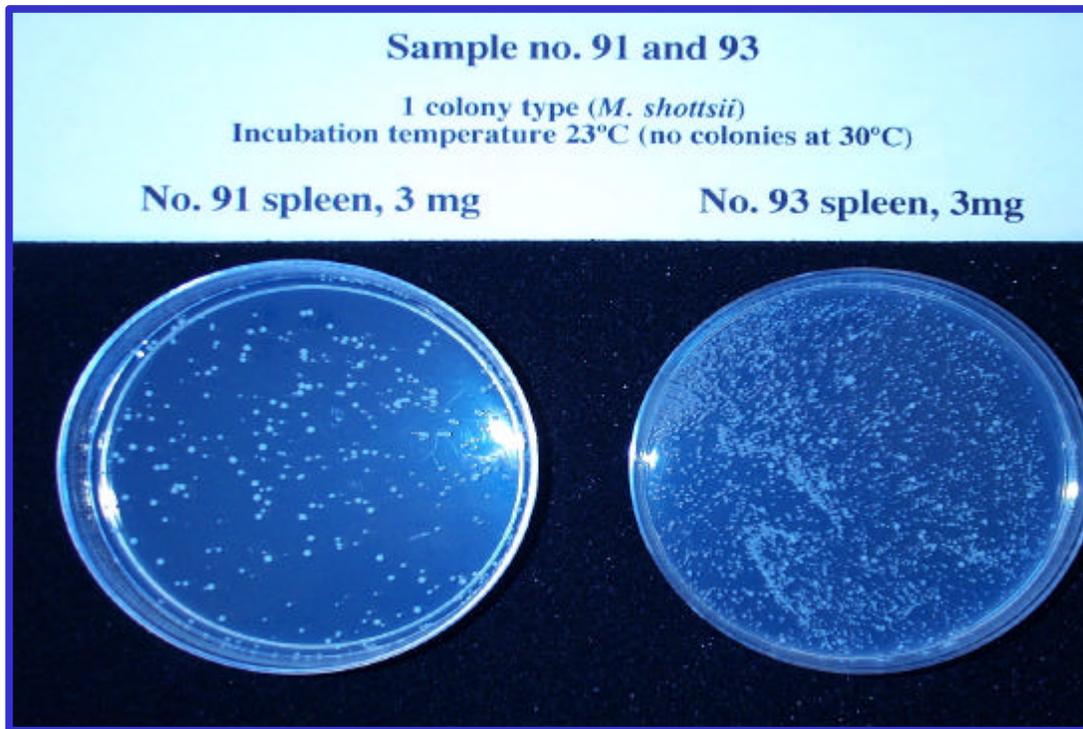


Fig. 6. Two quantitative microbiology (culture) plates of striped bass splenic tissue, incubated at 23°C; each with only *Mycobacterium shottsii* being isolated. Samples No. 91 and 93 each had $> 10^6$ CFU g⁻¹. Note that this isolate did not grow at 30°C (on duplicate, unshown plates.).



Fig. 7: Typical colony of *M. shottsii*, the predominant isolate from wild striped bass

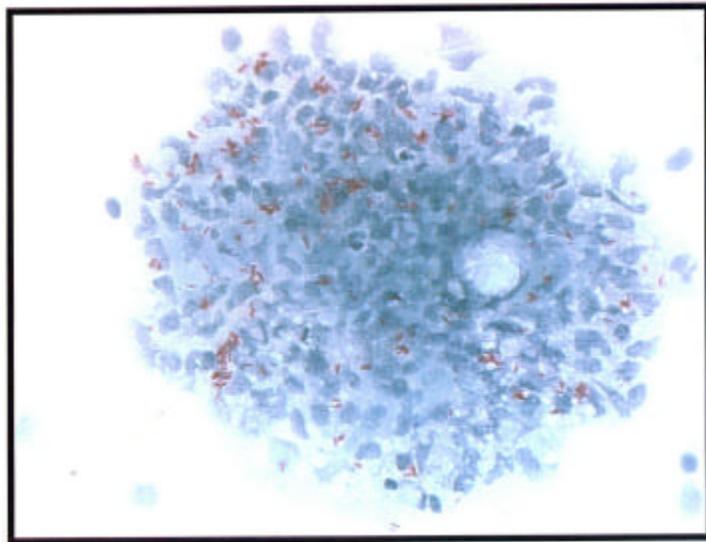


Fig. 8: Macrophage cell of wild striped bass containing numerous acid-fast (red-colored) *Mycobacterium* spp. (Ziehl-Neelsen stain)



Fig. 9. This wild striped bass (# MR122) was collected at the CCA Tournament. The exterior of the fish was healthy-appearing, but when the fish was surgically opened, the massive size and high number of granulomatous lesions in its spleen was obvious. *M. shottsii* was ultimately cultured from this spleen with a density of 150,000 CFU g⁻¹ and both histological and molecular methods were positive as well.

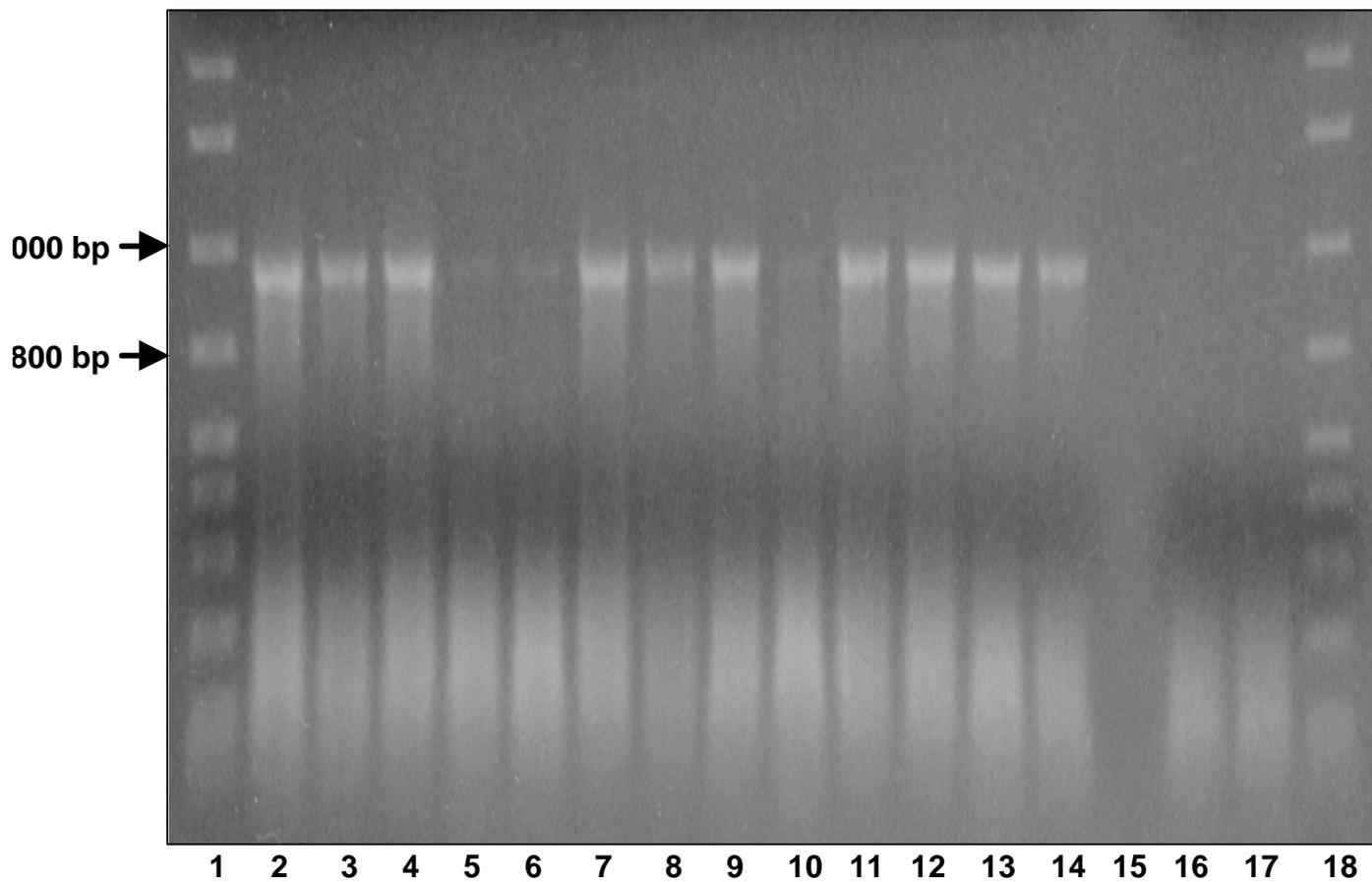


Fig. 10. Lanes 1 & 18: Molecular Weight Marker; 2: *M. flavescens*; 3: *M. gordonae*; 4: *M. interjectum*; 5: *M. marinum*; 6: *M. peregrinum*; 7: *M. scrofulaceum*; 8: *M. simiae*; 9: *M. szulgai*; 10: *M. terrae*; 11: *M. shottsii*; 12: *M. chelonae* (reference strain); 13: *M. fortuitum* (reference strain); 14: *M. marinum* (reference strain); 15: empty; 16: Negative control (no template); 17: Negative control (*Nocardia otitidiscaviarum*, ATCC 14629). *Mycobacterium* spp. in lanes 2-11 were isolated from fish and phenotypically resemble the listed bacteria. Reference strains in lanes 12-14 were obtained from the Virginia Department of Health and in lane 17 from the American Type Culture Collection (ATCC).

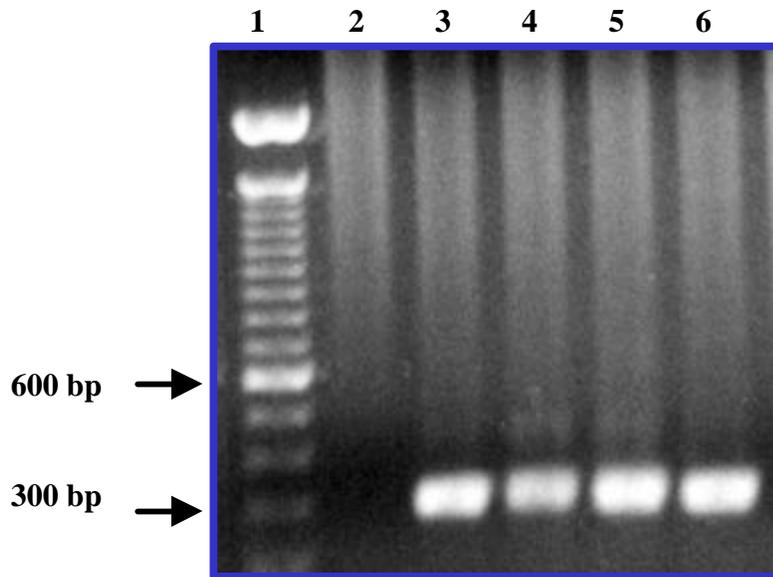


Fig. 11. Nested PCR reactions as visualized on 1% agarose gel, stained by ethidium bromide. Lane 1 = Molecular Weight Marker (100 bp ladder), Lane 2 = Negative control (no template DNA), Lane 3 = Positive control, Lane 4-6 = striped bass splenic DNA from MR 26, 27, & 28, respectively.

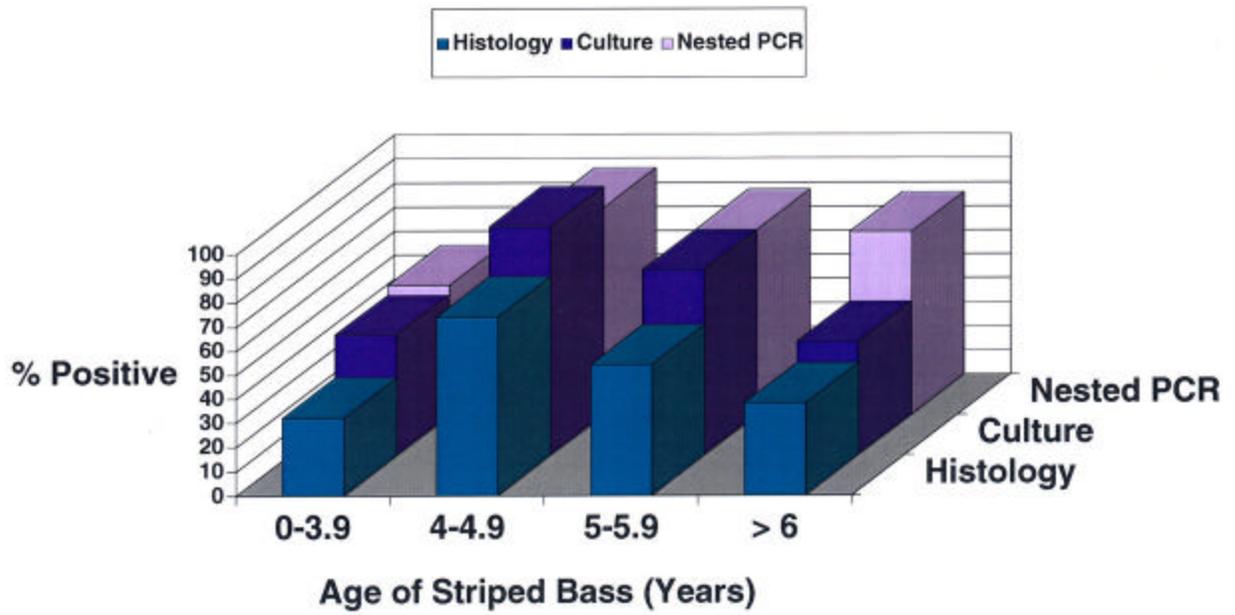


Fig. 12 Mycobacteriosis as detected by histology, culture, and nested PCR: Compared to age categories of striped bass.