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Biochemical, molecular, and virulence characteristics of select *Mycobacterium marinum* isolates in hybrid striped bass *Morone chrysops* × *M. saxatilis* and zebrafish *Danio rerio*

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ABSTRACT: A panel of 15 *Mycobacterium marinum* isolates was characterized by biochemical tests, sequencing the ribosomal DNA intergenic spacer (ITS) region and the heat shock protein 65 gene (*hsp65*) and pulsed-field gel electrophoresis (PFGE). The biochemical characteristics of all isolates were similar, except for Tween 80 hydrolysis. DNA sequence of *hsp65* for a subset of isolates were identical; however, at position 5 of the ITS rDNA, a single nucleotide polymorphism was identified. Isolates possessing a guanine residue at this position (G strains) were unable to hydrolyze Tween 80, while isolates that contained an adenine residue at this position (A strains) were positive for Tween 80 hydrolysis. PFGE successfully discriminated between the G and A strains; all G strains had identical *Ase*I restriction enzyme-cutting patterns while the A strains exhibited a variety of cutting patterns. Eight isolates (4 G and 4 A strains) were further characterized for virulence by experimental infection of hybrid striped bass (HSB) *Morone chrysops* × *M. saxatilis* and zebrafish *Danio rerio*. Seven of the 8 strains produced cumulative mortality ranging from 13.3 to 83.3% in the HSB virulence trial. The *M. marinum* reference strain ATCC 927^T did not produce mortality in HSB. HSB exposed to the G strains had significantly higher cumulative mortality than those exposed to the A strains. When these same isolates were tested in zebrafish, 6 of the 8 strains caused 100% cumulative mortality, with 2 of the A strains being the most pathogenic. In zebrafish, however, ATCC 927^T was virulent and produced 28.5% mortality. Collectively, we conclude that the *M. marinum* G strains are unique and may represent a distinct virulence phenotype in HSB, but this trend was not consistent in zebrafish.

KEY WORDS: *Mycobacterium marinum* · Hybrid striped bass · Zebrafish · Mycobacteriosis · Virulence

INTRODUCTION

Fish mycobacteriosis is a commonly diagnosed infectious disease in farmed, pet and research fish (Lansdell et al. 1993, Brocklebank et al. 2003, Broussard & Ennis 2006, Chang et al. 2006). The dramatic increase in the use of small fish species in research, particularly the zebrafish *Danio rerio*, has resulted in the recognition of fish mycobacteriosis as a concern to laboratory animal health management professionals (Astrofsky et al. 2000, Sanders & Swaim 2001, Kent et al. 2004, Matthews 2004, Watral & Kent 2006). The most common bacterial species associated with fish mycobacteriosis include *Mycobacterium chelonae*, *M. fortuitum*,

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M. abscessus and, less frequently, *M. marinum* (Teska et al. 1997, Chinabut 1999, Astrofsky et al. 2000, Kent et al. 2004). There is increasing evidence that while *M. marinum* is ubiquitous in freshwater and marine environments and can cause high mortality in captive fish, it does not appear to be common in zebrafish research facilities (Chinabut 1999, Astrofsky et al. 2000, Kent et al. 2004). Thus, efforts should be taken to avoid inadvertent introduction of this pathogen.

In wild striped bass *Morone saxatilis*, mycobacterial disease is also very common (Sakanari et al. 1983, Lansdell et al. 1993) and recent outbreaks in Chesapeake Bay striped bass populations have been associated with several species of *Mycobacterium* including *M. marinum* (Heckert et al. 2001, Rhodes et al. 2001, 2004, Vogelbein et al. 2001, Kaattari et al. 2005). The recreational and commercial importance of the striped bass fishery along the east coast of the USA has probably helped hybrid striped bass *Morone chrysops* × *Morone saxatilis* (HSB) aquaculture to become a rapidly growing sector of agriculture in the USA. At present, there is an annual HSB production of about 5 million kg with an approximate value of US $30 million (Carlberg et al. 2000). Approximately one-third of the HSB produced are reared in tanks in California, Florida and Pennsylvania; the remainder is reared extensively in ponds in North and South Carolina, Texas and Mississippi. With the rapid expansion of the HSB industry, disease control has been identified as one constraint to the future success of this industry (Carlberg et al. 2000).

Cultured striped bass and sea bass *Dicentrarchus labrax* are particularly sensitive to infections caused by *Mycobacterium marinum* (Hedrick et al. 1987, Colorni et al. 1998). This observation has been corroborated by laboratory studies that have shown striped bass to be more susceptible to infection by *M. marinum* than are tilapia (Wolf & Smith 1999). Furthermore, HSB reared in recirculating aquaculture systems seem to have increased susceptibility to Gram-positive bacterial infections (Stoffregen et al. 1996) including *Mycobacterium* sp. (Bowser et al. 2004). Systemic infection by *M. marinum* can produce severe disseminated disease in commercial HSB operations that, in the absence of approved products for the treatment or prevention of this disease, can cause widespread mortality and economic loss. Indeed, it has been estimated that *M. marinum* causes more than US $125 million in losses to aquaculture in the USA (Cirillo 1999). Future development of detection and control methods to contain the spread of *M. marinum* in USA aquaculture will require a better understanding of the epidemiology and virulence characteristics of this pathogen.

In this study we examine whether the virulence characteristics of *Mycobacterium marinum* isolates recovered from wild striped bass, intensively cultured HSB and tilapia, and zebrafish held in a research facility were associated with specific biochemical or molecular properties. Based on biochemical tests, sequencing ribosomal DNA (rDNA) and heat shock protein 65 gene (*hsp65*), pulsed-field gel electrophoresis (PFGE), and experimental pathogenicity studies using both HSB and zebrafish, our studies have identified a clone of *M. marinum* that can cause severe disease in HSB and, in some instances, zebrafish.

MATERIALS AND METHODS

Biochemical and genetic characterization. The source of the *Mycobacterium marinum* isolates used for this study is summarized in Table 1. The following biochemical tests were conducted as described by Kent & Kubica (1985): Tween 80 hydrolysis, NaCl tolerance, presence of arylsulfatase evaluated at either Day 3 or Day 14, presence of urease, niacin production, nitrate reduction, tellurite reduction, iron uptake, pyruvate, catalase and heat stable catalase.

Isolation of bacterial DNA was carried out using either the DNeasy Tissue Kit (QIAGEN) or the Mo Bio UltraClean™ Microbial DNA Isolation Kit (MO BIO Laboratories). Sequences were obtained by PCR and direct sequencing using conditions and primers as previously described (Ucko et al. 2002, Whipps et al. 2003, Poort et al. 2006). A fragment of approximately 500 bp was amplified using ITS primers 16SCF and 23S-23R, consisting of 250 nucleotides (nt) of ITS sequence and 250 nt of flanking SSU rDNA sequence. For *hsp65*, reverse-primer TB12 was used with either forward-primer TB11 or HS1F to generate a fragment approximately 400 or 550 bp, respectively. Sequencing primers were the same as those used for PCR.

PFGE was carried out following standard methods as guidelines (Philipp et al. 1998, Arbeit 1999, Holmes et al. 1999, Vanitha et al. 2003); cutting was conducted with restriction enzyme *Ase*I (New England Biolabs). Cultures were grown in 10 ml Middlebrook 7H9 supplemented with 0.05% Tween 80 at 30°C for 7 to 10 d. Optical density at 600 nm (OD_{600}) was determined; cells pelleted and then resuspended at 1 ml per 1 OD_{600} in TE+sucrose buffer (0.3 M sucrose, 25 mM Tris-HCl, 25 mM EDTA), i.e. a culture with an OD_{600} = 0.5 would be pelleted and suspended in 0.5 ml of buffer. Low-melting agarose plugs were prepared following manufacturer's instructions (Bio-Rad Laboratories). Plugs were placed in 2 ml of lysis solution (1 M NaCl, 100 mM EDTA, 0.5% Brij 58, 0.2% deoxycholate, 0.5% sodium dodecyl sulfate, 20 µg ml^{-1} RNase A, 5 mg ml^{-1} lysozyme) and incubated with gentle shaking overnight at 37°C. Plugs were placed in a new tube, rinsed

Table 1. *Mycobacterium marinum.* Isolates used for phenotypic, genetic and virulence trials in this study. +: strain was used for virulence testing (hybrid striped bass, HSB, or zebrafish) or was positive for Tween 80 hydrolysis; –: negative for Tween 80 hydrolysis. T = type strain; ND: not done; NA: not applicable; ITS: intergenic spacer, with G denoting base substitution with guanine and A denoting base substitution with adenine

Isolate	Fish species	Year of isolation	Host	Geographical region of USA	ITS	Tween 80	Virulence
KST687	HSB	1999	Farmed	Western	G	–	ND
KST94	HSB	2001	Farmed	Western	ND	–	ND
KST214	HSB	2002	Farmed	Western	G	–	+
KST417	HSB	2003	Farmed	Western	G	–	ND
KST458	HSB	2004	Farmed	Western	G	–	ND
KST266	Mozambique tilapia	2004	Farmed	Western	G	–	+
SH1	Zebrafish	2004	Farmed	Western	G	–	+
ATCC 927^T	Aquarium fish	1926	Unknown	Unknown	A	+	+
UCDavis	Striped bass	1987	Farmed	Western	A	+	+
VIMS	Striped bass	2001	Wild	Eastern	A	+	+
MSS1	HSB	1992	Farmed	Eastern	A	+	ND
MSS2	HSB	1997	Farmed	Eastern	A	+	ND
MSS3	HSB	1994	Farmed	Eastern	G	–	ND
MSS4	Human	1992	NA	Eastern	A	+	+
MSS5	HSB	1994	Farmed	Eastern	G	–	+

twice in ES buffer (0.5 M EDTA, 1% sodium dodecyl sulfate), and digested in 2 ml of ES buffer with 500 µg ml^{-1} of Proteinase K at 50°C overnight. Plugs were rinsed 4 times in 5 ml of TE buffer for 1 h at 37°C each, then rinsed in 1× restriction enzyme buffer for 1 h to remove excess EDTA. For each plug, 300 µl of restriction enzyme solution was prepared (1× buffer, 0.1 mg ml^{-1} BSA, 50 U *Ase*I), and incubated overnight at 37°C. A 1% gel was prepared and run following manufacturer's instructions and run on the Bio-Rad CHEF-DR® II PFGE System (Bio-Rad Laboratories) for 22 h at 14°C at 6 V cm^{-1}, with a linear ramp time of 1 to 20 s. The gel was stained in 0.1 µg ml^{-1} ethidium bromide for 30 min.

Virulence in HSB and zebrafish. Prior to beginning the *in vivo* virulence studies, each isolate was passed once through groups of 10 HSB fingerlings via an intraperitoneal (IP) injection with 0.1 ml of bacteria. Mortality was monitored for up to 28 d or until clinical disease was observed. From each tank, at least 3 representative moribund fish were selected, a necropsy performed and a spleen imprint was heat fixed and stained with the Kinyoun method (Hardy Diagnostics) to detect the presence of intracellular acid-fast bacilli. Upon confirmation of the presence of acid-fast bacteria, infected head kidney from each fish was aseptically streaked onto a Lowenstein Jensen slant (Gruft modification, LJ-G, Hardy Diagnostics) and incubated at 28°C for up to 4 wk until sufficient growth was obtained. If neither mortality nor clinical disease was observed during this period, all surviving fish were killed at the end of the experiment and the head kidney was cultured for the presence of *Mycobacterium marinum* as described. The axenic culture of yellow-pigmented colonies recovered from the head kidney was confirmed as *Mycobacterium* sp., based on cell morphology and acid-fast staining characteristics. For each strain, 2 to 3 bacterial colonies were aseptically transferred to 10 ml of Middlebrook 7H9 broth containing 1% albumin-dextrose-catalase (ADC, Hardy Diagnostics) and incubated for 72 h at 28°C on a rotary shaker (60 rpm). The cells were harvested by centrifugation, the pellet re-suspended in 1 ml of sterile distilled water containing 20% glycerol, divided into 100 µl aliquots and frozen in the vapor phase of liquid nitrogen. This stock preparation was used for the HSB and zebrafish *in vivo* exposure studies.

For the HSB infection challenge, all isolates were grown under biphasic conditions (i.e. a broth phase overlying a solid agar phase) to produce a sufficient quantity of cells for challenge. Frozen stock cultures of each isolate were removed from the vapor phase of liquid nitrogen and thawed at room temperature; a 10 µl aliquot was then streaked onto the surface of 50 ml tissue culture flask containing 12 ml of solidified LJ-G medium (Hardy Diagnostics). All flasks were incubated at 28°C for 3 to 4 wk until sufficient colony growth was obtained. Several colonies of each strain were removed and suspended in 10 ml of Middlebrook 7H9 broth containing 1% ADC and incubated for 48 h at 28°C on a rotary shaker (60 rpm). The broth culture was then aseptically transferred to another LJ-G flask and incubated horizontally under identical conditions for an additional 5 d. One day prior to challenge, the cells were harvested by centrifugation, the pellet resuspended in 3 ml of sterile distilled water, and the cell suspension held overnight at room temperature (24 to 26°C).

On the day of challenge, duplicate groups of HSB fingerlings (mean length and weight 14.6 cm and 40.0 g, respectively) were anesthetized with MS-222 (175 mg l^{-1} tricaine methanesulfonate, Argent) as per manufacturer's recommendations. The challenge isolate was diluted in sterile distilled water to OD_{600} of 0.7, giving a predicted dose of 1×10^7 bacteria and each fish received a 0.1 ml volume of a challenge isolate via an IP injection. Challenge dose was verified by colony counts using Columbia CNA agar supplemented with 5% sheep blood (CCNA, Hardy Diagnostics). Control HSB were treated in an identical manner except they received sterile distilled water. Following exposure, fish were returned to their respective holding tanks (in duplicate) for recovery from anesthesia. Each tank received single-pass, freshwater geothermal well water (constant 26 ± 1°C) and supplemental oxygenation. Each morning, water temperature and dissolved oxygen were measured and fish were fed a commercial trout ration (SilverCup) ad libitum. Morbidity and mortality were monitored twice daily for a total of 8 wk.

Wild-type zebrafish (AB strain, approximately 8 mo old) were obtained from the Zebrafish International Resource Center (ZIRC) at the University of Oregon. Groups of 20 fish were placed in static aquaria containing dechlorinated tap water that was maintained at 28°C. Ammonia and nitrate concentrations in the static aquaria were monitored with commercial test kits and were controlled by periodic water changes. In addition, each aquarium was equipped with a biological filter made from a box-type aquarium filter inside the tanks, which were filled with porous lava rock.

Each bacterial inoculum was prepared by removing colonies from the LJ-G slants, suspending them in 7H9 broth containing 1% ADC and 0.1% Tween 80 to prevent bacterial clumping and incubating them at 28°C for 5 d on a shaker. The bacterial concentration of each inoculum was determined using the McFarland Equivalence Turbidity Standards (Remel) and diluted to a predicted value of 2×10^6 bacteria ml^{-1}. For experimental infection, the zebrafish were anesthetized with MS-222 (130 mg l^{-1}) and each fish received a 25 µl IP injection (target dose of 5×10^4 bacteria per fish) using a 26-gauge needle attached to an Eppendorf Combitip placed on a repeat pipetter. Verification of injection dose was determined by colony counts using CCNA. All plates were incubated at 29°C for 10 d before enumeration. Data on strains KST214 and SH1 have been published previously (as OSU214 and SH1 respectively, Watral & Kent 2006), but were included here to evaluate their virulence in HSB. Twice daily, fish were fed a commercial ration (Tetramin, Tetra Holding) and monitored for evidence of morbidity and mortality for a total of 8 wk.

Histopathology. At 8 wk post exposure (PE), all surviving HSB were counted and a full necropsy conducted to visually assess the degree and extent of disease. Five fish were arbitrarily selected from each tank, euthanized, and a portion of each fish's liver, head kidney and spleen was placed in 10% buffered formalin. The following day the tissues from each fish were pooled, trimmed into the same cassette and processed for routine histopathology, after which sections were stained with haematoxylin and eosin (H&E) and Ziehl-Neelson acid-fast stain.

Most groups of zebrafish had 100% mortality by 8 wk PE; thus, histology samples were collected earlier. Five fish were collected from each tank (i.e. 10 fish per group) 10 d PE for all groups except SH1, VIMS and UCDavis. These groups had close to 100% mortality by 10 d PE, and thus histology was conducted on moribund fish in these groups between 5 and 10 d PE. Between 3 to 5 fish per tank were examined from these groups. Some fish exposed to ATCC 927^T and MSS4 survived until the termination of the experiment (8 wk PE), and thus 5 fish per tank were examined at this time for these groups. Fish were preserved whole in Deitrich's fixative, trimmed along the mid-sagittal plane and placed in a cassette. For decalcification prior to histological processing, the tissues were transferred to Cal-Ex (Fisher Scientific) for 48 h, thoroughly rinsed, transferred to 50% ethanol, processed, sectioned, and stained with H&E or Kinyoun's acid-fast.

Splenic mycobacterial quantification. At 10 d PE, 5 HSB from each tank were arbitrarily removed, euthanized by an MS-222 overdose (400 mg l^{-1}) and a piece of the spleen was excised and placed into a pre-weighed vial containing 1 ml sterile distilled water. The tissue was weighed to the nearest 0.1 mg, homogenized (Tissue Tearor, Biospec Products) and serially diluted to 10^{-6} to quantify the splenic bacterial load (expressed as colony-forming units per gram of splenic tissue, CFU g^{-1}). Duplicate 20 µl aliquots of the splenic homogenate were streaked onto CCNA and incubated at 28°C for 14 d until colony growth was sufficient to allow visual enumeration.

The splenic mycobacterial load was also quantified from the first 5 moribund HSB encountered from each tank during the 8 wk PE period. Moribund fish were euthanized in MS-222, a portion of the spleen placed into a pre-weighed tube, processed for bacteriological culture and duplicate aliquots streaked onto CCNA and enumerated as described. To confirm infection in the absence of clinical disease in tanks where no mortality was recorded, all fish were euthanized at the completion of the experiment, the abdomen aseptically opened and a 1 µl inoculation loop was inserted into the spleen, streaked onto CCNA and incubated as described to detect the presence of mycobacteria.

For zebrafish, the liver and spleen was removed from dead or moribund fish, placed in 100 µl of phosphate buffered saline (PBS) and 100 µl of a 1% CPC (cetylpyridinium chloride) solution was added. The tissues were homogenized and the samples were incubated overnight at room temperature for decontamination. The following day samples were rinsed twice with PBS, resuspended in 100 µl of PBS, streaked onto Mitchison 7H11 (Becton Dickinson) or CCNA plates and incubated at 29°C for 10 to 14 d, at which time bacterial growth was recorded.

Statistical analysis. The proportion of fish that died in each tank was determined by dividing the number that died by the number in the tank at the start of the study (minus those that were removed at Day 10). For each species, the proportion of fish that died in the different treatment groups was compared for statistical significance controlling for the tank effect using logistic regression. All statistical analyses were performed with Minitab 14.20 (MINITAB®). The Hosmer-Lemeshow statistic was used to evaluate the model's goodness-of fit. Pairwise comparisons of the number of fish that died were made between the G clone and A clone strains, and between strains within these 2 clones using a chi-square test with Yates' correction for continuity or Fisher's exact test when there were zeros in cells. p-values of less than 0.05 were considered significant.

Time to 50% mortality (in d) for zebrafish exposed to the different strains of *Mycobacterium marinum* was compared using a Kruskal-Wallis statistic. Individual comparisons between isolates were done using the Mann-Whitney *U*-test and the p-values were adjusted using a Bonferoni correction (the p-value was multiplied by the number of comparisons n = 7).

RESULTS

Biochemical characterization

Biochemical tests showed one consistent reaction that correlated to the 2 different strains. All the A strains were positive for Tween 80 hydrolysis, while the G strains were negative for this characteristic (Table 1). Aside from Tween 80 hydrolysis, the biochemical characteristics of all the isolates were very similar and showed the following profile: growth in NaCl was negative to weak positive; arylsulfatase was negative after 3 d but weak positive to positive after 14 d of incubation. The presence of urease was positive among all isolates while niacin production, nitrate and tellurite reduction, and pyruvate were negative. Catalase activity was positive for all isolates examined, but heat-stable catalase was negative. Iron uptake was considered inconclusive because all isolates gave a very weak positive reaction.

Molecular characterization

All *Mycobacterium marinum hsp65* sequences were identical to one another and to that of the *M. marinum* type strain ATCC 927^T (GenBank accession no. AF456470). However, at position 5 of the ITS rDNA, a single nucleotide polymorphism was identified (Table 1). Isolates possessing a guanine residue at this position were designated G strains and included all the HSB isolates from California (KST687, KST94, KST214, KST417, KST458), the isolates from the southeast USA (MSS3, MSS5), the zebrafish (SH1) obtained from an aquarium fish breeder and wholesaler, and the tilapia isolate (KST266) from California. The remaining isolates possessed an adenine residue at this same position and thus were designated as A strains (Table 1). These included the HSB isolates from the southeast USA (MSS1, MSS2), the striped bass isolate from California (UCDavis) and the wild striped bass (VIMS), the human *M. marinum* isolate (MSS4), and the *M. marinum* reference strain (ATCC 927^T). To define these isolates more precisely, PFGE was employed. All G strains had identical *Ase*I restriction enzyme-cutting patterns; however, the A strains exhibited a variety of cutting patterns (Fig. 1). Furthermore, PFGE was performed on 13 additional G strain isolates recovered from HSB over a 6 yr period and 5 additional G strains from zebrafish. All isolates had identical *Ase*I restriction enzyme-cutting patterns (data not shown). The A strains MSS1 from HSB and MSS4 from a human working at the same site where MSS1 was isolated showed

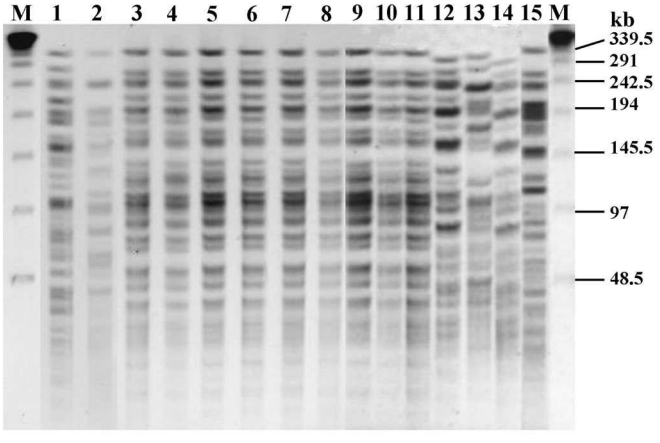

Fig. 1. *Mycobacterium marinum*. Pulse-field electrophoresis analysis of genomic DNA digested with *Ase*I (composite image). Lanes: M = lambda ladder, 1 = ATCC927^T, 2 = VIMS, 3 = KST687, 4 = KST94, 5 = KST214, 6 = KST417, 7 = KST458, 8 = KST266, 9 = SH1, 10 = MSS4, 11 = MSS5, 12 = MSS1, 13 = MSS2, 14 = MSS4, 15 = UCDavis. Fragment size of lambda ladder given in kb. Those designated G strains (Lanes 3–11) produced identical cutting patterns. MSS1 from hybrid striped bass (HSB) (Lane 12) and MSS4 from a human at the same site (Lane 14) showed the same cutting patterns

similar banding patterns (Fig. 1). Otherwise, all other A strains had different banding patterns and, thus, were different from each other.

Virulence studies in hybrid striped bass and zebrafish

At 8 wk PE, 7 of the 8 *Mycobacterium marinum* isolates used in this study produced mortality in HSB, with cumulative mortality ranging from 13.3 to 83.3% (Fig. 2). Mortality was not observed in control HSB or HSB that were injected with ATCC 927^T, the *M. marinum* type strain. In contrast, all *M. marinum* isolates produced mortality in zebrafish, with cumulative mortality ranging from 7 to 100% (Fig. 2). In zebrafish, 6 of the 8 isolates produced 100% mortality, 2 of which killed all fish by 4 wk PE. In contrast to HSB, however, the *M. marinum* isolate ATCC 927^T produced 28.5% mortality in zebrafish.

For comparative analysis of *Mycobacterium marinum* virulence in HSB and zebrafish, each isolate was ranked based on the cumulative % mortality for HSB and time (in d) to 50% mortality for zebrafish (Table 2). In HSB, isolate KST214 ranked first (highest mortality), while ATCC 927 was ranked eighth (no mortality). For zebrafish, the VIMS isolate was ranked first and MSS4 was ranked eighth in virulence. All strains but ATCC 927^T and MSS4 caused 100% mortality in both replicate tanks of zebrafish by 8 wk PE, with the VIMS strain demonstrating the shortest time to 50% mortality (Table 2).

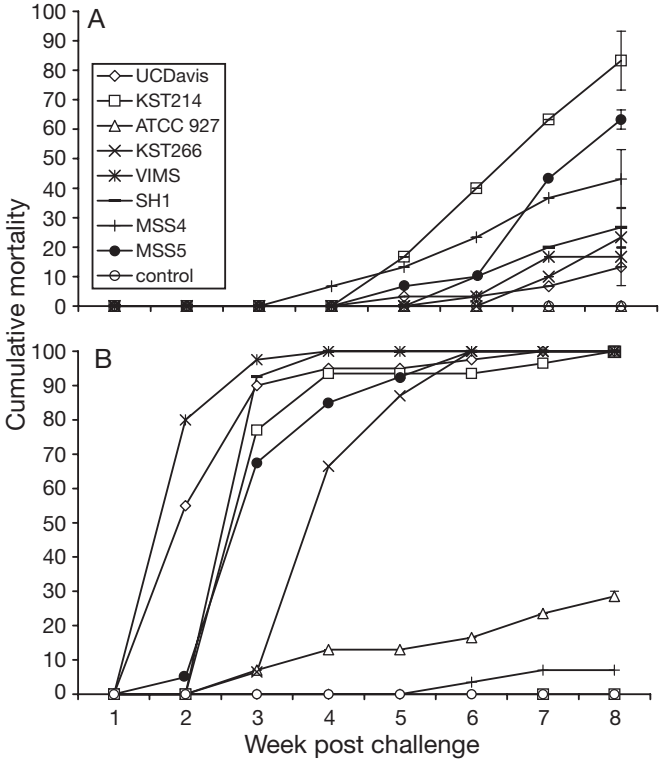

Fig. 2. *Morone chrysops* × *Morone saxatilis* and *Danio rerio*. Cumulative mortality (mean ± SD) of (A) hybrid striped bass or (B) zebrafish at 8 wk post exposure to various *Mycobacterium marinum* isolates

In the HSB, 3 of the 8 strains of mycobacteria tested in this study gave an 8 wk cumulative mortality greater than 40% (Table 2). The cumulative mortality ranged from 0% in the HSB exposed to ATCC 927^T to 83% in those exposed to the KST214 isolate. There was no significant difference in fish mortality (Z) between tanks exposed to the same strain of mycobacteria (Z = 0.16, p = 0.87); however, there was a significant difference between fish exposed to different strains (G_8 = 93.29, p < 0.001). The Hosmer-Lemeshow statistic indicated the model fit the data adequately (χ^2 = 4.43, p = 0.729).

HSB exposed to G strain isolates KST214 and MSS5 had a significantly higher cumulative mortality than fish exposed to the A strains (ATCC 927^T, VIMS, UCDavis, MSS4) (χ^2 = 24.15, p < 0.001). Cumulative mortality in HSB exposed to KST214 and MSS5 were not significantly different (χ^2 = 2.13, p = 0.144). HSB exposed to isolate KST266 had similar mortality to HSB exposed to SH1 (χ^2 < 0.001, p = 1.0); however, these fish had significantly lower mortality than the KST214, MSS5, or MSS4 isolates (χ^2 = 26.14, p < 0.001). The mortality rate of HSB exposed to the VIMS and UCDavis isolates were similar (χ^2 < 0.001, p = 1.0); however, the cumulative mortality experienced in HSB exposed to these 2 strains was significantly greater than fish exposed to ATCC 927^T (Fisher's exact test, p < 0.027) and significantly less than mortality caused by the MSS4 isolate (χ^2 = 7.23, p = 0.007). Furthermore, mortality rates of HSB exposed to strain MSS4 and ATCC 927^T were significantly different (χ^2 = 14.14, p = 0.002).

In the zebrafish virulence trial, exposure to 6 of the 8 isolates (KST214, VIMS, SH1, KST266, UCDavis, and MSS5) resulted in 100% mortality (Fig. 2). In contrast to the HSB infection experiment, the most virulent *Mycobacterium marinum* isolates in zebrafish were the A strains. Exposure of zebrafish to MSS4 resulted in 6.7% cumulative mortality, while exposure to strain ATCC 927^T resulted in 28.5% cumulative mortality over the 8 wk trial (Fig. 2). No tank effect was detected (Z = 0.35, p = 0.727) and the Hosmer-Lemeshow statistic indicated the model fit the data adequately (χ^2 = 0.0289, p = 1.00). There was a significant difference in zebrafish mortality depending on the isolate to which they were exposed (G_7 = 205.2, p < 0.001). Individual comparisons in zebrafish suggested 3 distinct groups based on time (d) to 50% mortality (Table 2); all isolates that caused 100% mortality (KST214, VIMS, SH1,

Table 2. *Mycobacterium marinum* infecting *Morone chrysops* × *Morone saxatilis* and *Danio rerio*. Ranking of *Mycobacterium marinum* isolates (intergenetic spacer, base substitution in brackets) 8 wk post exposure, based on cumulative mortality % (hybrid striped bass) or time (in d) to 50% mortality (zebrafish). Within each fish species, isolates with common superscripts indicates no significant difference in cumulative mortality ($p > 0.05$) based on Fisher's exact test or Yates' correction for continuity comparisons. Values in brackets following cumulative % mortality or time to 50% mortality denote mortality % in each replicate tank. NA: not applicable due to no mortality in control group (HSB or zebrafish), or 50% mortality not reached (zebrafish)

Hybrid striped bass				Zebrafish			
Isolate	Inoculum ($\times 10^4$ CFU)	Rank	Cumulative mortality (%)	Isolate	Inoculum ($\times 10^4$ CFU)	Rank	Time to 50% mortality (d)
KST214 (G)[a]	2500	1	83.3 (73, 93)	VIMS (A)[a]	0.9	1	5.5 (5,6)
MSS5 (G)[a]	1800	2	63.3 (67, 60)	UCDavis (A)[a]	1.3	2	6.5 (6,7)
MSS4 (A)[b]	750	3	43.3 (33, 53)	SH1 (G)[a]	3.4	3	9.5 (9,10)
SH1 (G)[c]	1100	4	26.7 (33, 20)	MSS5 (G)[a]	1.8	4	10.5 (10,11)
KST266 (G)[c]	4500	5	23.3 (27, 20)	KST214 (G)[a]	7.5	5	13.5 (13,14)
VIMS (A)[d]	250	6	16.7 (20, 13)	KST266 (G)[a]	7.5	6	21.5 (19,24)
UCDavis (A)[d]	260	7	13.3 (20, 7)	ATCC 927 (A)[b]	1.2	7	NA
ATCC 927 (A)[e]	630	8	0.0 (0, 0)	MSS4 (A)[b,c]	1.5	8	NA
Control[e]	0	NA	0.0 (0, 0)	Control[c]	0	NA	NA

KST266, UCDavis, and MSS5) had significantly higher mortality than the control group (Fisher's exact test, $p < 0.001$). Zebrafish exposed to strain ATCC 927^T had a significantly higher mortality rate than fish exposed to the MSS4 isolate ($\chi^2 = 4.01$, $p = 0.045$) or the control group ($\chi^2 = 8.37$, $p = 0.004$), but the mortality rate of fish exposed to MSS4 was not significantly different from the control group ($\chi^2 = 0.52$, $p = 0.47$).

For zebrafish, time to 50% mortality ranged from 5.5 (VIMS) to 21.5 d (KST266) while 2 isolates (ATCC 927^T, MSS4) failed to induce 50% mortality (Table 2). There was a statistically significant overall difference in the time to first mortality (in d) between the zebrafish exposed to *Mycobacterium marinum* and the unexposed controls (Kruskal-Wallis $H = 18.53$, $p = 0.018$); however, individual comparisons between *M. marinum* isolates were not statistically significant (data not shown).

Histopathology

A high prevalence of infection was evident in all HSB exposed to *Mycobacterium marinum* (Table 3) and moderate-to-severe mycobacteriosis was apparent in all fish infected with *M. marinum* at the completion of the experiment (56 d PE). The head kidney and spleen were the most severely affected organs, with less severe histopathological changes observed in the liver. Similarly, there did not appear to be any bacterial isolate-specific histopathological changes evident throughout the chronic inflammation of the visceral organs, except that acid-fast bacteria outside of granulomas were frequently observed in HSB exposed to KST214 (Fig. 3). In this instance, large regions of chronic inflammation surrounded numerous, discrete granulomas. Upon gross examination, *M. marinum* ATCC 927^T also produced numerous granulomas that were seen in large numbers during histopathological examination of infected tissues at 56 d PE (Fig. 3). Visceral granulomas and/or granulomatous inflammation were not observed in the control HSB.

All groups of zebrafish showed a high prevalence of infection (Table 3), and displayed 3 general patterns of visceral lesions (Fig. 3). The most common pattern of visceral lesions in zebrafish exposed to the less virulent strains (e.g. ATCC 927^T and MSS4) was a mixture of well-organized granulomas with epithelioid cells surrounding the periphery and multifocal aggregates of less organized macrophages. These occurred throughout the visceral organs, ovaries and kidney. For ATCC 927^T, 9 of 10 fish at 10 d PE were positive, 6 showing numerous granulomas and 3 with diffuse chronic peritonitis. Nine of 10 surviving zebrafish from the ATCC group exhibited numerous, well-organized granulomas throughout the viscera and kidney at 56 d PE. Similar lesions were found in zebrafish exposed to MSS4, with 3 of 9 fish positive at 10 d PE and 4 of 10 fish positive at 8 wk PE.

All moribund zebrafish or those collected at 10 d PE showed lesions consistent with severe mycobacteriosis caused by the more virulent isolates (i.e. the G strains, VIMS and UCDavis). The predominant histological change was severe, diffuse, chronic peritonitis, with chronic inflammation extending into the spleen, liver, and kidney. These fish also exhibited granulomas, but many were poorly organized. All 10 moribund zebrafish that were examined from the VIMS strain group and 2 of 8 of the UCDavis group exhibited severe, acute necrosis of mesenteries, pancreas and spleen, in addition to diffuse chronic inflammation in these organs. No acid-fast bacteria were detected in

Table 3. *Mycobacterium marinum* infecting *Danio rerio* and *Morone chrysops* × *Morone saxatilis.* Prevalence in zebrafish (by histology and culture) and hybrid striped bass (HSB, histology only) examined at 10 or 56 d post exposure (PE) or mortalities (Mort., no. of fish positive for *M. marinum*/no. of fish examined) after exposure to *M. marinum* isolates. All moribund HSB or those HSB selected at 10 d PE for splenic mycobacterial quantification were positive for *M. marinum* (see Table 4). HSB mortalities were not cultured. NA: not applicable

Isolate	Tank	Zebrafish						Hybrid striped bass
		Histology			Culture			Histology
		Mort.	10 d PE	56 d PE	Mort.	10 d PE	56 d PE	56 d PE
SH1	A	4/4	NA	NA	2/2	NA	NA	2/2
SH1	B	3/3	NA	NA	2/2	NA	NA	2/2
KST214	A	4/4	5/5	NA	2/2	5/5	NA	2/2
KST214	B	4/4	5/5	NA	2/2	5/5	NA	1/1
ATCC 927	A	NA	4/5	5/5	NA	4/4	1/2	2/2
ATTC 927	B	NA	5/5	4/5	NA	5/5	2/2	2/2
VIMS	A	4/4	NA	NA	7/7	NA	NA	2/2
VIMS	B	4/4	NA	NA	6/6	NA	NA	2/2
UCDavis	A	4/4	NA	NA	1/2	NA	NA	2/2
UCDavis	B	4/4	NA	NA	2/2	NA	NA	2/2
KST266	A	3/3	1/1	NA	2/2	5/5	NA	2/2
KST266	B	1/1	1/1	NA	2/2	5/5	NA	2/2
MSS4	A	NA	1/5	3/5	NA	5/5	1/2	2/2
MSS4	B	NA	2/4	1/5	NA	3/5	2/2	2/2
MSS5	A	2/2	4/4	NA	2/2	NA	NA	2/2
MSS5	B	2/2	5/5	NA	2/2	NA	NA	2/2
Control	A	NA	NA	0/10	NA	0/5	0/2	0/2
Control	B	NA	NA	0/10	NA	0/5	0/2	0/2

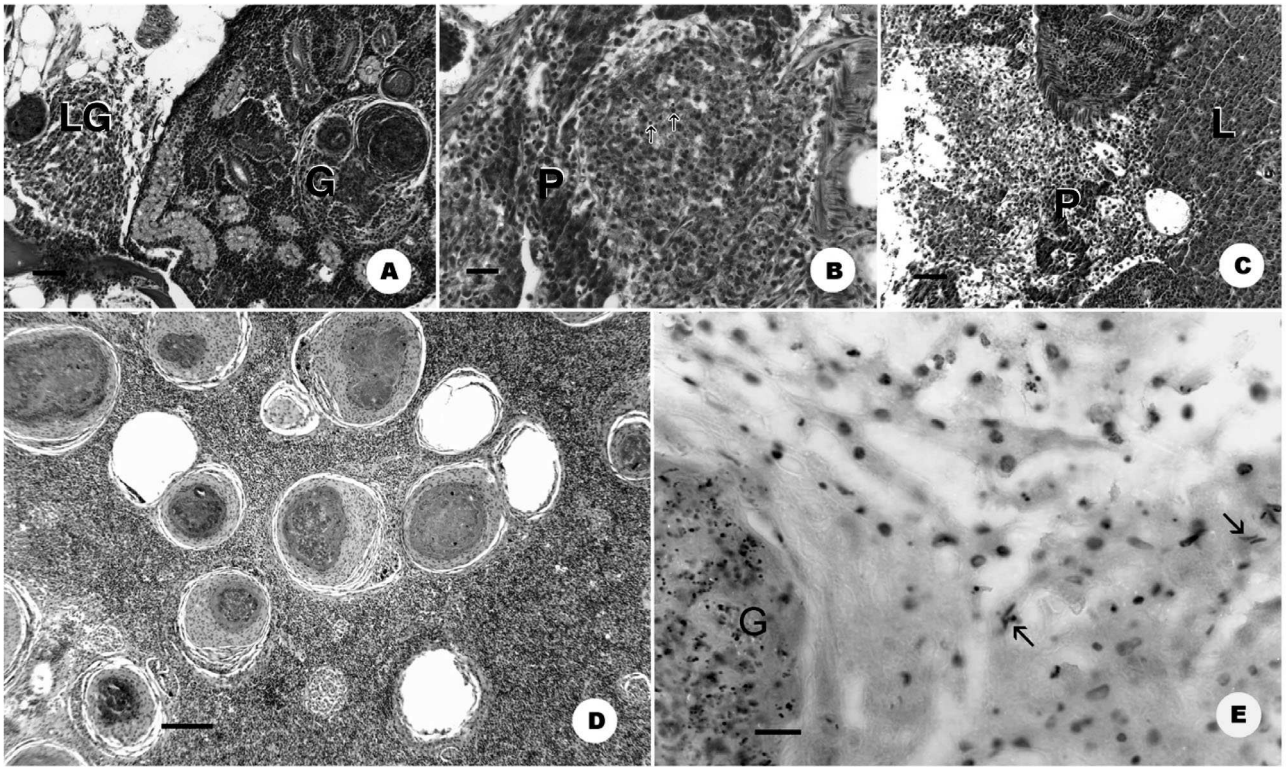

Fig. 3. *Mycobacterium marinum* infecting *Danio rerio* and *Morone chrysops* × *M. saxatilis.* Histological sections of zebrafish (A–C) and hybrid striped bass (D, E) infected with *Mycobacterium marinum* and stained with hematoxylin and eosin (H&E) (scale bar = 100 µm except E). (A) Well-organized granuloma in kidney and loose granuloma in mesenteries with ATCC 927^T infection (B) Infection with VIMS. Necrosis (arrows) in chronic inflammation in mesenteries. (C) Diffuse, chronic inflammation in mesenteries. (D) Multiple, well-organized granulomas in spleen. (E) High magnification of spleen showing bacteria (arrows) outside of granuloma (acid-fast, scale bar = 10 µm). G: granuloma, LG: loose granuloma, P: pancreas, L: liver

lesions, and it was determined subsequently that the Cal-Ex decalcification used for zebrafish histology interferes with the staining of bacteria (Kent et al. 2006a).

Splenic mycobacterial quantification in HSB

Based on a standard challenge dose with OD_{600} of 0.70, all experimentally infected HSB received between 0.25 and 4.5×10^7 CFU in a 0.1 ml injection dose volume (Table 2). At 10 d PE, all HSB were considered clinically healthy and displayed an aggressive feed response and the splenic mycobacterial load had increased roughly one order of magnitude and ranged from 0.59 to 28.7×10^8 CFU g^{-1} (Table 4). Over roughly the next 7 wk, moribund fish that were arbitrarily selected and removed for bacteriology displayed clinical symptoms of lethargy and anorexia, and contained approximately 10-fold greater CFU g^{-1} of spleen tissue, with counts ranging from 14.8 to 111.0×10^8 CFU g^{-1} (Table 4). HSB that were injected with ATCC 927^T did not experience mortality, but 33.3% of the fish were culture-positive for this pathogen at the completion of the trial. There were no mycobacteria recovered from the spleen tissue of the control fish at any of the sampling periods.

Mycobacterium marinum was isolated from virtually all zebrafish PE and was cultured in high numbers from all moribund fish (Table 3).

DISCUSSION

This research has shown that isolates of *Mycobacterium marinum* recovered from disease outbreaks affecting wild striped bass, intensively cultured HSB, tilapia, zebrafish and humans are capable of causing disease in both zebrafish and HSB. Our study also showed that the particularly virulent strain, the G strain, can be identified genetically by a single base substitution in the ITS region of the rDNA and that this substitution is associated with a phenotypic trait involving the inability to hydrolyze Tween 80. We have also determined that PFGE can help identify this particularly virulent strain of *M. marinum*, which has been recovered from severe outbreaks of disease in commercially reared HSB and zebrafish in different regions of the USA. While this research has shown that 3 of 4 *M. marinum* G strain isolates were highly virulent in HSB, this was not consistently the case, especially in zebrafish.

Mycobacterium marinum is commonly recovered from infections of cultured and wild striped bass and their hybrids (Sakanari et al. 1983, Hedrick et al. 1987, Heckert et al. 2001, Overton et al. 2003, Rhodes et al. 2004, Kaattari et al. 2005). Mycobacteriosis is one of the most common diseases of zebrafish held in research facilities, but *M. marinum* does not seem to be a common mycobacterial pathogen (Astrofsky et al. 2000, Kent et al. 2004, Watral & Kent 2006). There are few reports on host susceptibility in fish mycobacteriosis, but a growing number of experimental transmission studies indicate that zebrafish, striped bass and their hybrids, and several other freshwater and marine fish species are all highly susceptible to infection from *M. marinum*. Swaim et al. (2006) demonstrated that adult zebrafish are extremely susceptible to infection by *M. marinum*, and IP injections of as few as 5 bacteria caused persistent infections. Recently, Broussard & Ennis (2006) found that zebrafish were about 10-fold more susceptible to infection than were medaka. Similarly, Wolf & Smith (1999) showed that striped bass were more susceptible to *M. marinum* infections than were tilapia, and Colorni et al. (1998) reported that sea bass were very susceptible to infection by *M. marinum*. Using the same panel of challenge isolates, our studies have shown that zebrafish seem to be even more susceptible to infections than HSB with the *M. marinum* A strain isolates.

The disease we observed in zebrafish appeared to be more severe than that in HSB. Indeed, 6 of the 8 *M. marinum* isolates we tested caused 100% mortality in zebrafish but none of these isolates produced total mortality in HSB. The HSB weighed about 100 times more than the zebrafish, but the zebrafish were injected with about 200 times fewer bacteria (5×10^4 vs.

Table 4. *Mycobacterium marinum* infecting *Morone chrysops* × *Morone saxatilis*. Splenic colonization (expressed as mean colony forming units per gram, CFU g^{-1}, and SD in brackets) after experimental infection with each *Mycobacterium marinum* isolate at 10 d post exposure (PE) or in moribund fish throughout the 8 wk study. NA: not applicable as no mortality observed

Isolate	Mean CFU g^{-1} spleen ($\times 10^8$)			
	10 d PE	n	Moribund fish	n
KST214	28.7 (20.3)	10	111.0 (42)	10
MSS5	20.9 (15.4)	9	38.2 (24.5)	10
MSS4	5.7 (5.4)	10	23.2 (17.3)	10
SH1	13.1 (6.6)	10	34.2 (19.8)	3
KST266	8.3 (10.2)	10	47.5 (31.8)	5
VIMS	1.7 (1.6)	10	34.4 (33.1)	5
UCDavis	1.2 (1.1)	5	14.8 (6.7)	4
ATCC 927^T	0.59 (0.27)	9	NA	NA
Control	0.0	10	NA	NA

1×10^7 CFU). Of note, there seems to be an association between inoculum dose and cumulative mortality in HSB exposed to the *M. marinum* strains examined in this study. The challenge inoculum for the HSB virulence trial was standardized by optical density using a 7 d biphasic culture and viable dose was confirmed by colony counts. This association may reflect differences in *in vitro* growth characteristics of the specific isolates tested in this study. Nevertheless, regression analysis (Pearson's correlation coefficient of 0.35) failed to detect a significant relationship between inoculum dose and % cumulative mortality in HSB (data not shown).

In spite of the obvious differences in mortality between HSB and zebrafish, there were some consistencies between virulence of the various strains between these hosts. For example, the *Mycobacterium marinum* type strain ATCC 927^T was less virulent than all the other isolates, except for MSS4 in zebrafish (Table 2). Because ATCC 927^T was isolated roughly 80 yr ago, it is possible that it has lost some of its virulence factors over the numerous *in vitro* passages — although to help account for this, we did pass all isolates through HSB before the virulence trials began. Using our genotypic and phenotypic typing methods, however, the reduced virulence of *M. marinum* ATCC 927^T isolate in the HSB challenge model may also be explained by the fact that this isolate belonged to the A strain group, and not the more virulent G strain group, although this trend was less clear with the zebrafish challenge model under the experimental conditions employed.

The *Mycobacterium marinum* type culture ATCC 927^T has been used for disease research in a variety of fish species and there seems to be a marked difference in the challenge dose required to produce disease in different species of fish. At 15 d post injection, Pasnik & Smith (2005) produced total mortality in juvenile HSB that received an intramuscular injection of 8×10^5 bacteria g^{-1} body weight (average body weight of 40 to 50 g). Wolf & Smith (1999) reported high mortalities in juvenile striped bass with 1.6×10^6 CFU g^{-1} body weight (average body weight 4.8 g) of *M. marinum* ATCC 927^T, but the same dose and route of delivery failed to produce clinical or overt disease or mortality in tilapia (average body weight 6.3 g). Conversely, 4.5×10^8 CFU were required to produce 50% mortality in an acute goldfish infection model (Talaat et al. 1998). Despite the obvious differences in challenge dose, host- and age-related susceptibility, and culture conditions used to propagate the pathogen, it is likely that differences in infection route (intramusculature versus intraperitoneal) may also influence the onset of mortality and total mortality, although there are very few reports of these aspects with respect to *M. marinum* virulence studies. As we report here, ATCC 927^T was much less virulent in our zebrafish challenge model and failed to produce mortality in HSB, despite the use of a similar dose to that reported by Pasnik & Smith (2005). This raises the question as to whether ATCC 927^T is the best candidate for *M. marinum* virulence studies.

Van der Sar et al. (2004) reported that isolates of *Mycobacterium marinum* from humans were more pathogenic to zebrafish than were isolates recovered from fishes. This is contrary to our findings, which demonstrated that when tested in zebrafish, the human *M. marinum* isolate (MSS4) was the least pathogenic of the *M. marinum* isolates we examined. Previously, Kent et al. (2006b) found that fish isolates displayed improved growth, and survival in mice and human macrophages, compared to a single human isolate. Because of the limited numbers of fish and isolates used in these studies, we are unable to conclude whether there are significant virulence differences between human and fish isolates of *M. marinum* at this time.

Differences in histological changes between strains based on virulence agreed with previous transmission studies on zebrafish. Chronic diffuse peritonitis, rather than formation of well-organized granulomata, is often the primary histological change in zebrafish during severe outbreaks of mycobacteriosis (Kent et al. 2004, Watral & Kent 2006). In our study, the more virulent isolates (e.g. G strains, VIMS, and UCDavis) showed mostly diffuse chronic inflammation of the visceral organs rather than extensive granuloma formation. In addition, the VIMS strain showed prominent acute necrosis, consistent with the most virulent strains in the study by van der Sar et al. (2004). Our observations were consistent with those of Swaim et al. (2006), in which infections that produced higher mortality were associated with more diffuse inflammatory lesions. Prouty et al. (2003) reported that the most prominent histological change in zebrafish infected with ATCC 927^T was granuloma formation, and this is consistent with our findings regarding the least-virulent strains in zebrafish (i.e. MSS4 and ATCC 927^T). The histological presentation seen in HSB was similar to that reported in striped bass from either natural (Hedrick et al. 1987) or experimental infections (Wolf & Smith 1999, Gauthier et al. 2003), being characterized by numerous granulomas in various internal organs. Bacteria free of granulomas but within macrophages appeared most common in fish infected with KST214, which probably reflects that this isolate was the most virulent of those tested in HSB.

We have found DNA sequence-based identification to be particularly useful for delineation of piscine *Mycobacterium* species (Whipps et al. 2003, Kent et al. 2004, Poort et al. 2006). The *hsp65* gene appears to be quite reliable for species identification and may differ-

entiate between species that 16S rDNA sequence analysis cannot (Ringuet et al. 1999, Kim et al. 2005, McNabb et al. 2006). Even more variable sequences, such as the ITS, are used for *Mycobacterium* species identification and may be useful for differentiating between strains of some species (Roth et al. 1998, 2000). Here, the *M. marinum* isolates examined possessed identical *hsp65* sequences, yet in the ITS region, a single nucleotide polymorphism was noted that correlated well to PFGE types. All isolates designated as G strains had identical PFGE cutting patterns and therefore represent a widely distributed clone of *M. marinum* that is virulent to fish. Representatives included numerous clinical *M. marinum* isolates that were collected from intensively cultured HSB and tilapia from one facility in California over a 6 yr period and an isolate that infected HSB from the southeast USA (MSS5). Disparate isolates of this G strain clone produced significant differences in the degree of pathogenicity in the HSB virulence studies. Likewise, different isolates of the G strain clone produced differences in HSB splenic bacterial counts that generally correlated with virulence ranking and were greater than counts produced by the A strain isolates. Moribund HSB at 8 wk PE had correspondingly higher splenic counts than at 10 d PE, following the virulence ranking results of cumulative mortality. There were no significant differences in the G strain isolates when examined in the zebrafish virulence studies. However, zebrafish seemed more susceptible to disease and mortality when compared to HSB, necessitating a measurement of time to 50% mortality in the zebrafish compared to cumulative mortality over 8 wk with the HSB. These virulence study results suggest that there is a rich diversity of pathogenicity among the G strain clone isolates in HSB and between HSB and zebrafish. The differences in virulence examined in this study may represent various degrees of host/mycobacterial adaptation occurring over time and are most likely due to factors not addressed in this study. Surprisingly, the G strain isolate recovered from zebrafish (SH1) originated from an aquarium fish wholesale facility that had previously reared white bass *Morone chrysops*, suggesting that the G strain may have been inadvertently introduced with the white bass. PFGE analysis indicated that 2 A strains, 1 from HSB (MSS1) and 1 from a human (MSS4) working at this facility, were identical. To our knowledge, this is the first demonstration of infection in humans and fish by the same clone of *M. marinum*.

There is an increasing amount of evidence that suggests the potential for mycobacterial clonality may exist, based on amplified fragment length polymorphism analysis (van der Sar et al. 2004, Ucko & Colorni 2005) and (in the present study) the use of PFGE restriction enzyme-cutting patterns, *hsp65* gene sequences and rDNA ITS-sequence analysis. Considering that the strain or clone of *Mycobacterium marinum* we have identified possesses high virulence for HSB in laboratory experiments and has been recovered from (and continues to be recovered from) severe outbreaks of mycobacteriosis in aquacultured HSB from different regions of the USA, this information could be used to develop screening methods to detect carrier animals, determine the origin of the highly pathogenic clone and thus avoid the inadvertent introduction and spread of this pathogen throughout aquaculture in the USA.

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