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Distribution and genetic characterization of *Mycobacterium chelonae* in laboratory zebrafish *Danio rerio*

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ABSTRACT: During routine screening of zebrafish at a research facility, histological changes consistent with mycobacteriosis were observed, prompting an investigation to determine the background prevalence and distribution of *Mycobacterium* species throughout the facility. Infection status was evaluated in 240 zebrafish representing 9 genetic lines, using histology, culture and PCR. Environmental sources were also tested for the presence of mycobacteria. Prevalence in zebrafish by culture and PCR was 10% (24/240), 21 of which were TU line fish. All isolates from fish were identified as *M. chelonae* by *hsp65* DNA sequencing; subsequent DNA fingerprinting delineated 3 strains, designated H1E1 (1/24), H1E2 (22/24), and H1E3 (1/24). From external sources, tank or tubing surface biofilms were positive by culture (13/32) with multiple species and strains isolated including *M. neoaurum*, *M. phocaicum,* and identical strains of *M. chelonae* that were found in zebrafish: H1E1 (2/13) and H1E2 (8/13). Comparing diagnostic methods, acid-fast stained histological sections showed substantial agreement with plate culture and PCR for detection of mycobacteria in fish. Observation of granulomas in hematoxylin and eosin-stained sections was a less reliable predictor of mycobacteriosis, as uninfected females with egg-associated inflammation and hyperplasia were misdiagnosed. These data revealed background levels of mycobacteriosis in a healthy and well-managed facility. Infected populations were removed, although the apparent ability for *M. chelonae* to remain viable in environmental reservoirs may make it difficult to eradicate completely. This highlights the importance of an animal-health monitoring program and good husbandry practices to prevent disease in zebrafish research laboratories.

KEY WORDS: *Mycobacterium chelonae* · Zebrafish · *Danio rerio* · Biofilms · Enterobacterial repetitive intergenic consensus PCR · ERIC-PCR · Randomly amplified polymorphic DNA · RAPD

INTRODUCTION

Mycobacteriosis is recognized as a significant disease problem in zebrafish research facilities (Astrofsky et al. 2000, Kent et al. 2004, Whipps et al. 2007a). Findings range from overt disease outbreaks with high mortality to incidental findings where few fish show signs of disease. Several species of *Mycobacterium* have been identified, and experimental exposures indicate dramatic differences between species and strains (Watral & Kent 2007). Some *M. marinum* strains cause more disease than others (Ostland et al. 2008), and even modest exposures to *M. haemophilum* causes overt disease and mortality (Whipps et al. 2007a). We have also isolated *M. chelonae*, *M. abscessus*, and *M. peregrinum* from zebrafish during disease outbreaks (Kent et al. 2004), but little disease or mortality occurred when fish were exposed experimentally (Watral & Kent 2007). Thus, although some species of mycobacteria cause outright disease, other species

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infect with low virulence and the opportunistic manifestation of disease may be a result of suboptimal environmental conditions.

Even in the absence of mortalities, chronic underlying infections are a threat to the efficacy of results derived from experiments that employ zebrafish. As with any research animal (Baker 2003), it is imperative that disease be minimized. Acute diseases causing severe morbidity and mortality in zebrafish have been reported (Kent et al. 2004, Whipps et al. 2007a), but little is known about the distribution and prevalence of sub-clinical infections. Good husbandry practices likely minimize the potential spread of pathogens in a system, and when standard practices include a health monitoring program, infected stocks can be identified early and isolated or removed. Case in point, in January 2006, during routine monitoring at a zebrafish research laboratory, a few fish exhibiting lesions consistent with mycobacteriosis were identified and the infection was confirmed by histology. This prompted the removal of the stock and further evaluation of the presence of background levels of *Mycobacterium* spp. infections throughout the facility.

MATERIALS AND METHODS

Facility. All fish that were introduced into the facility were derived from eggs disinfected with chlorine as described by Westerfield (2000). The facility has a traditional configuration with multiple tanks receiving recirculated water from a filtration system in which nitrogenous wastes were removed with large sand-based biological filters; then water was disinfected by ultraviolet sterilization. Water was held at 28 to 28.5°C, pH 6.8 to 7.2, conductivity 500 µS. Fish were fed twice daily, once with brine shrimp nauplii and once with a commercial flake diet mix.

Survey. Between January and April 2006, 240 zebrafish representing 9 genetic lines (TU, TL, *mitfa*, *sparse*, *cct2*, *brg1*, *Tg*(*hsp70l:Gal4*)*1.5kca4*, WIK, and AB) were tested for mycobacterial infections by histology, culture and PCR (Table 1). Individuals were euthanized with an overdose of tricane methanesulfonate (Argent Laboratories) and examined for clinical signs of mycobacteriosis, i.e. presence of dermal lesions, emaciation and raised scales. The external surface was then cleaned with ethanol and a laboratory tissue in preparation for necropsy. For each fish, the peritoneal cavity was opened along the lateral line with sterile instruments, being careful to avoid contact with visceral organs. At this point, the presence of any visible granulomas was noted. Using another set of sterile instruments, a portion of the spleen and liver were removed and placed in a tube containing 100 µl 1% cetyl pyridinium chloride (CPC) for culture. An additional piece of spleen or liver was removed and frozen at –20°C for PCR. The remainder of the zebrafish was placed in Dietrich's fixative for histological processing.

Environmental sources. Food supply, tank surfaces, tubing, water reservoirs, and sources outside of the building were examined for the presence of mycobacteria (Table 2). Water was collected in 2 ml tubes, contents pelleted at 10 000 × *g* for 10 min, supernatant carefully removed, and pellets treated overnight in 100 µl 1% CPC prior to culture. Surfaces were tested using a sterile swab, which was placed directly in 200 µl of 1% CPC overnight and processed for culture.

Culture and histology. All samples treated with 1% CPC overnight were processed for culture following Kent & Kubica (1985). Cultures were grown on Middlebrook (MB) 7H10 biplates and Lowenstein-Jensen growth medium (LJ), incubated at 29°C, and monitored for growth for up to 8 wk. Rapid fungal overgrowth in water samples collected from outside of the facility required a second attempt at culture on MB 7H10, supplemented with 0.25 µg ml^{-1} amphotericin B.

For histology, fish were preserved whole in Dietrich's fixative. For decalcification prior to histological processing, the tissues were transferred to trifluoroacetic acid (5%) for 24 h. Fish were then processed using standard methods and stained with hematoxylin and eosin (H&E), Ziehl-Neelsen's acid-fast, or Kinyoun's acid-fast. Multiple sagittal sections were prepared from each fish, allowing visual evaluation of essentially all visceral organs. Agreement between methods was evaluated using Cohen's unweighted Kappa (Cohen 1960), and categorized by the standards proposed by Landis & Koch (1977) as follows: Kappa coefficient K: ≤0 = poor, 0.01 to 0.2 = slight, 0.21 to 0.4 = fair, 0.41 to 0.6 = moderate, 0.61 to 0.8 = substantial, and 0.81 to 1 = almost perfect.

Molecular. Genomic DNA from biofilms and spleen or liver tissue was extracted using the DNeasy Tissue Kit (QIAGEN) following pretreatment with 20 mg ml^{-1} lysozyme (Sigma) for 1 h at 37°C. For cultures, DNA was extracted from 3 individual colonies grown in 7H9 broth, using the UltraClean microbial DNA isolation kit (MoBio Laboratories). For extracted tissues and cultures, PCR was used to amplify heat shock protein 65 (*hsp65*) genes using conditions and primers as described previously (Kent et al. 2004, Poort et al. 2006, Whipps et al. 2007b). Following gel electrophoresis, PCR products were gel-extracted using the QIAquick Gel Extraction Kit (QIAGEN). Direct sequencing was carried out using amplification primers with the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1, using the ABI PRISM® 3730 DNA Analyzer (Applied Biosystems). Enterobacterial repetitive intergenic consensus (ERIC) PCR was performed following the methods of Sampaio et al. (2006). Additional ran-

Table 1. *Danio rerio* examined for *Mycobacterium* spp. infection with percent positive for histology, culture and PCR combined (Positive by any test). Number of male (M) and female (F) fish for each sample is indicated. Results from histological sections stained with hematoxylin and eosin are indicated: GRAN, granulomas; EAIF, egg-associated inflammation and fibroplasia; AERO, aerocystitis. Results from acid-fast stained sections are: AFB, acid-fast bacilli observed; SB AFB, acid-fast bacilli on the swim bladder wall. Mycobacteria (Myco ID) were identified by *hsp65* gene sequencing, and strains of *M. chelonae* (Mc strain) were further characterized by enterobacterial repetitive intergenic consensus (ERIC) and randomly amplified polymorphic DNA (RAPD) PCR. na: not applicable

Date (d/mo/yr)	Line	Positive by any test (%)	Fish no.	M, F	GRAN	EAIF	AERO	AFB	SB AFB	Culture	Tissue PCR	Myco ID	Mc strain
13/1/06	TU	25 (5/20)		13,7						2/20			
			ZF-23	M	+	na	+	+	+	+	+	*chelonae*	H1E2
			ZF-24	M	+	na	+	+	+	Neg	Neg	na	na
			ZF-26	F	Neg	Neg	Neg	Neg	Neg	+	+	*chelonae*	H1E2
			ZF-37	F	+	+	Neg	Neg	Neg	Neg	Neg	na	na
			ZF-39	M	+	na	+	+	+	Neg	Neg	na	na
31/1/06	TU	35 (7/20)		16,4						5/20			
			ZF-63	M	+	na	+	+	+	+	+	*chelonae*	H1E2
			ZF-70	F	+	+	Neg	Neg	Neg	Neg	Neg	na	na
			ZF-71	F	+	+	Neg	Neg	Neg	Neg	Neg	na	na
			ZF-72	M	+	na	+	+	+	+	+	*chelonae*	H1E2
			ZF-75	M	+	na	+	+	+	+	+	*chelonae*	H1E2
			ZF-79	M	+	na	+	+	+	+	+	*chelonae*	H1E2
			ZF-80	M	+	na	+	+	+	+	+	*chelonae*	H1E2
31/1/06	TU	20 (4/20)		16,4						3/20			
			ZF-45	F	+	+	Neg	Neg	Neg	Neg	Neg	na	na
			ZF-48	M	Neg	na	Neg	Neg	Neg	+	+	*chelonae*	H1E2
			ZF-55	F	Neg	Neg	Neg	Neg	Neg	+	+	*chelonae*	H1E1
			ZF-60	F	+	+	Neg	+	Neg	+	+	*chelonae*	H1E2
07/3/06	TL	5 (1/20)		7,13						1/20			
			ZF-84	M	Neg	na	Neg	Neg	Neg	+	+	*chelonae*	H1E2
13/3/06	*mitfa*	0 (0/20)		14,6						0/20			
13/3/06	*kita*	10 (2/20)		7,13						1/20			
			ZF-126	F	+	+	Neg	+	Neg	Neg	Neg	na	na
			ZF-128	F	+	+	Neg	+	Neg	+	+	*chelonae*	H1E2
21/3/06	*cct2*	0 (0/20)		15,5						0/20			
21/3/06	TU	55 (11/20)		20,0						11/20			
			ZF-163	M	+	na	+	+	+	+	+	*chelonae*	H1E2
			ZF-164	M	+	na	Neg	+	Neg	+	+	*chelonae*	H1E2
			ZF-167	M	+	na	+	+	+	+	+	*chelonae*	H1E2
			ZF-170	M	+	na	+	+	+	+	+	*chelonae*	H1E2
			ZF-173	M	+	na	+	+	+	+	+	*chelonae*	H1E2
			ZF-175	M	+	na	+	+	+	+	+	*chelonae*	H1E2
			ZF-176	M	Neg	na	Neg	Neg	Neg	+	+	*chelonae*	H1E3
			ZF-177	M	+	na	+	+	+	+	+	*chelonae*	H1E2
			ZF-178	M	+	na	+	+	+	+	+	*chelonae*	H1E2
			ZF-179	M	+	na	+	+	+	+	+	*chelonae*	H1E2
			ZF-180	M	+	na	Neg	+	+	+	+	*chelonae*	H1E2
04/4/06	*brg1*	10 (2/20)		4,16						0/20			
			ZF-195	F	+	+	Neg	Neg	Neg	Neg	Neg	na	na
			ZF-200	F	+	+	Neg	Neg	Neg	Neg	Neg	na	na
04/4/06	*Tg*(*hsp70l:Gal4*) *1.5 kca4*	0 (0/20)		4,16						0/20			
11/4/06	WIK	10 (2/20)		12,8						1/20			
			ZF-225	M	Neg	na	Neg	Neg	Neg	+	+	*chelonae*	H1E2
			ZF-231	F	+	+	Neg	Neg	Neg	Neg	Neg	na	na
20/4/06	AB	5 (1/20)		10,10						0/20			
			ZF-250	F	+	+	Neg	Neg	Neg	Neg	Neg	na	na
Total positive		35/240		135,105	29	11	16	21	17	24/240	24/240		

Table 2. Locations of environmental and biofilm collections, cultured on MB 7H10. Multiple colony types were initially screened by acid-fast staining to select mycobacteria for subculture. Species were identified by *hsp65* gene sequencing, and strains of *Mycobacterium chelonae* (Mc strain) were further characterized by ERIC and RAPD PCR. AFB: acid-fast bacilli; na: not applicable (1 gal = ca. 3.8 l)

Sample	Location	Colony types	Colony no.	*hsp65* sequence	GenBank accession no.	Mc strain
1	Paramecium culture	None	BF-1	na	na	na
2	Brine shrimp cone	None	BF-2	na	na	na
3	Tank, no fish (20 gal)	None	BF-3	na	na	na
4	Side of tank (20 gal)	Multiple	BF-4A	Not AFB	na	na
			BF-4B	*neoaurum*	EU478698	na
			BF-4C	*neoaurum*	EU478697	na
5	Algae/detritis on tank bottom (20 gal)	Multiple	BF-5A	*chelonae*	DQ866784	H1E2
			BF-5B	*phocaicum/peregrinum*	EU478699	na
6	Side of tank (1 gal)	Multiple	BF-6A	*chelonae*	DQ866785	H2E2
			BF-6B	*phocaicum/peregrinum*	EU478699	na
7	Uncleaned tank meniscus (20 gal)	None	BF-7	na	na	na
8	Debris on tank bottom (20 gal)	None	BF-8	na	na	na
9	Side of tank (20 gal)	Single	BF-9	*chelonae*	DQ866784	H1E2
10	Drain bulkhead (20 gal)	Single	BF-10	*chelonae*	DQ866784	H1E1
11	Side of tank (20 gal)	Multiple	BF-11A	*chelonae*	DQ866784	H1E2
			BF-11B	Not AFB	na	na
12	Fungal mat on tank bottom (20 gal)	Single	BF-12	*chelonae*	DQ866784	H1E2
13	Clean tank, no fish (1 gal)	None	BF-13	na	na	na
14	Side of tank (1 gal)	Single	BF-14	*chelonae*	DQ866784	H1E2
15	Side of sump	Single	BF-15	*chelonae*	DQ866784	H1E2
16	Side of sump	Single	BF-16	*chelonae*	DQ866784	H1E2
17	Clean sump	None	BF-17	na	na	na
18	Side of sump	Single	BF-18	*chelonae*	DQ866784	H1E2
19	Side of tank (4 gal)	Single	BF-19	*phocaicum*	EF551431	na
20	Side of tank (1 gal)	None	BF-20	na	na	na
21	Main water supply ball valve 1	None	BF-31	na	na	na
22	Main water supply ball valve 2	None	BF-32	na	na	na
23	Norprene tubing from tank	Single	BF-33	*chelonae*	DQ866784	H1E1
24	Air filter	None	BF-34	na	na	na
25	Air pump	None	BF-35	na	na	na
26	Mud puddle	None[a]	BF-36	na	na	na
27	River bank	Single	BF-37	Not AFB	na	na
28	Creek 1	None[a]	BF-38	na	na	na
29	Creek 2	None[a]	BF-39	na	na	na
30	Lunchroom tap water	None	BF-40	na	na	na
31	Sodium thiosulfate tank	Single	BF-41	Not AFB	na	na
32	Inside dishwasher	None	BF-42	na	na	na

[a]Initial cultures on MB 7H10 were dominated by fungal growth and subsequently tested on media containing amphotericin B

domly amplified polymorphic DNA (RAPD) primers OPA2, OPA18, IS986-FP and INS-2 (Zhang et al. 1997) were also tested. Amplification patterns were compared visually and relatedness categorized as in Zhang et al. (1997).

RESULTS

Survey

While overall mortalities routinely observed in the facility were very low, occurrence of mycobacteriosis in a few fish in a population of some 200 TU zebrafish prompted further investigation. Forty zebrafish from this tank were selected at random for disease screening, 7 of which (17.5%) were positive for mycobacteria by culture and PCR testing. Fish from the same stock in an adjacent tank, but not exhibiting signs of disease were also tested and 3/20 (15%) were positive. When an additional group of 20 TU fish originating from a separate stock held elsewhere in the facility were tested, 11 (55%) were infected with mycobacteria. Of the remaining groups of zebrafish tested (TL, *mitfa*, *kita*, *cct2*, *brg1*, *Tg*(*hsp70l:Gal4*)*1.5 kca4*, WIK, and AB), only 3/160 (1.9%) of these zebrafish were positive

by culture (Table 1). In total, 240 zebrafish were examined with 24 (10%) positive for infection based on culture and PCR of the organism.

Environmental reservoirs

We were able to culture mycobacteria from a variety of sources around the facility (Table 2). Tank surfaces at the interface of air and water yielded positive results most often, with a variety of *Mycobacterium* spp. identified. Food preparations (brine shrimp, paramecia) were negative, as were cultures from the main water supply. Multiple species of *Mycobacterium* were identified in biofilms (see Table 2).

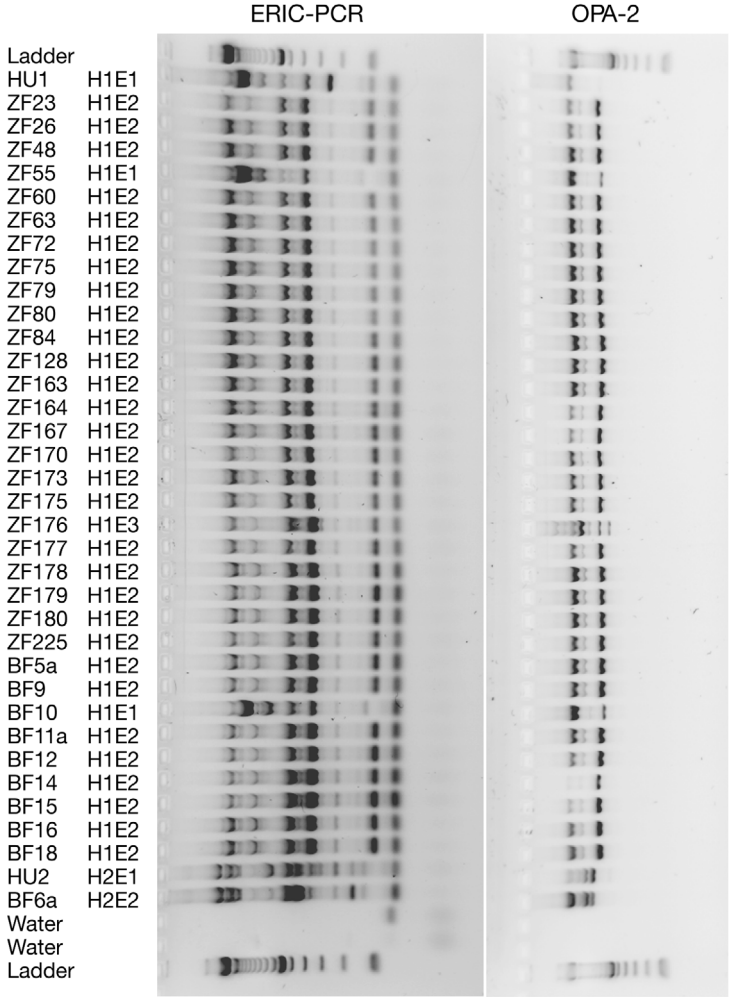

Fig. 1. *Mycobacterium chelonae* infecting *Danio rerio.* Amplification products from enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and randomly amplified polymorphic DNA (RAPD) with primer OPA-2 of 36 *M. chelonae* isolates from zebrafish. HU1 and HU2 were described previously (Kent et al. 2004). Samples designated ZF were colonies isolated from zebrafish, BF were from biofilms. 100 bp DNA ladder in first and last lanes

Molecular characterization

In all cases where mycobacteria were isolated from zebrafish, *hsp65* DNA sequencing identified the isolates as *Mycobacterium chelonae* (Table 1). Furthermore, all were identical to the *hsp65* sequence of *M. chelonae* isolate HU1 (GenBank # DQ866784) obtained from a zebrafish in our previous work (Kent et al. 2004, Whipps et al. 2007b). All isolates were analyzed further using ERIC-PCR and RAPD primer OPA-2 and could be categorized into 3 strains, designated H1E1 (1 isolate), H1E2 (22 isolates), and H1E3 (1 isolate) (Fig. 1). RAPD primers OPA-18, IS986-FP, and INS-2 yielded equivalent or less resolution (data not shown).

From biofilms, multiple species of *Mycobacterium* (*M. chelonae*, *M. phocaicum*, *M. neoaurum* and an unclassified species) were identified by *hsp65* sequencing (Table 2). The *hsp65* sequences from these *M. chelonae* isolates was identical to those previously isolated from zebrafish, strains HU1 (GenBank # DQ866784) and HU2 (GenBank # DQ866785). Subtyping analysis (Fig. 1) identified the same strains of *M. chelon*ae in biofilms, H1E1 (2 isolates) and H1E2 (8 isolates), that were isolated from zebrafish. *Mycobacterium* species other than *M. chelonae* were not analyzed by ERIC-PCR or RAPD.

Histopathology

Twenty-nine of 240 zebrafish surveyed exhibited chronic inflammatory changes consistent with mycobacteriosis (Table 1). Most were males (n = 18) exhibiting moderate to severe, chronic, diffuse inflammation and 16/18 displayed necrosis of the swim bladder (aerocystitis). No aerocystitis was observed in females. The affected swim bladders showed severe necrosis of the epithelium, with inflammatory changes extending through all layers of the swim bladder wall, and accompanying diffuse peritonitis. Acid-fast stains revealed numerous, free acid-fast bacilii (AFB) in the lumen of the swim bladder and often numerous bacteria in inflammatory cells in the swim bladder wall and surrounding peritoneum (Fig. 2).

The 11 females presenting with chronic inflammatory changes all had lesions in the ovaries and were associated with degenerated eggs (referred to egg-associated inflammation and fibroplasia, EAIF). In only 3 of these 11 zebrafish did acid-fast staining of sections reveal mycobacteria.

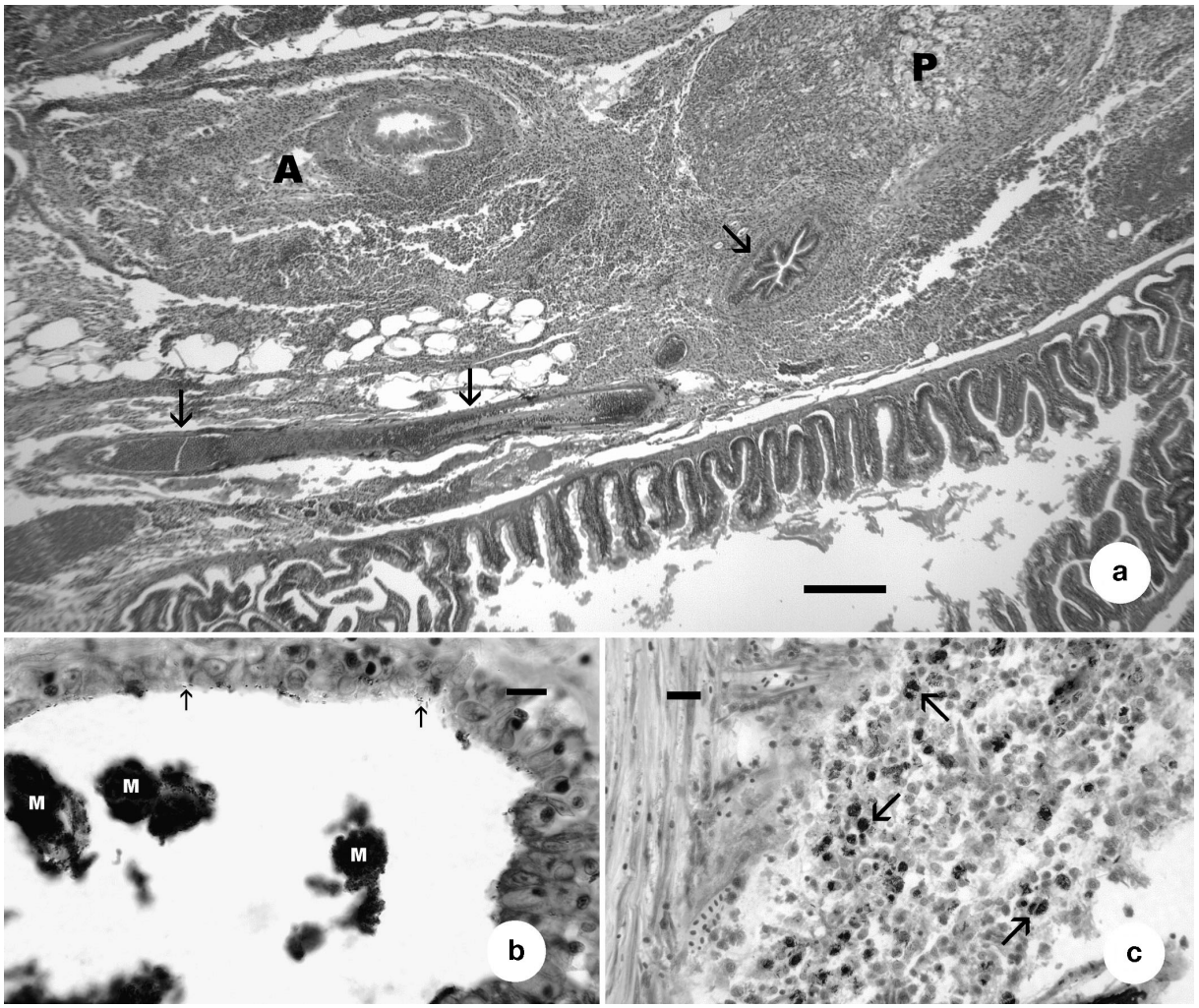

Fig. 2. *Mycobacterium chelonae* infecting *Danio rerio*. (a) Sagittal section showing severe, chronic aerocystitis with *M. chelonae* infection. Anterior (A) and posterior (P) chambers of the swim bladder exhibit massive infiltration of inflammation. Arrows = pneumatic duct connecting to esophagus. Scale bar = 20 µm. (b) High magnification showing masses of mycobacteria in lumen (M) and in swim bladder wall (arrows). Acid-fast. Scale bar = 10 µm. (c) Mycobacteria within phagocytes in swim bladder wall (arrows). Scale bar = 10 µm

Comparison of diagnostic methods

Histology, culture and PCR all performed comparably well for diagnosis of mycobacteriosis. Histological diagnosis based on H&E-stained sections was less reliable than acid-fast-stained sections, as diagnosis depended on the observation of granulomas or generalized chronic inflammation, which were present in females with EAIF in the absence of AFB (Table 1). Nevertheless, agreement between H&E histology and culture was modest (K = 0.6398). Diagnosis of mycobacteria by acid-fast-stained sections had substantial agreement (K = 0.7794) to culture results. Culture and PCR appear to be more sensitive than observations from tissue sections, as no AFB were observed in 6 of 24 zebrafish positive by PCR or culture. PCR directly from tissues and growth by culture were correlated perfectly.

DISCUSSION

Our investigation of zebrafish mycobacteriosis identified *Mycobacterium chelonae* in all positive individuals and the same subtype of *M. chelonae* associated with all but 1 infection. The zebrafish that made up the majority of our culture positive samples (21/24) were the TU genetic line, suggesting these fish are more susceptible to infection. The 3 TU populations were euthanized, and replaced with a new line of TU. This

was accomplished by following routine procedures for introduction of new lines, in which zebrafish are spawned in a quarantine facility and only progeny from chlorine-disinfected eggs are introduced. The remaining 3 infected zebrafish were from the TL, *kita*, and WIK lines. Overall, prevalence of mycobacteria in the facility was 10%. However, when TU zebrafish were excluded, prevalence was much lower, at 1.25%. With regards to the higher prevalence of mycobacteriosis in the TU line, the TU line or this particular stock may be genetically more susceptible to mycobacteriosis. Swaim et al. (2006) found that zebrafish lacking the *rag1* gene were hypersusceptible to *M. marinum* infection, supporting a genetic basis for susceptibility. In cattle, breed susceptibility to *M. avium* subsp. *paratuberculosis* has been reported (Koets et al. 2000), but found to have little effect in other studies (Pavlik et al. 2000). As pointed out by Chiodini et al. (1984), breed susceptibility may be difficult to dissect from unique exposure events, leading to an alternate explanation, namely that this particular stock of zebrafish was exposed to high levels of *M. chelonae*, perhaps early in development. Until more is known on the transmission of different mycobacteria in fish, the interpretations of such observations and control of spread is difficult.

Comparing diagnostic methods, there was substantial agreement between culture and histology when sections were stained with H&E ($K = 0.6398$). However, in some zebrafish the presence of degenerating eggs and EAIF as described by Kent et al. (2002) led to a misdiagnosis of mycobacteriosis (i.e. granuloma formation is present in both conditions). This serves as a reminder that diagnosis by observation of granuloma is presumptive at best. Conversely, the absence of granulomas would result in an under-appreciation of infection determined by H&E sections, as fish with mycobacteriosis may exhibit diffuse inflammatory lesions without discrete granulomas (Kent et al. 2004, Swaim et al. 2006). In a similar cross-sectional study of mycobacterial infection in striped bass, Kaattari et al (2005) observed much higher prevalence by PCR or culture than with observation of granuloma in H&E-stained sections.

When sections were stained acid-fast, the presence or absence of mycobacteria could be clearly distinguished and comparison of culture versus acid-fast-stained sections had substantial agreement and an increased Kappa coefficient ($K = 0.7794$). Thus, histology with acid-fast staining remains a reliable tool for identification of infected animals; where species identifications are required, culture and PCR-based methods can be employed. Where culture and PCR were negative, only 3 zebrafish were observed to have AFB. These were less severe infections and the discrepancy may be explained by an uneven distribution of the bacterium throughout the viscera. Unequal distribution may also explain the 6 culture-positive zebrafish that were AFB negative by histology. Alternatively, these positives could have been a result of environmental contamination during necropsy, as the same strains of *M. chelonae* are present outside of fish. We believe this unlikely for 2 reasons: (1) during necropsy the body surface was wiped with alcohol and great care was taken to avoid puncture of the gut; and (2) other *Mycobacterium* species isolated from biofilms (Table 2) were never isolated from zebrafish samples. Thus, we are confident of the potential for PCR for the rapid screening of zebrafish mycobacteria in the future.

A major advantage of PCR is its ability to identify *Mycobacterium* species quickly. A common method for genetic differentiation of mycobacteria is 16S small subunit (SSU) rDNA sequencing (Tortoli 2003). We have successfully used SSU rDNA to identify zebrafish mycobacteria from either cultures or directly from DNA extracted from infected host tissues (Kent et al. 2004). However, a limitation of using SSU alone is that a few species have identical SSU rDNA sequences (Tortoli 2003). More variable sequences, such as the internal transcribed spacer (ITS), may be useful for species and strain identification (Roth et al. 1998, 2000) and we have found differences in ITS sequence from 2 *M. chelonae* isolates from zebrafish that had identical SSU rDNA sequences (Kent et al. 2004). We have also identified a particularly virulent strain of *M. marinum* that possesses a single nucleotide polymorphism at position 5 of the ITS rDNA, which can be used to distinguish it from other isolates (Ostland et al. 2008). For routine identification, however, the heat shock protein 65 gene (*hsp65*) appears to be the most reliable (Kim et al. 2005, McNabb et al. 2006), differentiating species that SSU rDNA sequence cannot (Ringuet et al. 1999) and providing greater phylogenetic resolution than the ITS (Whipps et al. 2007b). Here we were able to identify 2 *hsp65* sequence types of *M. chelonae* in zebrafish or biofilms. Both *hsp65* types were consistent with those of *M. chelonae* we previously isolated from zebrafish, cases 13 and 19 of Kent et al. (2004), subsequently designated HU1 (GenBank # DQ866784) and HU2 (GenBank # DQ866785) by Whipps et al. (2007b). Nonetheless, to subdivide strains more thoroughly, additional steps must be taken.

Molecular typing by pulsed-field gel electrophoresis (PFGE) is often used for species of *Mycobacterium*, including *M. chelonae* (Vanitha et al. 2003). Other methods, such as amplified fragment length polymorphism (AFLP), have also been used to conduct epidemiological studies of mycobacteria (Motiwala et al. 2003) and yield results comparable to PFGE (Willems et al. 2000, D'Agata et al. 2001). In our experience,

PFGE is reliable but excessively time-consuming (Ostland et al. 2008), while AFLP is inconsistent and difficult to interpret. As a result, we have switched to the more rapid ERIC-PCR. Compared to PFGE, ERIC provides equal, if not greater resolution, and results can be obtained quickly (Sampaio et al. 2006). Furthermore, such analyses can be performed by any lab with a thermocycler and results are interpreted by visual inspection, comparing amplification patterns of various isolates on a single gel. To supplement ERIC-PCR, RAPD analysis is also performed using primers of Zhang et al. (1997). We have found the OPA-2 to be the most discriminatory, as in other studies (Esteban et al. 2007), yielding results equivalent to ERIC-PCR. These are the methods we employ on a routine basis and recommend for delineation of mycobacteria with identical *hsp65* genes.

The same strain of *Mycobacterium chelonae* (H1E2) was identified in all but 2 infected zebrafish, which were infected with *M. chelonae* H1E1 and H1E3. The predominance of a single strain suggests a common source of infection, possibly environmental. In an attempt to identify potential sources or reservoirs of infections in zebrafish, we tested several biofilms for mycobacteria. These included tank surfaces, drains, sump tanks, and Norprene tubing. *Mycobacterium chelonae* (H1E2) was identified on several tank surfaces, drains, the sump and tubing feeding water to tanks. Fortunately, no mycobacteria were detected in fish food, the main incoming water supply, or from the city water supply to the dishwasher or kitchen faucet. Increased sampling volumes may yet reveal some mycobacteria in these sources. However, for now our results suggest that *M. chelonae* is endemic at the facility, although its pathogenicity and virulence in most lines of zebrafish may be minimal under standard conditions.

Previous experimental transmission studies conducted with *Mycobacterium chelonae* strains that we have collected and tested to date suggest that *M. chelonae* is less virulent than *M. marinum* and *M. haemophilium* (Watral & Kent 2007, Whipps et al. 2007a, Ostland et al. 2008). Although we have not yet evaluated H1E2 (the most common isolate from this study) by experimental exposure, the isolate HU1 (type H1E1; also present in the facility) caused little disease following intraperitoneal injections. Harriff et al. (2008) evaluated several strains of *M. chelonae* (including H1E1) and a closely related organism from salmon (*M. salmoniphilum*) with *in vitro* macrophage proliferation assays and a microarray. Corresponding to our *in vivo* study (Watral & Kent 2007), the H1E1 grew poorly in macrophage cell lines and lacked a newly discovered pathogenicity island (Harriff et al. 2008). The prevalent strain in the present study (H1E2) should be evaluated with these *in vivo* and *in vitro* assays.

The manifestation of infection showed a consistent pattern. Histological examinations of infected zebrafish revealed that the occurrence of disease was largely confined to male fish with chronic aerocystitis. Zebrafish have a physostomus swim bladder with a pneumatic duct connecting it to the esophagus (Finney et al. 2006). Harriff et al. (2007) showed that the primary route of infection by mycobacteria in zebrafish is oral; thus, connection with the digestive tract via this duct may provide a route for swim bladder infections. None of the 3 infected females in the current study showed signs of infection in the swim bladder. Thus, the reason for the apparent predisposition of mycobacterial aerocystitis in males here is unknown. In a review of the Zebrafish International Resource Center diagnostic cases, aerocystitis was diagnosed in 35 zebrafish (21 females, 14 males) from 20 groups of fish submitted from research laboratories between 2003 and 2007. AFB were found in the swim bladder lesions in 51% (18/35) of these fishes. Other cases were associated with Gram-negative bacterial or fungal infections (Kent et al. 2002).

Ultimately, the control of mycobacteriosis in zebrafish colonies first requires a thorough understanding of the virulence diversity, prevalence, distribution and potential modes of transmission for *Mycobacterium* species found in research facilities. We have seen significant differences in virulence between *Mycobacterium* species infecting zebrafish, highlighting the importance of species identification (Watral & Kent 2007, Whipps et al. 2007a). Preventing the introduction of the highly virulent strains and species, particularly *M. marinum* and *M. haemophilum*, to fish research facilities is paramount, because once established, mycobacteria can be difficult to eliminate (Francis-Floyd & Yanong 1999). In contrast, *M. chelonae* may be very common in water and has been isolated from aquaria in the absence of disease (Beran et al. 2006). With these low virulent, ubiquitous strains, dramatic measures such as entire euthanasia of the affected population may not be warranted. However, even with these strains, it would be prudent to minimize the spread of infections. Antibiotic treatment is usually discouraged due to lengthy treatment times and varying susceptibility of *Mycobacterium* spp. to different antibiotics (Decostere et al. 2004). However, in circumstances where a stock of fish is very valuable, identification of the *Mycobacterium* sp. by culture or PCR, followed by treatment with the appropriate antibiotic, based on known vulnerability or susceptibility testing, might be advisable upon the recommendation of the facility's laboratory animal veterinarian. Depopulation and disinfection of the facility may be necessary when

all other measures fail. This unfavorable course of action may be avoidable by minimizing the chances of introduction and spread within a facility. Some measures we recommend are summarized in Kent et al. (2008) and include sentinel programs, quarantine of incoming fish and eggs, regular monitoring, minimizing circulating mycobacteria through regular cleaning and maintenance, and upkeep of the UV sterilization system.

In conclusion, this survey of background levels of *Mycobacterium* species in a zebrafish facility revealed that *M. chelonae* was the only species isolated from zebrafish. The same strains were identified in surface biofilms, implicating a potential environmental reservoir of infection. Overall, levels of infection in the facility were very low, with the exception of TU zebrafish, which represented all but 3 of our positive fish. Thus, we suspect that TU zebrafish are more susceptible to infection. Consistent with results from our earlier virulence experiments (Watral & Kent 2007), virulence of *M. chelonae* appeared to be low, i.e. few fish showed signs of overt mycobacteriosis. This study demonstrates that *M. chelonae* infections can persist in the background, even at very well-managed facilities. These results emphasize the importance of good husbandry and fish health monitoring in zebrafish research facilities to minimize the risk of a disease outbreak, with the ultimate goal of greatly reducing or eliminating these infections from laboratory-reared populations.

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