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Comparison of fixatives and fixation time for PCR detection of *Mycobacterium* in zebrafish *Danio rerio*

Tracy S. Peterson[1,*], Michael L. Kent[1,2], Jayde A. Ferguson[4], Virginia G. Watral[1], Christopher M. Whipps[3]

[1]Department of Microbiology, Oregon State University, Corvallis, Oregon 97331, USA
[2]Department of Biomedical Sciences, Oregon State University, Corvallis, Oregon 97331, USA
[3]Department of Environmental Science and Forestry, State University of New York College of Environmental Science and Forestry, SUNY-ESF, 1 Forestry Drive, Syracuse, New York 13210, USA
[4]Alaska Department of Fish and Game, Commercial Fisheries Division, Pathology Laboratory, Anchorage, Alaska 99518, USA

ABSTRACT: Mycobacteriosis is a common disease of laboratory zebrafish *Danio rerio*. Different infection patterns occur in zebrafish depending on mycobacterial species. *Mycobacterium marinum* and *M. haemophilum* produce virulent infections associated with high mortality, whereas *M. chelonae* is more widespread and is not associated with high mortality. Identification of mycobacterial infections to the species level provides important information for making management decisions. Observation of acid-fast bacilli in histological sections or tissue imprints is the most common diagnostic method for mycobacteriosis in fish, but only allows for diagnosis to the genus level. Mycobacterial culture followed by molecular or biochemical identification is the traditional approach, but DNA of diagnostic value can also be retrieved from paraffin blocks. Here we investigated the type of fixative, time in fixative before processing, species of mycobacteria, and severity of infection as parameters to determine whether the *hsp* gene PCR assay (primer set HS5F/hsp667R) could detect and amplify mycobacterial DNA from paraffin-embedded zebrafish. Whole zebrafish were experimentally infected with either *M. chelonae* or *M. marinum*, and then preserved in 10% neutral buffered formalin or Dietrich's fixative for 3, 7, 21, and 45 d. Subsequently, fish were evaluated by hematoxylin and eosin and Fite's acid-fast stains to detect mycobacteria within granulomatous lesions. The PCR assay was quite effective and obtained PCR product from 75 and 88% of the *M. chelonae*- and *M. marinum*-infected fish, respectively. Fixative type, time in fixative, and mycobacterial species showed no statistical relationship with the efficacy of the PCR test.

KEY WORDS: PCR · *Mycobacterium marinum* · *Mycobacterium haemophilum* · 10% NBF · Formalin · Dietrich's · Zebrafish · Infection severity · Diagnostics

INTRODUCTION

Piscine mycobacteriosis is a well-characterized disease, with several mycobacterial species infecting multiple genera and species of fish (Decostere et al. 2004, Lewis & Chinabut 2011). Zebrafish *Danio rerio* are now a widely used vertebrate animal model in biomedical research (Lele & Krone 1996, Dooley & Zon 2000, Grunwald & Eisen 2002, Rubinstein 2003, Aleström et al. 2006, Allen & Neely 2010) with an ever-increasing number of academic and private laboratories using zebrafish. Since the emergence of the zebrafish as a pre-eminent animal model, there has been a corresponding interest in diseases that may occur in this species within a laboratory setting. The Zebrafish International Resource Center (ZIRC)

*Email: fishpathman@gmail.com

in Eugene, Oregon, USA (http://zebrafish.org/zirc/home/guide.php), has provided a diagnostic service to the zebrafish research community since 2000. Mycobacteriosis, based on observation of acid-fast bacteria in histological sections, has been detected at 41% of submitting facilities in about 150 cases with over 500 fish (http://zebrafish.org/zirc/health/diseaseManual.php).

Several species of *Mycobacterium* have been reported to cause disease in zebrafish, including *M. chelonae* (Astrofsky et al. 2000, Kent et al. 2004, Murray et al. 2011), *M. peregrinum* (Kent et al. 2004), *M. haemophilum* (Whipps et al. 2007, 2012), and *M. marinum* (Ostland et al. 2008). Based on observations reported in diagnostic cases (Astrofsky et al. 2000, Kent et al. 2004, Whipps et al. 2008, 2012, Murray et al. 2011) and laboratory transmission studies (Watral & Kent 2007, Whipps et al. 2007, Ostlander al. 2008), the severity of mycobacteriosis is usually related to the *Mycobacterium* species causing the infection. *M. chelonae* is relatively widespread and causes chronic infections but minimal mortalities (Whipps et al. 2008, Murray et al. 2011). There have been 6 outbreaks of *M. haemophilum,* which caused severe infections with high but chronic mortality (Whipps et al. 2007). *M. marinum* is uncommonly isolated from cases of mycobacteriosis in zebrafish, but when infection occurs it is associated with acute disease and high mortalities (Watral & Kent 2007, Ostland et al. 2008).

Histology is the primary diagnostic method that we use with zebrafish, but diagnosis of mycobacterial infections by histology only allows for identification of the bacteria to the genus level. Of the over 150 cases diagnosed histologically by ZIRC, identification of the bacteria to the species level using culture or molecular methods has been achieved in fewer than 20 cases (Watral & Kent 2007, Whipps et al. 2007, 2008, 2012). Mycobacterial DNA can be amplified from human and animal (including fish) tissues from paraffin blocks (Ghossein et al. 1992, Miller et al. 1997, Marchetti et al. 1998, Zink & Nerlich 2004, Pourahmad et al. 2009a). Efforts to develop PCR assays that would reduce the time required for diagnosis as well as increase both the specificity and sensitivity of detecting mycobacteria in formalin-fixed, paraffin-embedded tissues have been ongoing, and are mostly focused on human mycobacteriosis (Pao et al. 1988, 1990, Fiallo et al. 1992, Hardman et al. 1996, Rish et al. 1996, Osaki et al. 1997, Salian et al. 1998, Whittington et al. 1999, Singh et al. 2000, Baba et al. 2008) and to a lesser extent, mycobacterial infections of animals including fish (Gyimesi et al. 1999, Puttinaowarat et al. 2002, Pourahmad 2009a,b). Results have been mixed with this approach, and time in fixative before processing into paraffin blocks appears to be an important factor for successfully obtaining mycobacterial DNA from tissues (Tokuda et al. 1990, Greer et al. 1991).

Fixative formulations, especially those containing acids or alcohol at various concentrations, can also influence the ability to retrieve DNA (Eltoum et al. 2001). Most human and veterinary laboratories use 10% neutral buffered formalin as a fixative, which is also used for preservation of zebrafish and tissues from other fishes (Ferguson 2006, Harper & Lawrence 2011). However, zebrafish have been traditionally preserved in Dietrich's fixative, a mixture of chemicals that contains acetic acid and 95% alcohol in addition to formaldehyde. The ZIRC zebrafish diagnostic program is largely based on histologic evaluation of whole fish specimens; however, there is a need to identify mycobacterial infections to the species level in order to allow for informed management of these infections. Subsequently, we developed a PCR test for retrieval of mycobacterial DNA and evaluated the influence of time and fixative (either 10% neutral buffered formalin or Dietrich's) on the ability to recover DNA for PCR assays. This was achieved by experimentally infecting zebrafish with either *Mycobacterium chelonae* or *M. marinum*, and preserving the infected fish in both fixatives for various time points up to 45 d.

MATERIALS AND METHODS

Mycobacterial cultures used for inoculation were prepared in the following manner. Stock cultures from the Kent laboratory reference collection of *Mycobacterium marinum* OSU 214 (*M. marinum*) and *M. chelonae* H1E2 (*M. chelonae*) were incubated on Columbia colistin and nalidixic acid 5% sheep blood and Middlebrook 7H10 agar (Remel) for 14 d at a temperature of 28°C. Fresh culture material from agar plates was loop-inoculated into sterile phosphate-buffered saline (PBS), washed 3 times to eliminate carry-over of antimicrobial chemicals from the agar plates and then adjusted in sterile PBS to an optical density of 1.0 based on the MacFarlane scale, yielding an approximate dose of 1×10^6 bacteria per 25 µl for the final injection amount.

AB strain adult zebrafish of mixed sex were obtained from the Sinnhuber Aquatic Research Laboratory at Oregon State University as experimental animals. Fish were housed in a biosafety-

level 2 laboratory and maintained for 12 wk after inoculation, allowing for progression of infection, in triplicate flow-through tanks at 25 to 27°C with supplemental aeration and a 14:10 h light:dark photoperiod. For inoculation with *Mycobacterium marinum* and *M. chelonae*, fish were anesthetized with 100 mg l^{-1} tricaine methanosulfonate (MS-222) and then aseptically given intraperitoneal injections in the right flank, to avoid accidentally lancing the spleen, with 25 µl of either sterile PBS (sham-inoculated control group) or the prepared *M. marinum* and *M. chelonae* inocula. At the end of 12 wk, moribund fish and all surviving fish were euthanized by overdose of MS-222, the operculae were removed, and fish were incised along the ventrum in order to expose viscera to the fixative solutions, then immediately placed into 15 ml of either Dietrich's fixative or 10% neutral buffered formalin (NBF). Fish were held in Dietrich's fixative or 10% NBF for 3, 7, 21, and 45 d (Table 1).

Table 1. *Danio rerio* infected with *Mycobacterium* spp. Proportion of PCR positives (primer set HS5F/hsp667R) and mean granuloma intensity in paraffin-embedded zebrafish. Fish were preserved in either 10% neutral buffered formalin (NBF) or Dietrich's fixative for various times (days, in parentheses) before processing. There was no statistical significance in prevalence between mycobacterial species, fixatives, or duration of fixation (all $p \geq 0.3$, Fisher's exact test). Granulomas $fish^{-1}$ are reported as the mean for the total group, and the mean of PCR negative and positive fish

Group	PCR +ve/ no. tested	Granulomas $fish^{-1}$		
		Total	PCR−ve	PCR+ve
M. chelonae				
NBF (3 d)	5/5	14.4	–	–
NBF (7 d)	4/5	6.2	9.0	5.5
NBF (21 d)	3/5	15.4	19.5	12.7
NBF (45 d)	1/5	5.6	5.5	6.0
Dietrich's (3 d)	4/5	8.4	14.0	7.0
Dietrich's (7 d)	4/5	8.0	1.0	9.75
Dietrich's (21 d)	4/5	11.8	15.0	11.0
Dietrich's (45 d)	5/5	8.6	–	–
M. marinum				
NBF (3 d)	5/5	14.6	–	–
NBF (7 d)	5/5	35.0	–	–
NBF (21 d)	5/5	38.0	–	–
NBF (45 d)	5/5	20.2	–	–
Dietrich's (3 d)	4/5	36.2	26.0	38.75
Dietrich's (7 d)	4/5	33.0	25.0	35.0
Dietrich's (21 d)	4/5	20.8	16.0	22.0
Dietrich's (45 d)	3/5	29.0	22.5	33.3

Histology and PCR

Decalcification was performed on the fixed zebrafish samples as follows. Fish preserved in Dietrich's fixative were decalcified in 5% trichloroacetic acid overnight (following the ZIRC protocol), while fish preserved in 10% NBF were placed in CalExII (Fisher Scientific) for 48 h. After decalcification and rinsing for 30 min with de-ionized water, all fish were transferred to 70% ethanol and held no longer than 48 h before processing. Following fixation, zebrafish were processed for paraffin embedding by routine procedures. Sections examined for histology were cut at 5 µm and stained with hematoxylin and eosin as well as Fite's acid-fast stain for detection of acid-fast bacilli (Luna 1968).

PCR scrolls were made from dry tissue sections cut from blocks at 5 µm thickness after decontamination and prepared for DNA extraction in the following manner. Prior to sectioning the scrolls, each tissue block was wiped down with 1 DNA AWAY™ wipe (Molecular BioProducts), then faced with a new microtome blade. The block face was wiped with a fresh DNA AWAY™ wipe, the microtome blade changed, and 12 sections (5 µm) cut in a scroll. Two scrolls, handled with clean wooden toothpicks, were placed into a sterile, pre-labeled 1.5 ml microfuge tube. Microtome blades were discarded after cutting scrolls for each tissue block, in order to avoid cross-contamination. The entire microtome was then wiped down with xylene (to remove excess paraffin waste) and 100% ethanol, allowed to air dry, and then wiped down with a DNA AWAY™ wipe and air-dried. Gloves were changed between microtome cleaning, blade changes, and tissue block handling. After every 5 blocks, scrolls were obtained from a block containing a negative zebrafish from the Sinnhuber Aquatic Research Laboratory specific pathogen-free colony. All tissue blocks and PCR scrolls were prepared in an identical manner.

Paraffin-embedded tissue sections were washed twice with 1.2 ml of xylene and then twice with the same volume of 100% ethanol, which followed the recommended protocol for the Qiagen DNeasy Blood & Tissue Kit. The tissue pellet was suspended in 375 µl of ATL buffer, and Antifoam A (Sigma-Aldrich) was added to a final concentration of 1%. This was transferred to a 0.5 ml screw cap tube containing 300 µl of 0.1 mm Zirconia/Silica Beads (BioSpec Products) and placed on a Mini-BeadBeater 16 (BioSpec Products) for 3 min. The bead beating step was incorporated, as preliminary studies showed that this yielded more positive results. Thirty µl of 20 mg ml^{-1} Proteinase K were added to this disrupted

tissue and digested overnight in a 45°C water bath. The sample was centrifuged, and then 200 µl were removed and DNA was extracted following the manufacturer's instructions. PCR was carried out with primers HS5F (GTC ATC ACC GTC GAG GAG) and hsp667R (Selvaraju et al. 2005), yielding a 156 bp product of *hsp65* sequence. The new primer HS5F was designed to be a *Mycobacterium*-general primer based on sequence alignments of approximately 80 representative sequences. The primer locations are as follows, based on the reference sequence from *Mycobacterium leprae* (GenBank accession M14341): HS5F (655–672) and hsp667R (792–810). The short fragment size of the target region was used to maximize efficiency of amplification of the potentially low quality and cross-linked DNA in preserved specimens. Amplifications were performed on a C1000™ Thermal Cycler (BioRad Laboratories) with initial denaturation at 95°C for 3 min, followed by 35 cycles of 94°C for 30 s, 54°C for 45 s, 68°C for 60 s, and a final extension at 68°C for 7 min. Product amplification was evaluated by observation on a 2% agarose gel. To confirm the specificity of the PCR reactions, products from a subset of 3 positives each of *M. chelonae* and *M. marinum* samples were sequenced. Amplification products were purified using the E.Z.N.A. Cycle Pure Kit (Omega Bio-Tek), and direct sequencing was performed using primer HS5F on the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1, using the ABI3730xl Genetic Analyzer (Applied Biosystems).

Severity of infection

Individual infected fish were scored as light or heavy infections by enumerating granulomas containing acid-fast bacteria in 2 whole-body sagittal sections per fish per slide. For both *Mycobacterium marinum* and *M. chelonae*, light and heavy infections were classified as 1–6 and >6 granulomas, respectively.

Statistics

All statistical analyses were conducted with R version 2.7.2 (R Development Core Team). Significance was set at $p \leq 0.05$, and p-values are 2-tailed. Differences in PCR prevalence between *Mycobacterium* species, fixative types, and fixation duration were tested with Fisher's exact test. A generalized linear regression model was used to evaluate the influence that fish sex, *Mycobacterium* species, fixative type, fixation time, and number of granulomas (indicating level of infection) detected by histology had on the ability of the PCR (primer set HS5F/hsp667R) to successfully detect *Mycobacterium* DNA. Logistic regression was the most appropriate technique to evaluate these effects because the response of PCR detection is a binary variable. This model had a logit link function, and an interaction effect of fixative and fixation time was explored. The likelihood ratio test was used to determine how well the model fit the data compared to the null model of just the intercept. Individual variables and combinations thereof were also tested using a backwards elimination technique.

RESULTS

DNA was successfully recovered from a large number of the histology-positive samples, irrespective of total fixation time, type of fixative, or *Mycobacterium* spp. (Table 1). There was no significant interaction effect of fixative and fixation time ($p > 0.99$), so this variable was removed, and the resulting model that contained the variables of interest did not fit the data as well as the null model ($p = 0.37$; $\chi^2 = 7.5$, df = 7), meaning that none of these variables explained the variation in the detection of mycobacterial DNA by PCR. Similar results were obtained by evaluating individual variables and combinations thereof. Fixation time of 45 d showed a trend towards fewer PCR positives, and 1 of the 4 groups at this time period (*M. chelonae* in 10% NBF) had only 1 of 5 positive samples. Although this time in fixative was not a statistically significant influence, it was suggestive of having an effect ($p = 0.08$). None of the histology-negative control fish that were included throughout the evaluations was positive by PCR. Sequencing of 3 *M. marinum* and 3 *M. chelonae* PCR-positive samples confirmed their identity.

Severity of infection was also evaluated as an explanatory variable. Mortality occurred in the *Mycobacterium marinum* group, and overall these fish exhibited about 3 times the number of granulomas containing acid-fast bacteria than the *M. chelonae*-infected fish (29.6 versus 9.4 granulomas fish^{-1}, respectively) that were positive by PCR (Table 1). The *M. chelonae* groups yielded fewer positive fish by PCR, but there was no significant relationship with PCR compared to either *Mycobacterium* species or number of granulomas fish^{-1}. There was also no significant difference between fixative types.

DISCUSSION

Evaluation of DNA sequences has become a cornerstone in bacterial species identification. This is certainly the case for *Mycobacterium* species, as they are often fastidious, grow slowly, and typically provide few culture-specific or biochemical traits by conventional methods that facilitate species-level identification (Daniel 1990, Cousins et al. 1992). It is often more useful to obtain sequences for diagnoses directly from infected tissues, particularly for *Mycobacterium* species that are slow growing or require specialized media. This approach has been used to diagnose mycobacterial infections directly from frozen or freshly infected fish (Whipps et al. 2003, 2007, Kaattari et al. 2005, Poort et al. 2006). Often only formalin-preserved tissues embedded in paraffin are available, which has led to attempts to obtain mycobacterial DNA from paraffin-embedded tissues following observation of either chronic lesions (i.e. granulomas) or the presence of acid-fast bacteria in tissues that are indicative of mycobacterial infection (Ghossein et al. 1992, Miller et al. 1997, Marchetti et al. 1998, Zink & Nerlich 2004, Pourahmad et al. 2009a,b).

Fixatives used to preserve tissues (regardless of whether they are coagulant or non-coagulant fixatives) and the total time the tissues are held in a fixative can result in degradation of DNA and RNA within tissues (Dubeau et al. 1986, Fiallo et al. 1992, Foss et al. 1994). Exposure of mycobacterial DNA to formalin causes production of Schiff bases on the free amino groups of nucleotides (Fraenkel-Conrat 1954, Dubeau et al. 1986), and subsequent cross-linking between tissue proteins and DNA (Jackson & Chalkey 1974). Therefore, time in formalin is considered to be among the most important criteria for successful DNA retrieval and PCR amplification following tissue processing for histology (Ben-Ezra et al. 1991, Greer et al. 1991). Once the tissues are embedded in paraffin wax, this degradation process is slowed, and subsequent amplification of short DNA sequences is successful (Shibata et al. 1988). Fish are often preserved at a research laboratory, aquaculture facility, or in the field, and then shipped many days later to another laboratory for histology processing. Hence, there was particular interest in the effects of time in fixative. Positive results were consistently obtained with fish held in fixative up to 21 d, and the same occurred at 45 d with the exception of 1 group. This positive result at the later time points was somewhat surprising. *Mycobacterium* spp. has a unique, waxy cell wall comprised of lipids and fatty acids (Kolattukudy et al. 1997), which may exclude aldehydes to some extent. Perhaps this characteristic plays a role in preserving DNA more than in non-acid-fast bacteria exposed to formalin-based fixatives.

Dietrich's, Davidson's, and Bouin's fixatives are commonly used with fish tissues. These contain acid and alcohol, both of which cause denaturation and coagulation of proteins and nucleic acids within tissue specimens by dehydration and disruption of electrostatic and hydrogen bonding (Fournie et al. 2000, Eltoum et al. 2001). Tissues preserved in Bouin's solution (which contains both picric and acetic acid) are particularly problematic for DNA retrieval (Greer et al. 1991). Interestingly, there was no reduction in positive samples with Dietrich's compared to 10% buffered formalin.

Some samples were negative in both fixatives at some of the early time points. The inability to retrieve DNA logically would be influenced by the amount of bacterial DNA. Incorporation of bead beating enhances retrieval of DNA from formalin-preserved samples to liberate more DNA (Tripathi & Stevenson 2012). This was included with the PCR protocol because from previous experiences it was found to consistently yield more positive results with PCR from paraffin-embedded tissues. Nevertheless, there was no significance in the ability to detect mycobacterial DNA based on severity of infection. The results were similar to previous transmission studies regarding the virulence of *Mycobacterium chelonae* compared to *M. marinum* in zebrafish (Watral & Kent 2007, Ostland et al. 2008, Whipps et al. 2008). The *M. marinum* isolate was highly virulent, and several fish became moribund or died over the 8 wk period, and these fish were included in 45 d samples. In contrast, all fish injected with *M. chelonae* became infected, but none exhibited morbidity or mortality. The *M. marinum*-infected fish had 3 times the number of granulomas with acid-fast bacteria, but the recovery and detection of DNA was not different than with fish infected with *M. chelonae*.

Most zebrafish cases submitted to the ZIRC and the Kent Laboratory (OSU) for health screening and diagnosis consist of multiple fish, which are usually processed and embedded within 3 wk. Hence, even with only about 50 to 70% ability to obtain mycobacterial DNA, diagnosticians can confidently use PCR on routinely processed and paraffin-embedded zebrafish to identify these infections to the species level for many of the fish within a particular case. This is important, as the severity and distribution of these various *Mycobacterium* spp. found in zebrafish are quite different, and fish health managers and cli-

nicians with species identifications in hand would be able to make more informed decisions. For example, facilities with *M. haemophilum*-infected zebrafish often euthanize infected populations, disinfect the aquaria, and repopulate (Kent et al. 2009, 2011, Whipps et al. 2012), and this approach is similarly recommended for *M. marinum* outbreaks. In contrast, *M. chelonae* infections are presently managed by cleaning tanks more frequently (Murray et al. 2011) or using different wild-type strains of zebrafish (Whipps et al. 2008). Acid-fast bacteria are frequently observed in the intestinal lumen of zebrafish, with and without infections in the visceral organs. Therefore, a concern with amplification of DNA using whole fish sections may be confusion of mycobacteria in granulomas versus intraintestinal mycobacteria that may be associated with other constitutive microbiota in the intestinal tract. A solution for the potentially confounding problem of intraintestinal bacterial contaminants would be to use histologic sections as a template to guide in the removal of specific cores from the tissue block that correspond to confirmed areas of infected organs (Sfanos et al. 2008) or, even more precisely, by using laser capture microdissection to select individual intralesional granulomas containing mycobacteria for PCR assays (Ryan et al. 2002, Zhu et al. 2003, Selva et al. 2004). Both methods would allow for accurate selection of infected tissue sites within the infected zebrafish prior to PCR analysis for mycobacterial identification and PCR primer-specific molecular identification of *Mycobacterium* spp.

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