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Development of Quantitative Real-Time PCR Assays for Postmortem Detection of *Mycobacterium* spp. Common in Zebrafish (*Danio rerio*) Research Colonies

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***Mycobacterium* spp. infections are common in zebrafish kept in research facilities. These comorbidities can substantially modulate the responses of these fish to external and internal stimuli. Therefore, diagnostic tests to detect *Mycobacterium* spp. infections in zebrafish colonies prove essential. Here, we outline the development of quantitative simplex real-time PCR assays to detect the 3 *Mycobacterium* species most commonly identified in laboratory zebrafish. The assays targeted the heat-shock protein 65 gene of *M. marinum*, *M. chelonae*, and *M. haemophilum*. The assays are both highly specific and sensitive for fresh-frozen samples and highly specific and moderately sensitive for formalin-fixed paraffin-embedded (FFPE) samples. Two sampling techniques for FFPE samples of sagittally sectioned zebrafish were evaluated. Both paraffin cores targeting granulomas containing bacteria and scrolls from the entire fish yielded DNA of equivalent quantity and purity. The diagnostic sensitivity of cores was superior to that of scrolls for *M. chelonae* and *M. haemophilum* but not *M. marinum*. The assays are cost-effective and ideally suited to diagnosing common *Mycobacterium* spp. infections in zebrafish.**

Abbreviations: C_t, cycle threshold; FFPE, formalin-fixed paraffin-embedded; qPCR, quantitative real-time polymerase chain reaction; T_m, melting temperature

Zebrafish (*Danio rerio*) are quickly gaining popularity in the research setting as a preclinical animal model. To remove confounding comorbidities and prevent the loss of vital resources and study animals, diagnostic and screening tests are needed to detect some of the most common and devastating infections in zebrafish: mycobacterioses. Although numerous *Mycobacterium* spp. can infect zebrafish, 3 species in particular are of concern due to their high morbidity or mortality or subclinical infection with high bacterial loads. *M. marinum* and *M. haemophilum* have both been documented as causing widespread outbreaks in research settings;[28,29] *M. chelonae* infections are more common in zebrafish[1,7,26,27] and usually result in low mortality but are of concern because apparently healthy (subclinically infected) fish may have extensive internal lesions with high bacterial loads. Mycobacterial infections in experimental animals also pose a health threat to laboratory personnel. Given the importance of these infections and the differences in severity of clinical disease due to mycobacterial species, it is important to develop rapid, sensitive, and specific diagnostic tests for these 3 pathogens. Molecular diagnostic methods are now the most practical and efficient approach to detect and identify mycobacteria to the species level, because many species are difficult to grow in culture and because biochemical tests are often not informative.[33] These PCR methods may involve amplification of DNA from fresh, frozen, or ethanol-preserved tissues and target areas of high discriminatory power, such as *hsp65*, *rpoB*, and the 16S–23S internal transcriber spacer. This approach has been used with piscine *Mycobacterium* species that are difficult to culture, such as *M. haemophilum* in zebrafish and *M. triplex*-like organisms in rockfish, swordtails, and mollies.[19,27-29,37] Alternatively, species and strain identifications can be rapidly and accurately obtained by using PCR tests of bacteria isolated in culture.[37] In zebrafish, PCR testing for mycobacteria by using formalin-fixed or paraffin-embedded (or both) material is particularly useful, because histopathology is generally the primary diagnostic method used for this species, and fresh tissues and cultures are often unavailable.[9,12,17]

The purpose of the current study was to develop simplex (that is, using a single primer or probe set to detect a particular species), real-time quantitative PCR (qPCR) assays for each of the aforementioned mycobacterial species of importance to zebrafish for 3 types of samples: bacterial isolates, fresh-frozen fish, and formalin-fixed paraffin-embedded (FFPE) fish. The resulting assays have aided in the identification of mycobacterial outbreaks and their specific causes. Furthermore, the assays will serve as screening tools to develop and maintain SPF zebrafish lines.[8]

Materials and Methods

Bacterial strains and cultures. Mycobacterial cultures used for experimental infection were prepared as described following. Three isolates each of *M. marinum* and *M. chelonae* (Figure 1) were obtained from the Kent laboratory at Oregon State University (Corvallis, OR). Specifically, 2 isolates of *M. marinum* (MmE and MmW) were obtained from zebrafish from outbreaks at 2 large research facilities, and the third (OSU214) was isolated from hybrid striped bass and identical to isolates

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Test isolates	Identification	Reference
M. marinum	M.m. OSU214	Ostland et al. 2008
	M.m.W	Kent Laboratory, Oregon State University
	M.m.E	Kent Laboratory, Oregon State University
M. chelonae	M.c. H1E1	Whipps et al. 2008
	M.c. H1E2	Whipps et al. 2008
	M.c. H1E3	Whipps et al. 2008
M. haemophilum	M.h. ATCC 29548	
Control Isolates	**Identification**	**Reference**
BCG Pasteur	BCG	Institute Pasteur, Paris
K-10 M. *avium* ssp. *paratuberculosis*	K-10	Williams et al. 1999
Nocardia sp.	Noc	VDL Reference isolate[a]
Pasteurella sp.	Past	VDL Reference isolate[a]
Corynebacterium sp.	Coryne	VDL Reference isolate[a]
Streptococcus spp.	Strep1	VDL Reference isolate[a]
	Strep4	VDL Reference isolate[a]

[a]Internal reference isolates, Veterinary Diagnostic Laboratory, Oregon State University, Corvallis, OR

Figure 1. Bacterial isolates used to develop qPCR assays for mycobacteria commonly diagnosed in zebrafish research colonies.

from zebrafish based on pulsed-field gel electrophoresis.[15,26] The 3 *M. chelonae* isolates originated from zebrafish with low-level mortality.[29] The single isolate of *M. haemophilum* was obtained through ATCC (no. 29548, Vienna, VA). The *M. marinum* and *M. chelonae* isolates were streaked onto Middlebrook 7H10 media and incubated for 14 d at 32 °C; the *M. haemophilum* isolate was streaked on Middlebrook 7H10 media enhanced with hemin and incubated for 28 d at 32 °C.[32] Cultured colonies from each isolate were transferred to appropriate broth media by using loop inoculation to generate serial dilutions. Fresh culture material was placed in sterile PBS to obtain a stock solution of approximately 3×10^8 cells per mL by using the MacFarland optical density of 1.0 as a reference.[16] A 1:10 dilution of these stocks (3×10^7) was used to infect fish by injecting 10 µL (approximately 3×10^5 organisms per fish). Control isolates (Figure 1) were as follows: attenuated BCG Pasteur from the Institute Pasteur (Paris, France) and K10 *M. avium* subsp. *paratuberculosis* from Raul Barletta (University of Nebraska, Lincoln, NE) were provided by the laboratory of Luiz Bermudez at Oregon State University; *Streptococcus inia*, *Nocardia nova*, *Pasteurella multocida*, and *Corynebacterium pseudotuberculosis* isolated from diagnostic samples and phenotypically characterized were provided by the Veterinary Diagnostic Laboratory at Oregon State University.[25,31]

Zebrafish. Adult wild-type zebrafish of both sexes were acquired from the Sinnhuber Aquatic Research Laboratory (Oregon State University), a facility that is SPF for *Pseudoloma neurophilia* and has a very low background infection with *M. chelonae*.[8] The AB/5D strain was used for infection with *M. marinum* and *M. haemophilum*, the TU/5D strain was used for infection with *M. chelonae.* Both the AB and 5D strains are SPF for *Pseudoloma neurophilia* and *M. marinum*, *M. haemophilum*, and *M. chelonae*. The TU strain is not certified SPF, but this colony of TU/5D crosses has repeatedly tested negative for those pathogens. The fish were maintained in a Biosafety Level 2 facility in flow-through tanks according to an Animal Care and Use Protocol approved by the IACUC at Oregon State University. For inoculation, zebrafish were anesthetized with 100 mg buffered 1^{-1} tricaine methanosulfonate (MS222) and injected with either 10 µL PBS (sham inoculation) or prepared *M. marinum* (OSU214), *M. chelonae* (H1E1), and *M. haemophilum* inocula by using aseptic technique. Zebrafish were maintained at 25 to 27 °C with sufficient aeration and a 14:10-h light:dark photoperiod for 12 wk to allow for infection to develop. Dead fish were collected daily, and all surviving zebrafish were euthanized at the end of the 12-wk period. Euthanized zebrafish were either fresh-frozen (–80 °C) or fixed in 10% neutral-buffered formalin for 7 d followed by decalcification in CalExII (ThermoFisher Scientific, Waltham, MA) for 48 h. A total of 15 fish from each isolate per species were fresh-frozen, and another 15 from each isolate were fixed for paraffin embedding, although only 6 were used in the experiments. Fixed zebrafish were held in a 70% ethanol solution until processing into paraffin blocks for a maximum of 8 wk at the Veterinary Diagnostic Laboratory (Oregon State University). For evaluation by light microscopy, 3-µM sections were stained with hematoxylin and eosin (ThermoFisher Scientific) or Kinyoun acid-fast stain (Kinyoun carbol fuchsin, VWR, Radnor, PA) according to standard protocols and operating procedures.[2]

Primer and probe design. Primers were designed based on published sequences of the bacterial heat-shock protein 65 gene, which is highly conserved within the *Mycobacterium* genus but variable enough to distinguish between *Mycobacterium* species.[10] Sequences of the *HSP65* gene were obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/) and analyzed by using Primer Express 3.0 Software to generate primer and probe sets.[21] Primer and probes were: *M. marinum* (GenBank accession no., AF547855 #6) MmForward, 5′ CAA CCC GCT CGG TCT GAA 3′ (melting temperature [T_m], 59 °C; GC content, 61%); MmReverse, 5′ CGA CCT CTT TGG CCG ACT T 3′ (T_m, 59 °C;

GC content, 58%); and MmProbe, 5′ TCA CCG AGA CCT TGC 3′ (T_m 69 °C; GC content, 60%); *M. chelonae* (DQ866784 #31) McForward, 5′ AAG GAA GTT GCC AAG AAG ACT GA 3′ (T_m 58 °C; GC content, 43%); McReverse, 5′ CAG AGC CTG GGC AAG CA 3′ (T_m, 58 °C; GC content, 65%); and McProbe, 5′ ACG GCA CTA CTA CCG C 3′ (T_m, 69 °C; GC content, 63%); and *M. haemophilum* (GQ245967#2) MhForward, 5′ GTT AAG GTG GCG TTG GAA GCT 3′ (T_m, 58 °C; GC content, 50%); MhReverse, 5′ TCC AGC CCG GAG TTG AAG 3′ (T_m, 58 °C; GC content, 61%); and MhProbe, 5′ CGC TGA AGC AGA TCG 3′ (T_m, 69 °C; GC content, 60%)

Primer and probe sets were analyzed for sequence similarity to other *Mycobacterium* species and were used in a BLAST search to identify sequence similarity across all bacterial genera. In addition, each primer and probe set was examined for sequence similarity with the other sets. Selected sets had a low penalty score (Primer Express 3.0 Software) and high specificity as determined by sequence similarity analysis (Table 1).

Sample preparation and DNA extraction. Aliquoted isolate samples were centrifuged at 6000 × *g* for 5 min, the supernatant removed, and the pellet resuspended in 200 µL PBS; 20 µL lysozyme (10 mg/mL; ThermoFisher Scientific) was added to each sample to optimize the assays and improve diagnostic yield. After incubation at 37 °C for 60 min, the DNA in 50 µL of the solution was extracted (MagMAX Total Nucleic Acid Isolation Kit, Life Technologies, Grand Island, NY) according to the manufacturer's instructions. Different concentrations of primers and probes used in the Master Mix were tested to optimize diagnostic yield. For primers, 0.2, 0.4, 0.6, and 0.8 µM concentrations were tested; for probes, 120 and 240 nM concentrations were tested.

Fresh-frozen samples of whole fish were thawed at room temperature and minced on individual culture dishes by using new scalpel blades for each fish to prevent cross-contamination. Screw-top centrifuge tubes containing 0.1 g of tissue sample and zirconia–silica beads (0.1 mm, Biospec Products, Bartlesville, OK) in 1 mL PBS were placed on a Mini-BeadBeater 16 (Biospec Products) for 3 min. After centrifugation at 1000 × *g* for 1 min, the supernatant was used for DNA extraction as described in the preceding paragraph.

Prior to testing of FFPE samples, their respective corresponding slides were evaluated to locate lesions for core sampling. If the sample quality was poor (due to advanced autolysis as determined by a board-certified veterinary pathologist [CVL]), that sample was omitted from the test run. Scrolls of embedded zebrafish were generated by sectioning paraffin blocks (thickness, 5 µm; model 820 II rotary microtome, Reichert–Jung, Depew, NY); sections were immediately transferred into sterile microfuge tubes. Between each block, the microtome blade was changed and the microtome wiped down with xylene followed by 90% ethanol. Three negative (that is, paraffin only) blocks were sampled after every 5 test blocks for *M. chelonae* and *M. haemophilum* and after every 3 test blocks for *M. marinum* to prevent and allow for testing for cross-contamination.[17] To generate cores, acid-fast–stained slides of infected fish were analyzed to identify granulomas containing mycobacterial organisms in situ. Lesions were marked on each slide and matched to the location in the paraffin-embedded zebrafish (Figure 2). Core samples were collected by using a new, sterile 16-gauge injection needle for each fish. DNA was extracted by using the RecoverAll Total Nucleic Acid Kit for FFPE (Life Technologies) according to manufacturer's instructions. For optimal yield, the protease digestion step was performed overnight (approximately 20 h) at 50 °C.

Table 1. Sequence homology between primers and probes to mycobacterial heat-shock protein 65

	MmForward	MmReverse	MmProbe
MmForward	NA	5/18 (27.8%)	4/15 (26.7%)
MmReverse	NA	NA	5/15 (33.3%)
MmProbe	NA	NA	NA
	McForward	**McReverse**	**McProbe**
McForward	NA	4/17 (23.5%)	4/16 (25.0%)
McReverse	NA	NA	7/16 (43.7%)
McProbe	NA	NA	NA
	MhForward	**MhReverse**	**MhProbe**
MhForward	NA	3/18 (16.7%)	2/15 (13.3%)
MhReverse	NA	NA	3/15 (20.0%)
MhProbe	NA	NA	NA

Mm, *M. marinum* OSU214; Mc, *M. chelonae* H1E1; Mh, *M. haemophilum*; NA, not applicable

Data are given as the number of base pair matches between the 2 sequences / the number of nucleotides present in the shorter of the 2 sequences, starting at the 3′ end (percentage of homology)

Real-time qPCR assays. *Isolates.* Samples were PCR-amplified (model 7500 Fast Real-Time PCR System, Applied Biosystems, Foster City, CA) by using an initial uracil-N glycosylase incubation cycle of 50 °C for 2 min, followed by an Amplitaq (Life Technologies, Grand Island, NY) activation cycle of 95 °C for 10 min and 40 cycles of denaturation at 95 °C for 15 s with an annealing–extension step at 57 °C for 1 min. PCR products were visualized by using GelRed (Biotium, Hayward, CA) in a 2.5% agarose gel to determine specificity and to obtain samples for sequencing (Center for Genome Research and Biocomputing, Oregon State University) to confirm that the desired product was obtained.

To determine analytical specificity as well as reproducibility and repeatability of the PCR assays, both inter- and intra-assay comparison runs were performed for all simplex assays using serial dilutions of isolate samples of each test species along with the control isolates (attenuated BCG and K10; *Streptococcus iniae*, *Nocardia nova*, *Pasteurella multocida*, and *Corynebacterium pseudotuberculosis*). When multiple isolates were available for a species, a single isolate was chosen on which to perform intra-assay comparisons. Serial dilutions ranging from 10^1 to 10^8 (see following) were tested 4 times. The mean cycle threshold (C_t) values for each dilution were plotted on a line graph to determine linear regression (R^2), with an R^2 value greater than 0.95 being ideal (Excel 2010, Microsoft, Redmond, WA). The coefficient of variation was expressed as a percentage and is a measure of repeatability or precision; CV values greater than 4% are considered to be too variable, or imprecise, to be useful. For interassay comparison, all isolates were tested 4 times (except for *M. haemophilum*, for which only one isolate was available and therefore could not be compared with other isolates), and C_t values plotted on a line graph to determine linear regression with an R^2 value greater than 0.95 being ideal.

The analytical sensitivity of the simplex qPCR assays, as defined by limit of detection, was determined by using 10-fold serial dilutions made from stock solutions of each isolate. The stock solutions were generated by using a MacFarland optical density of 1, which reflects an approximate bacterial cell density of 3×10^8/mL, as a starting point. The 1:10-diluted solutions

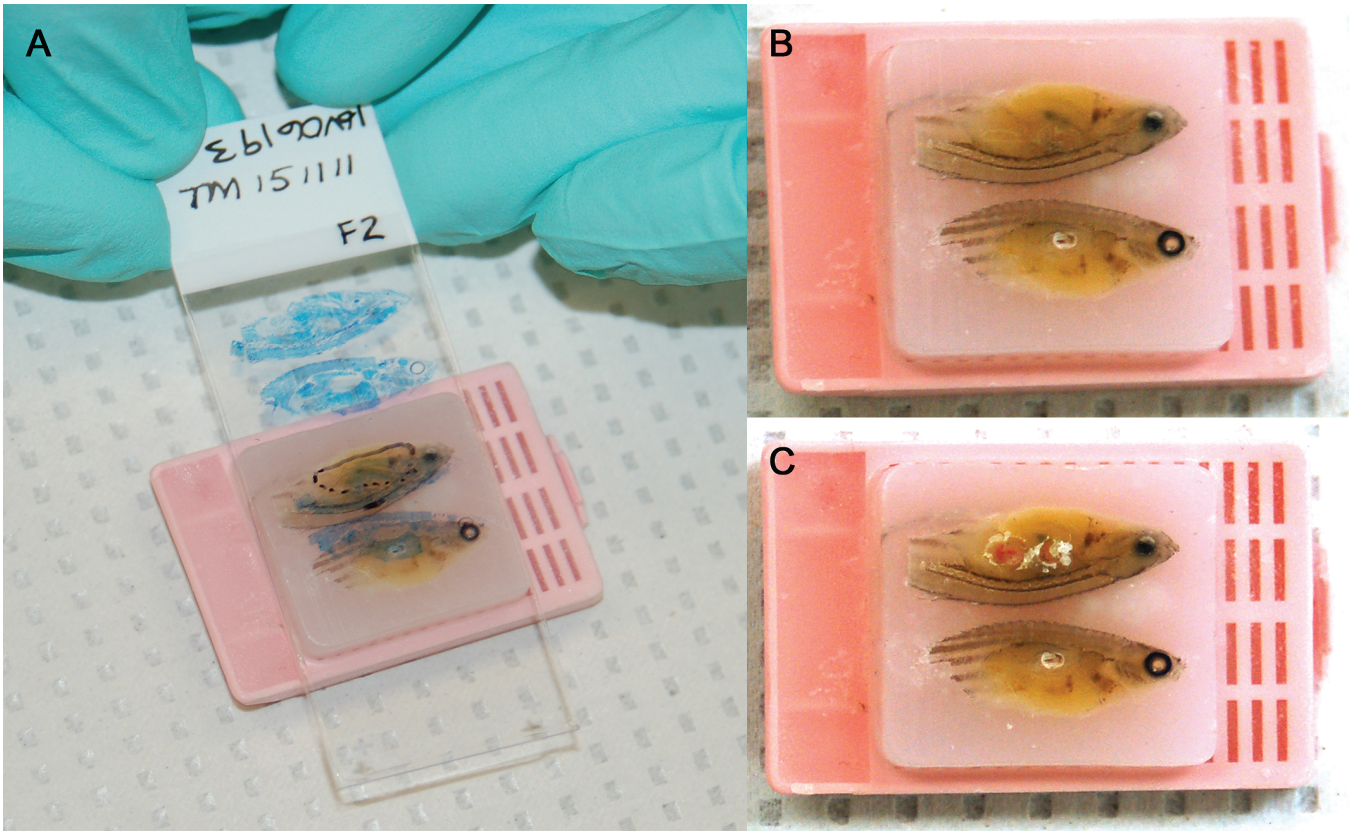

Figure 2. (A) Paraffin block of an embedded zebrafish diagnosed with mycobacteriosis according to histologic examination. The corresponding acid-fast–stained microscopic slide with marked-up mycobacterial granulomas is overlaid to select specific areas for coring. Same paraffin block as in panel A but (B) before and (C) after collection of core samples. Images were modified to adjust white balance.

of these stocks (3×10^7/mL) were used to infect fish with 3×10^5 organisms by the injection of 10 µL. More dilute solutions (specifically 3×10^4, 3×10^3, and 3×10^2 cells/mL) were plated to confirm that the expected number of colony-forming units was obtained. All dilutions of an isolate were amplified on the same plate in the same qPCR run to determine the lowest number of colony-forming units detectable.

Fresh-frozen samples. Once the analytical specificity–sensitivity and repeatability–reproducibility of the assays were evaluated by using bacterial isolates and no cross-reactivity identified, the assays were used on fresh frozen tissue samples as described for isolates. To assess specificity and sensitivity, 9 fresh-frozen fish per species were used, taking 3 from each isolate for *M. marinum* and *M. chelonae*, given that 3 isolates were available for these species. When testing repeatability and reproducibility, 4 fish per isolate were tested. The same thermal cycling parameters were used as for the isolates.

FFPE samples. After the specificity–sensitivity and repeatability–reproducibility of the simplex assays were established for fresh-frozen samples, each simplex assay was tested on FFPE samples on both scroll and matched core samples for each of the 3 mycobacterial species. The same thermal cycling parameters were used as described for the isolates, except that 50 cycles of the denaturation and annealing–extension steps were performed to increase yield. Both the purity and concentration of the DNA isolated from FFPE samples were determined by UV spectrometry (Nandrop 2000, ThermoScientific; Table 2).

Statistics. Detection limits (that is, the lowest number of colony-forming units that yielded a positive PCR result) were calculated for all mycobacterial isolates under investigation. Reproducibility and repeatability were calculated (Excel 2010, Microsoft) as the mean, 1 SD, and coefficient of variance by using the C_t values of serial dilutions of representative isolates of each mycobacterial species. C_t values were tabulated for a minimum of 3 replicates. Diagnostic specificity and sensitivity were calculated (Excel 2010, Microsoft) on samples from experimentally infected zebrafish by using the current 'gold standard' of diagnosis: detection of acid-fast bacteria in granulomas in situ. Diagnostic and analytical sensitivity were calculated as the number of true positives divided by the number of all positives, whereby 'positive' refers to a samples or dilution, respectively. Diagnostic and analytical specificity was calculated as number of true negatives divided by all negatives, whereby 'negative' refers to a sample or dilution, respectively.

Results

Histopathology. Findings from microscopic examination of tissue samples stained with hematoxylin and eosin or acid-fast stain are detailed in Table 2. Zebrafish infected with *M. marinum* (n = 6) had few, scattered acid-fast bacilli; acid-fast bacteria were present in the spleen of all zebrafish. Other common body sites were kidney, liver, and ovaries and within or adjacent to the swim bladder. Infection with *M. chelonae* (n =7) presented in about half of the fish with large granulomas in the ovary or adjacent to the swim bladder; in the other half, acid-fast bacteria lined the lumen of the swim bladder. *M. haemophilum*-infected zebrafish (n = 14) had large granulomas, which were located in the kidney in all fish as well as (in decreasing prevalence) adjacent to the swim bladder, in the liver, or in the pericardium.

Table 2. Paraffin-embedded zebrafish and control blocks: granuloma distribution and mycobacterial load according to microscopy; DNA quantity and quality; and qPCR results

					DNA					
					Concentration (ng/µL)		Purity[b] (260:280 nm)		qPCR	
Isolate	Fish no.	Internal fish no.	Lesions	Category[a]	scrolls	cores	scrolls	cores	scrolls	cores
M. marinum OSU214	1	R12-MVT-73	Large granulomas in kidney, spleen	2	210.7	230.1	1.68	1.83	+	+
	2	R12-MVT-70	Numerous acid-fast bacilli scattered in spleen, adjacent to swim bladder; poorly preserved granulomas adjacent to swim bladder	2	126.7	170.2	1.83	1.75	–	–
	3	R12-MVT-67	Numerous acid-fast bacilli scattered in spleen, adjacent to swim bladder; focal granuloma in pericardium	2	169.4	185.7	1.76	1.73	+	–
		N1[d]	Not applicable	NA	141.1	NA	1.87	NA	–	NA
		N2	Not applicable	NA	119.7	NA	1.85	NA	–	NA
		N3	Not applicable	NA	182.5	NA	1.73	NA	–	NA
	4	R12-MVT-64	Numerous large granulomas and scattered acid-fast bacilli in ovaries, kidney, spleen, liver and pericardium	3	160.8	228.3	1.83	1.69	+	+
	5	R12-MVT-63	Numerous large granulomas and scattered acid-fast bacilli in spleen, kidney, liver, ovaries and adjacent to swim bladder	3	178.0	292.9	1.75	1.74	+	–
	6	R12-MVT-61	Very few (~10-20) acid-fast bacilli scattered in spleen, ovary and liver; one discrete granuloma dorsal to ovaries	1	NA	218.2	NA	1.65	NA	–
M. chelonae H1E1	7	1	3 or 4 large granulomas in ovary and scattered acid-fast bacilli in ovary	2	1.88	1.82	139.7	163.7	–	–
	8	2	3 or 4 large granulomas in ovary	2	1.79	1.71	176.5	259.5	–	+
	9	5	Lining swim bladder	1	1.83	1.81	151.4	156.6	–	+
		N1[c]	Not applicable	NA	1.73	NA	123.7	NA	–	NA
		N2	Not applicable	NA	1.75	NA	127.1	NA	–	NA
		N3	Not applicable	NA	1.71	NA	133.6	NA	–	NA
	10	6	Large granulomas in ovary and scattered acid-fast bacilli in ovary	2	1.83	1.69	169.8	449.1	–	+
	11	7	Lining swim bladder	1	1.85	1.77	143.5	198.3	+	–
	12	9	Focal granuloma adjacent to swim bladder	1	1.86	1.81	154.0	148.6	–	+
	13	11	Focal granuloma adjacent to swim bladder	1	NA	NA	NA	NA	NA	NA
	14	12	Lining swim bladder	1	NA	NA	NA	NA	NA	NA
M. haemophilum	15	1	Large granulomas adjacent to swim bladder; kidney	2	136.9	264.9	1.89	1.68	–	+
	16	2	Large granulomas adjacent to swim bladder; kidney; liver	3	138.9	189.0	1.80	1.71	–	–

Table 2. Continued

Isolate	Fish no.	Internal fish no.	Lesions	Category[a]	DNA Concentration (ng/μL) scrolls	DNA Concentration (ng/μL) cores	DNA Purity[b] (260:280 nm) scrolls	DNA Purity[b] (260:280 nm) cores	qPCR scrolls	qPCR cores
	17	3	Large granulomas in liver; kidney	2	148.7	739.0	1.79	1.72	NA	NA
	18	4	Large granulomas adjacent to swim bladder; kidney	2	125.8	203.5	1.83	1.72	NA	NA
	19	5	Numerous large granulomas adjacent to swim bladder; kidney; liver	3	168.2	180.9	1.78	1.71	NA	NA
	20	6	Indiscrete granulomas; lymphoplasmacytic inflammation in kidney; liver	1	123.7	141.1	1.82	1.79	NA	NA
	21	7	Large granulomas in spleen, kidney, adjacent to swim bladder	3	122.7	197.3	1.82	1.73	NA	NA
	22	8	Large granulomas in kidney, liver, adjacent to swim bladder	3	125.6	297.0	1.81	1.66	NA	NA
	23	9	Large granulomas adjacent to swim bladder, pericardium, kidney	3	127.5	142.9	1.91	1.78	+	+
	24	10	Large granulomas in kidney, adjacent to swim bladder, pericardium	2	142.4	166.0	1.76	1.74	–	+
		N1	Not applicable	NA	136.9	NA	1.86	NA	–	NA
		N2	Not applicable	NA	134.3	NA	1.81	NA	–	NA
		N3	Not applicable	NA	124.7	NA	1.79	NA	–	NA
	25	11	Lymphoplasmacytic inflammation in pericardium, kidney; smaller granulomas adjacent to swim bladder	1, 2	145.6	198.6	1.76	1.71	+	–
	26	12	Large granuloma adjacent to swim bladder, kidney; lymphoplasmacytic inflammation in pericardium	3	136.2	117.7	1.87	1.89	+	+
	27	13	Lymphoplasmacytic inflammation in kidney, adjacent to swim bladder ± pericardium	1	NA	NA	NA	NA	NA	NA
	28	14	Lymphoplasmacytic inflammation adjacent to swim bladder, kidney	1	NA	NA	NA	NA	NA	NA
	29	15	Numerous granulomas in kidney, liver, adjacent to swim bladder; lymphoplasmacytic inflammation in pericardium	3	NA	NA	NA	NA	NA	NA

[a]Category denotes severity of infection: 1, mild; 2, moderate; 3, severe
[b]Purity of DNA preparation according to the 260:280-nm absorbance ratio of the sample
[c]N denotes an empty paraffin block used between sectioning specimens for scrolls to prevent and control for cross-contamination

qPCR analysis of bacterial isolates: analytical specificity, sensitivity, and reproducibility. Analytical specificity was confirmed for all qPCR assays. Each assay proved to be specific and yielded the expected PCR amplicon for the tested mycobacterial species (data not shown). None of the simplex assays yielded any amplification products for the control isolates (attenuated BCG and K10; *Streptococcus iniae*, *Nocardia nova*, *Pasteurella multocida*, and *Corynebacterium pseudotuberculosis*). An assay was determined specific when only isolates of the particular *Mycobacterium* species yielded an amplification product of the expected size and when the sequence of the amplification product matched the reference genome by at least 99%.

Analytical sensitivity, defined as the limit of detection of 10-fold serial dilutions made from stock solutions (3×10^8/mL) of each *Mycobacterium* spp. isolate, ranged from 3000 to 300,000 (Table 3). For *M. marinum* isolates, detection limits differed by as much as 10-fold. The *M. chelonae* isolates showed a maximum of a 100-fold difference in the detection limit when the same dilution was used for each test species. The addition of lysozyme decreased (improved) the C_t values from approximately 36 to approximately 31. The optimal primer and probe concentrations were 0.4 µm and 120 nM, respectively.

To determine analytical reproducibility and repeatability, both intra- and interstrain (that is, isolate) comparison runs were performed by using serial dilutions of multiple isolates wherever possible; only a single isolate of *M. hemophilum* was available. For intrastrain comparison runs, serial 10-fold dilutions of individual isolates for each *Mycobacterium* spp. were made and then run on the same plate, with 4 replicates of each dilution. The qPCR results for the intrastrain analysis are provided in Table 4 and Figure 3. For interstrain analysis, serial dilutions were created for each isolate of *M. marinum* and *M. chelonae* to compare the sensitivity of each simplex assay for each isolate at various concentrations. The qPCR results are provided in Table 5 and Figure 4.

qPCR analysis of fresh-frozen samples. The assays detected the respective organism in 100% of the infected fish. Of noteworthy mention, the C_t values were lower (that is, bacterial loads were higher) with chronic infections (infected fish that survived until euthanized, approximately 3 wk on average) than in zebrafish that died within days of injection (data not shown). For example, for fresh-frozen fish infected with *M. chelonae*, early deaths led to C_t values as high as 36, whereas chronic infections (approximately 8 wk) resulted in a C_t value as low as 31.

qPCR analysis of FFPE samples: scrolls compared with cores. The DNA quantity and quality and the results from the 3 PCR assays are provided in Table 2. The same 6 test samples for each *Mycobacterium* spp. were evaluated by using both the scroll and core sampling technique. None of the control samples taken from empty paraffin blocks interspersed between infected samples tested positive in any of the simplex qPCR assays (Table 2); thus diagnostic specificity was 100% for both scrolls and cores. In general, the yield from paraffin-embedded material was fairly low for each qPCR assay, with the lowest levels obtained from *M. chelonae*-infected fish (Figure 5). In core samples, each mycobacterial infection was detected in at least some of the fish, yielding a diagnostic sensitivity of 33.3% (2 of 6) for *M. marinum*, 50% (3 of 6) for *M. chelonae*, and 66.7% (4 of 6) for *M. haemophilum*. The diagnostic sensitivity for detection of mycobacterial infections in scrolls was slightly more variable between species: *M. marinum*, 66.7% (4 of 6); *M. chelonae*, 16.7% (1 of 6), and *M. haemophilum*, 50% (3 of 6).

Table 3. Detection limits of simplex PCR assays for mycobacterial isolates

Isolate	Lowest dilution at which a signal was detectable[a]	CFU[b]
M. marinum MmE	10^5	30,000
M. marinum MmW	10^5	30,000
M. marinum OSU214	10^6	300,000
M. chelonae H1E1	10^4	3000
M. chelonae H1E2	10^6	300,000
M. chelonae H1E3	10^4	3000
M. haemophilum	10^5	30,000

[a]Based on serial 10-fold dilutions, starting at 10^8 (with an estimated 3×10^8 cells per mL).
[b]Number of colony-forming units obtained after plating of 100 µL.

Discussion

The primary goal of this project was to develop simplex real-time qPCR assays to detect the 3 most problematic mycobacterial species for screening and epidemiologic purposes in zebrafish husbandry and research. A secondary goal was to optimize the assays for a variety of specimen types—bacterial isolates, fresh-frozen zebrafish, and FFPE zebrafish—from both tissue sections (scrolls) and by targeting lesions in embedded zebrafish (cores).

The developed simplex qPCR assays we described here are specific for the examined mycobacterial species (*M. marinum, M. chelonae* and *M. haemophilum*) because none generated amplification products from other bacterial isolates of species within and outside of their genus. For bacterial isolates, the limit of detection varied among isolates within a species, likely due to variant genetic variation in *M. marinum* and *M. chelonae hsp65* sequences. Nonetheless, an acceptable limit of detection ranging from 30 to 3000 CFU was obtained regardless of the isolate or species being tested. Mycobacteria, in general, are known to have a high limit of detection in PCR assays (that is, require more organisms to be detected), given that robust cell walls protect their DNA.[5,14] Even with a lysozyme step added prior to nucleic acid isolation, it is difficult to obtain a large quantity of DNA.[17] Therefore, the number of colony-forming units required to detect mycobacteria by using PCR assays is greater than those documented for other bacteria.[5] Alternatively, perhaps the calculation of CFU counts by using the MacFarland method overestimated the bacteria present in the stock solution, such that sensitivity is actually higher than reported.

For fresh-frozen samples, each simplex assay achieved 100% analytical sensitivity, because we obtained positive results from all of the experimentally infected fish. The C_t values differed with chronicity of infection, such that lower C_t values were seen with chronic (established) infections, averaging about 5 cycles fewer than those of acute infection (that is, C_t of 31 instead of 36). This finding suggests a greater number of bacterial organisms in chronic infections, whereas fewer organisms are present in acute infections before substantial bacterial replication and dissemination to organs. Alternatively, mortalities that occurred within days after inoculation might have resulted from stress associated with anesthesia and intraperitoneal injection, even though all fish were handled with great care. These findings are of importance with regard to the duration of quarantine and timing of testing of quarantined fish. False negative results may occur when testing fish that die shortly after arriving at a facility and may warrant later testing (that is, in 2 to 3 w) of any fish cohoused with the early mortalities.

Using paraffin scrolls, one group obtained PCR product from 75% and 88% of *M. chelonae*- and *M. marinum*-infected zebrafish, respectively.[17] In addition, although these PCR tests were directed toward heat-shock protein DNA, the assay was not quantitative. We found somewhat more variable results, with positive samples ranging from 33.3% to 66.7% for core samples and 16% to 80% for scrolls. Detection of *M. haemophilum* was the most reliable of the 3 examined assays. A previous protocol used a different nucleic acid isolation kit and had different cycling parameters for a conventional PCR assay, as compared with the real-time PCR assay we developed in the current project.[17] These differences might account for the varying percentages of detection. Moreover, the primers and probes used in our assays were created initially in the hope of developing a multiplex PCR

Table 4. Intraassay comparison of qPCR assays for mycobacteria

Dilution	Replicate number 1	2	3	4	Mean	1 SD	CV (%)
M. marinum (mME)							
10^2	not done	39.40	not done	not done			
10^3	not done	not done	37.41	36.00	36.70	0.99	2.72
10^4	32.29	33.90	33.75	32.68	33.15	0.79	2.39
10^5	29.98	29.94	29.56	29.73	29.80	0.19	0.66
10^6	26.93	26.24	27.91	26.34	26.86	0.76	2.86
10^7	23.02	22.97	23.63	22.58	23.05	0.43	1.89
10^8	19.89	20.09	19.69	19.27	19.73	0.34	1.77
M. chelonae (H1E1)							
10^5	36.03	35.57	36.00	35.21	35.70	0.38	1.09
10^6	33.54	32.85	33.10	33.04	33.13	0.28	0.87
10^7	29.31	28.39	29.19	28.50	28.85	0.46	1.61
10^8	25.19	25.10	25.48	not done	25.26	0.19	0.78
M. haemophilum							
10^2	not done	39.58	not done	38.70	38.70	0.62	1.61
10^3	35.44	36.44	34.95	35.76	35.65	0.62	1.75
10^4	32.57	32.00	31.69	32.74	32.25	0.49	1.52
10^5	28.79	28.38	28.45	28.35	28.49	0.20	0.71
10^6	25.77	25.61	24.45	24.96	25.20	0.60	2.40
10^7	21.71	21.85	21.32	21.65	21.63	0.22	1.03
10^8	17.45	18.02	18.69	17.98	18.04	0.50	2.82

Data are given as the C_t value.

assay (data not shown), which places stricter requirements on primers and probes than does a simplex PCR assay. The percentage homology between primer and probe sets is lower for multiplex than simplex PCR assays, to avoid the production of undesired PCR products such as primer dimers.

Decreased diagnostic sensitivity is a known challenge for qPCR tests on FFPE samples and most likely is due to DNA degradation during the fixation process.[3] A previous study found that fixative type, time in fixative, and mycobacterial species showed no statistical relationship with efficacy.[17] In that study, more than half the fish held for 45 d in fixative before being processed into paraffin were positive. However, some known positives held for only 3 d in fixative yielded a negative PCR result. Tissue decalcification can similarly negatively affect DNA quantity and quality, but this step is unavoidable when preparing whole zebrafish for histologic examination. The effect is highly variable and depends on the specific acid used, with EDTA and formic acid being least detrimental.[23] The decalcification solution applied to fixed zebrafish in the current study uses formic acid. Other factors that can reduce the sensitivity of PCR-based assays are the binding of primers or probes to the DNA of other bacteria present in the samples (such as the gut and skin microbiome) and the copy number of the targeted gene. None of the runs of our assays yielded primer dimers or nontarget amplification products. Because bacterial 16S rRNA codon copy numbers typically range from 1 to 15, targeting the 16S–23S rRNA internal transcribed spacer has the potential to increase sensitivity when determining bacterial loads.[35] However, most of the mycobacterial species examined to date, including *M. marinum*, contain only a single copy of the 16S rRNA gene (ribosomal RNA database at the University of Michigan).[6] Assays targeting the 16S rRNA gene for speciation generally rely on posthoc analyses, such as sequencing or capillary gel electrophoresis, which are unlikely to be available to diagnostic units and increase the cost of testing.[4] A *Mycobacterium* spp.-general PCR assay will differentiate FFPE samples that are negative with the specific tests and contain other species of mycobacteria from those that are negative due to inadequate, degraded, or absent DNA in the sample.[20]

In an effort to improve DNA yield, we tested different sampling techniques—scrolls compared with cores—but neither technique was superior. One reason for this finding may be the manner in which different *Mycobacterium* spp. distribute throughout infected zebrafish. Both *M. marinum* and *M. haemophilum* infections in zebrafish caused widespread granuloma formation as previously reported.[24,28] In contrast, *M. chelonae* infection in zebrafish results in a more limited or focal pattern of infection, with acid-fast bacilli aggregating along the lining of the swim bladder and pericardial cavity as well as in ovaries and eggs.[29] Scrolls may prove more efficient for organisms that are scattered in a variety of tissues, whereas core samples may work best with organisms that typically form large aggregates or have a known affinity for or an increased prevalence in specific organs or tissues. The core technique offers 2 major advantages. First, it eliminates the risk of cross-contamination during sectioning of paraffin blocks and thus the need for negative controls between sampling of specimens.[34] Second, zebrafish for histology are usually divided into 2 pieces by using a midsagittal cut after fixation; the pieces are embedded together and positioned so that sections are taken from this location. Acid-fast stained slides can therefore be matched so that cores can be obtained from precise locations in any organ where infected tissue is identified. Mycobacteria, including nonpathogenic strains, are common in freshwater aquaria.[22,30,36] The intestinal lumen of zebrafish is often colonized by these bacteria; consequently, scrolls from a fish with extraintestinal mycobacteriosis likely could be contaminated by gut mycobacteria that are not involved with the disease.[18]

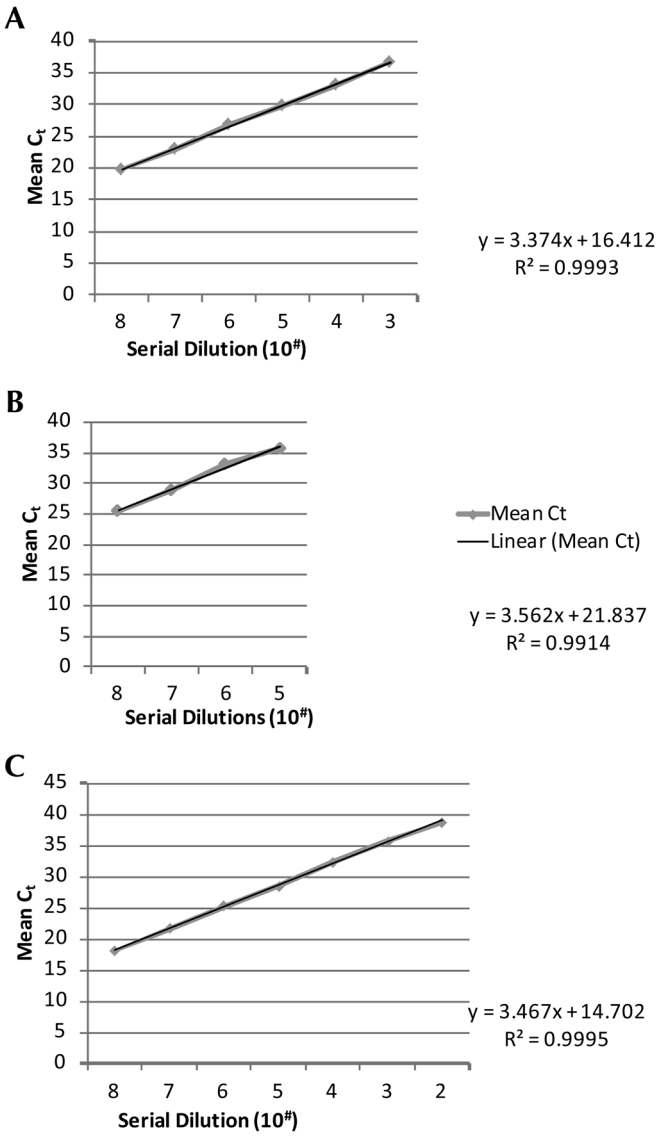

Figure 3. Intraassay comparisons for mycobacterial simplex qPCR assays. Shown are qPCR cycle threshold (C_t) values (*y* axis) for serial 10-fold dilutions of the titer (CFU; *x* axis) of single isolates. For each isolate, all dilutions were run in 4 replicates on the same plate. All assays showed high reproducibility and repeatability, with *r* values that exceeded 0.99. The calculated formulas for each qPCR assay are provided in the respective graph. Note the different lengths of the log scale for individual qPCR assays and mycobacterial species. Sensitivity was highest for *M. haemophilum* and lowest for *M. chelonae*. (A) *M. marinum*, MmE isolate. (B) *M. chelonae*, H1E1 isolate. (C) *M. haemophilum*.

We also evaluated both the purity and concentration of DNA isolated from FFPE samples to determine whether these factors affected the ability of the assay to detect infection on the basis on sampling technique. However, neither DNA purity nor DNA concentration rendered one sampling technique superior over the other. Because of the relatively low diagnostic yield from FFPE samples, we recommend testing multiple (5 or 6) zebrafish for detection of infection in a population, in view of our current results. This strategy is practical because typically multiple zebrafish from a given population are submitted for histology for evaluation of sentinels or when an outbreak occurs. We are providing these tests to the zebrafish community currently, and they were recently used in pathogen survey studies at 2 large facilities.[11,13]

Table 5. Sensitivity of qPCR assay across isolates

	Isolate		
Dilution	MmE	MmW	OSU214
M. marinum			
10^8	29.58	28.00	29.55
10^7	33.44	31.67	32.72
10^6	36.66	34.74	37.25
10^5	38.82	37.28	not done
M. chelonae	H1E1	H1E2	H1E3
10^8	24.54	29.44	27.14
10^7	27.64	32.89	30.32
10^6	34.62	36.00	33.28
10^5	37.38	not done	38.19

Data are given as the C_t value.

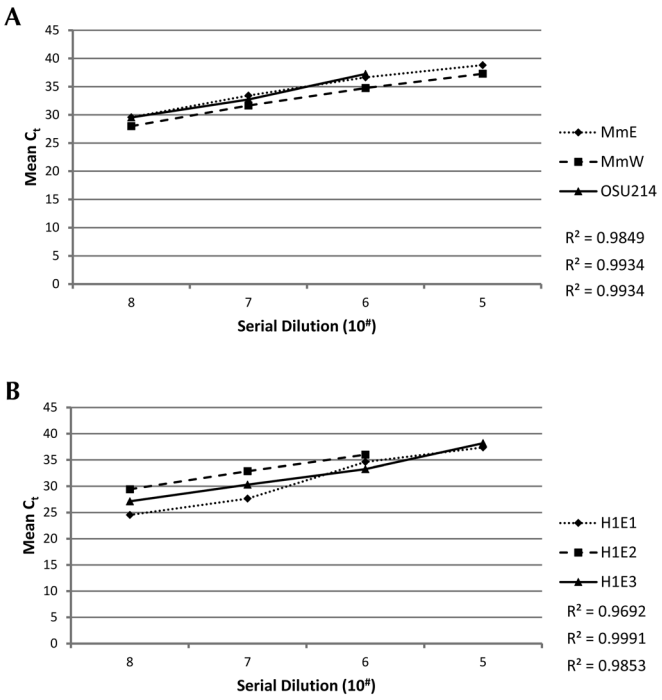

Figure 4. Sensitivity of simplex qPCR assays across *M. marinum* and *M. chelonae* isolates. Shown are qPCR C_t values (*y* axis) for serial 10-fold dilutions of titers (CFU; *x* axis) of 3 isolates per mycobacterial species. For each isolate, all dilutions were run in 4 replicates on the same plate and are depicted as decreasing concentrations. (A). The sensitivity of the *M. marinum* qPCR assay was nearly identical among all isolates: MmE (dotted line and diamonds), MmW (dashed line and squares), and OSU214 (solid line and triangles). (B) The sensitivity of the *M. chelonae* qPCR assay varied slightly among isolates from testes (listed from least to most sensitive): H1E2 (dashed line and squares), H1E3 (dotted line and triangles), and H1E1 (solid line and diamonds).

In conclusion, the simplex qPCR assays we developed are sensitive, specific, repeatable, and reproducible for the detection of the 3 most pertinent *Mycobacterium* pathogens of zebrafish. Future applications for these assays include screening for newly acquired fish to improve biosecurity and as a diagnostic test for laboratories with high rates of mortality or morbidity. To further minimize the resources needed for detecting infection,

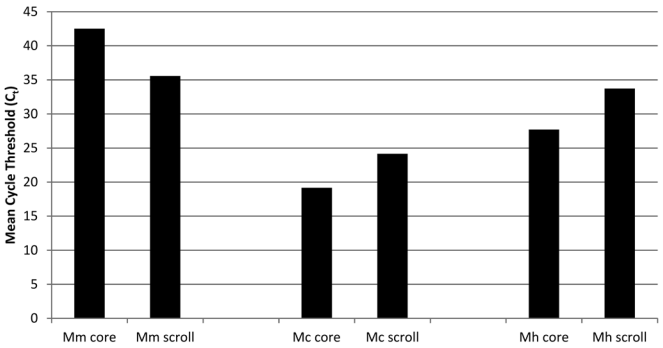

Figure 5. Mean C_t values of cores compared with scrolls of FFPE fish experimentally infected with *Mycobacterium* spp. Shown are mean C_t values for cores compared with scrolls obtained by respective simplex quantitative PCR assays for *M. marinum* (OSU214), *M. chelonae* (H1E1), and *M. haemophilum*.

the development of a multiplex qPCR would be beneficial, because it is a more efficient and economical approach than are simplex assays.

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