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Comparison of single-round polymerase chain reaction (PCR) and pepsin-trypsin digest (PTD) methods for detection of *Myxobolus cerebralis*

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ABSTRACT: Single-round polymerase chain reaction (PCR) and pepsin-trypsin digest (PTD) methods were compared for the detection of *Myxobolus cerebralis*. Parallel tests were conducted on a total of 1743 free-ranging and 400 hatchery-reared salmonids. Concurrent results were found in 84.6% of the free-ranging fish samples, and 83.5% of the hatchery samples. PCR identified *M. cerebralis* more frequently than did PTD, and did so in many geographic locations previously considered free of the parasite. Average myxospore count by PTD among both free-ranging and hatchery fish increased significantly ($p < 0.001$) with a subjective evaluation of amplicon staining intensity.

KEY WORDS: Whirling disease · *Myxobolus cerebralis* · Pepsin-trypsin digest (PTD) · Polymerase chain reaction (PCR) · Testing methods

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INTRODUCTION

Myxobolus cerebralis, the causative agent of salmonid whirling disease, was first identified in the USA in the 1950s, and now occurs in at least 21 states (Bergersen & Anderson 1997). Spread of *M. cerebralis* has been accelerated by inadvertent stocking of infected fish thought to be free of the parasite into natural populations. This is due in part to the lack of sensitivity in currently used testing procedures for *M. cerebralis*. In 1992 and 1996, fish were stocked into 226 high elevation Colorado waters from infected hatcheries thought to be free of the parasite (Schisler 1999).

A widely used presumptive test for diagnosis of subclinical *Myxobolus cerebralis* infections among adult fish is the pepsin-trypsin digest (PTD) method (Markiw & Wolf 1974). This technique involves the microscopic observation of mature myxospores that have been isolated and concentrated from enzymatically digested bone and cartilage. Another procedure for isolating myxospores is the plankton centrifuge method (O'Grodnick 1975).

A confirmatory test of *Myxobolus cerebralis* infection is usually conducted after myxospore isolation. The confirmatory test most commonly used is histological sectioning and microscopic verification of spores and trophozoites in skeletal tissues. Fluorescent antibody test (FAT) may also be used as a confirmatory test (Markiw & Wolf 1978, Thoeson 1994). While these techniques are useful, very lightly infected fish, or fish carrying immature spores and trophozoites, may not be identified if the presumptive test fails to isolate mature myxospores.

Andree et al. (1998) described a nested polymerase chain reaction (PCR) test for the detection of *Myxobolus cerebralis* that is currently in use by several fish disease laboratories. This method involves the amplification of a 415 bp segment of the 18S rRNA gene from *M. cerebralis*. The primary advantage of this technique over traditional testing methods is its ability to identify

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the parasite in all life stages, thus eliminating reliance on isolation of mature myxospores. The procedure is also much less labor-intensive and more efficient than traditional methods.

The single-round PCR method is a modification of the nested PCR procedure published by Andree et al. (1998) that is reported to have the advantages of decreased likelihood of contamination and reduced expenses associated with time, reagents, materials and labor (Epp & Wood 1998). This method relies on the use of a modified Tr 5-16 primer (Tr 5-16m) and the Tr 3-17 primer from the nested procedure. Tr 5-16 is used as the forward primer in the first round of the nested PCR procedure, while the Tr 5-16m primer is used as the sole forward primer in the single-round PCR procedure. Tr 3-17 is used as the reverse primer in the second round of the nested PCR procedure, and as the sole reverse primer in the single-round PCR procedure.

PCR testing has not been benchmarked as a standard diagnostic technique for detecting *Myxobolus cerebralis*, and its practicality and reliability is the subject of much debate (United States Animal Health Association 1998). Much of the controversy surrounding PCR techniques is the ability of the procedure to detect genomic material of the target organism that may or may not have been derived from a living pathogen. Many people have argued that genomic evidence is not biologically significant. The extreme sensitivity of the technique also makes it prone to contamination resulting in false positive results. Further concern over the use of the technique is the possibility that DNA fragments from related organisms could be amplified and falsely identified as the target organism. Free-ranging fish stocks are often, and many times needlessly, considered at risk of collapse once identified as *M. cerebralis* positive. Fish rearing units producing fish thought to be infected with the parasite are considered tainted, and even a suggestion of *M. cerebralis* infection greatly reduces the value and marketability of these fish. PCR is being used for detection of many bacterial fish pathogens, including *Aeromonas salmonicida* (Hiney et al. 1992, Hiney 1994), *Renibacterium salmoninarum* (Brown et al. 1994, 1995, McIntosh et al. 1996), *Yersinia ruckeri* (Argenton et al. 1996) and others. As with PCR testing for *M. cerebralis*, these methods have not been benchmarked as standard diagnostic tests.

Over the course of the 1998 and 1999 field seasons, a study of *Myxobolus cerebralis* distribution in Colorado's high elevation waters provided us with the opportunity to field test the single-round PCR technique in parallel with the standard PTD method. The objective of this study was to test the single-round PCR method on a large scale, and determine how well the technique performs when compared to the traditional PTD method as a presumptive test for *M. cerebralis* infection in a field sampling scenario.

MATERIALS AND METHODS

Free-ranging fish samples. Resident rainbow trout *Oncorhynchus mykiss*, brown trout *Salmo trutta*, brook trout *Salvelinus fontinalis*, and cutthroat trout *Oncorhynchus clarki* were sampled by electrofishing, hook-and-line, and gill nets in a variety of habitats with various stocking histories and ranging in elevation from 2103 to 3840 m. Fish kept for testing were at least 1 yr old, ensuring that mature myxospores would be present for the PTD testing. A total of 72 lakes and streams were sampled in this manner and a total of 1900 fish were collected. Parallel PCR and PTD tests were conducted on 1743 of these fish.

Fish were frozen and transported to the Colorado Cooperative Fish and Wildlife Research Unit Fish Laboratory, where heads from each fish were removed immediately caudal to the operculum and split sagittally along the longitudinal axis, using new disposable scalpels and cutting surfaces for each fish. One half of each head was placed into either a centrifuge tube or a 177 to 354 ml collection cup, depending on its size, and refrozen. The other half of each head was placed into an individual plastic bag, then into plastic storage boxes and refrozen.

Sagittal halves of heads placed in centrifuge tubes or collection cups were tested for *Myxobolus cerebralis* DNA with single-round PCR. DNA from samples was prepared with a spin column purification procedure (DNeasy Tissue kit, Qiagen, Valencia, CA) with the following minor modifications. Fish samples were heated to 95°C in deionized H_2O for 15 min in screw capped polypropylene test tubes. The heads were defleshed using clean scalpels and the bone and cartilage was placed in microfuge tubes containing 180 µl ATL tissue lysis buffer, 20 µl Proteinase K solution (both from DNeasy Tissue kit, Qiagen, Valencia, CA), 150 mg small (710 to 1180 µm) and 3 large (3 mm) acid washed glass beads. The samples were incubated at 55°C for 60 min with occasional vortexing. They were then centrifuged at 16 000 × *g* for 5 min, and 200 µl of the aqueous supernatant was transferred to clean microfuge tubes. From this point, the procedure continued according to the 'tissue lysis protocol' included in the DNeasy kit, with a final elution volume of 200 µl.

The PCR reaction mix (20 µl total volume) consisted of 10 mM Tris-HCl, pH 8.8, 23°C, 3.5 mM $MgCl_2$, 25 mM KCl, 0.8 mM dNTPs, 0.8 µM forward primer, Tr 5-16m (5'-GCA TTG GTT TAC GCT GAT GTA GC-3'), 0.8 µM reverse primer, Tr 3-17 (5'-GGC ACA CTA

CTC CAA CAC TGA ATT TG-3'), 0.4 units *Taq* polymerase and 2.0 µl template. The thermal cycling parameters consisted of a denaturing cycle at 95°C for 2 min, followed by 35 cycles of 95°C for 30 s, 67°C for 1.5 min, and 74°C for 2.5 min.

Visualization of the PCR results by agarose gel electrophoresis and fluorescent staining was performed using 2% Metaphor agarose, 7 cm (width) × 10 cm (length) × 5 mm (depth) submarine gels, with wells 1.0 mm thick × 2.6 mm wide, in TAE buffer. Samples were 6 µl of each PCR reaction mixed with 1.5 µl loading buffer/dye. The gel was run at 12.5 V cm^{-1} for 35 min in a coolant-filled submarine gel electrophoresis unit (Hoefer HE 33, Amersham Pharmacia Biotech, Piscataway, NJ) then stained with SYBR Green dye (1/10000 dilution into TAE buffer) for 45 min. After staining, gels were photographed on a UV trans-illuminator with 254 nm short wavelength bulbs, using Polaroid 667 film and Yellow 15 and UV glass filters. Varying amplicon band intensities were routinely observed with the single-round method, and were rated as 1 of 5 categories: negative, weak positive, positive, strong positive, and very strong positive. Amplicon band intensities were determined subjectively, by comparison with amplicons from previously assigned strong positive and weak positive control sample DNA preparations. The categories for amplicon band strength and control DNA preparations were initially assigned according to the following subjective criteria: weak positive, very low band intensity near the limit of detection; positive, easily visible, sharply defined band with no broadening or smearing upward; strong positive, brightly visible band with obvious broadening and smearing; very strong positive, brightly visible band with extensive and bright upward smearing. The standardized conditions described above were used for PCR reaction parameters, agarose gel electrophoresis, post-electrophoresis staining, and UV trans-illumination and photography. Although amplicon band intensities were scored subjectively, the scores were found to be very similar between 2 individuals independently scoring the gel photographs, and were reproducible when repeated in another PCR run.

The corresponding sets of half heads were delivered to the Colorado Aquatic Animal Health Laboratory in Brush, Colorado for spore concentration. The samples were defleshed by first soaking at 45°C for as long as was necessary to soften the tissues. Soft tissues were then separated from skeletal elements by agitation in a wrist-action electric shaker using glass marbles as hammers, then decanted through disposable 190 µm calculi filters. The rinse water was added back to the skeletal elements for purification and concentration by PTD (Markiw & Wolf 1974) and quantification (O'Grodnick 1975).

Hatchery fish samples. Fish exposed to *Myxobolus cerebralis* were collected from the Chalk Cliffs State Trout Rearing Unit, Nathrop, Colorado as positive controls. Presumed-negative fish were obtained from 2 separate hatcheries outside Colorado that were free of *M. cerebralis*. These fish were processed in the same way as the free-ranging fish, and all fish were assigned random numbers to provide the laboratories with blind tests of known-exposed and presumed-negative fish.

DNA sequencing. *Myxobolus cerebralis* was identified with PCR on many occasions from fish populations with no history of whirling disease and/or past stocking of infected fish, and with no positive results from PTD testing. To verify that these positive PCR results were indeed *M. cerebralis*, an attempt was made to re-amplify and determine the nucleotide sequence of the amplicon from 40 fish, identified as 'weak positive', from remote areas where presence of the organism was not expected. DNA sequencing of the amplicons was performed by direct sequencing of a purified PCR amplicon band, excised from the gel and purified with a commercial PCR cleanup kit (QIAquick PCR Purification Kit, Qiagen, Valencia, CA). Dideoxynucleotide chain termination sequencing was performed by a commercial DNA sequencing facility (Macromolecular Resources, Ft Collins, CO).

Statistical analysis. McNemar's tests (Marascuilo & McSweeney 1977) were conducted on the comparison results to determine if significant differences occurred between the 2 testing methods. Regression analysis was used to determine if a correlation existed between PCR band strength and numbers of spores recovered from PTD testing. Square-root transformations were used to account for the proportional increase in variation with average spore count.

RESULTS

PCR identified 31.3% of the free-ranging fish as *Myxobolus cerebralis* positive, while PTD identified only 14.5% of the free-ranging fish as positive. Although 201 of the 400 hatchery-reared fish were considered exposed to *M. cerebralis*, only 31.0% of the hatchery fish tested positive with PTD, and 42.5% tested positive with PCR. Lower-than-expected positive results with both tests in the hatchery fish may have been due to limited exposures in the Chalk Cliffs State Hatchery, which is currently undergoing a cleanup program to reduce infection levels at the facility.

PCR and PTD results were concurrent in 1475 (84.6%) of the 1743 free-ranging fish tested (Table 1). A total of 1309 fish were identified as negative and 166 fish were identified as positive with both techniques.

Table 1. Concurrent and non-concurrent results for pepsin-trypsin digest (PTD) and single-round polymerase chain reaction (PCR) tests for the detection of *Myxobolus cerebralis*

Free-ranging fish				Hatchery-reared fish			
PCR	PTD Negative	PTD Positive	Total	PCR	PTD Negative	PTD Positive	Total
Negative	1309	24	1333	Negative	220	10	230
Positive	244	166	410	Positive	56	114	170
Total	1553	190	1743	Total	276	124	400

Twenty-four fish were identified as positive with the PTD method but negative with the PCR method; 244 fish were identified as positive with the PCR method but negative with the PTD method. The PCR and PTD results in this study were significantly different ($p < 0.0001$) among the free-ranging fish samples.

Concurrent results were found in 334 (83.5%) of the 400 hatchery fish tested (Table 1). Although the 199 negative control fish in this study were collected from presumed-negative hatcheries, 5 of these fish were identified as 'weak positive' with the PCR method, and 5 fish were identified as positive with the PTD method. None of the negative control fish identified as positive with the PCR test were identified as positive with the PTD test and vice-versa. The PTD-positive fish from the presumed-negative hatcheries were designated as such from the finding of a total of 11 spores in the 5 samples. Preserved filtrate from presumed-negative samples identified as positive with PTD were re-examined microscopically after the results were known, and myxospores could not be found in any of the samples.

Of the 40 samples resulting in 'weak positive' PCR results that were re-amplified, only 2 produced enough DNA for sequence analysis. These 2 samples were positively identified as *Myxobolus cerebralis*. A common alternative method of sequence identification, Southern hybridization, was considered for testing the 38 samples that did not re-amplify. However, this approach was rejected because Southern hybridization with a labeled probe is only useful for distinguishing 2 or more known sequences, and cannot differentiate between a single known sequence and other closely related, but unknown sequences.

An additional 120 fish were sampled from 1 of the presumed-negative units and re-tested for *Myxobolus cerebralis*. These fish were collected from the same lot of fish as the first samples. Sixty fish were tested with plankton centrifuge in 5 fish pools, and another 60 were tested with PTD at 2 separate fish disease diagnostic laboratories outside Colorado. No positive results were found in any of the samples. Contrary results from the first and second inspections could have arisen from very low spore content of the samples, or due to misidentification of the spores during the initial inspection. These results demonstrate the importance of using a confirmatory test when testing fish for high-profile pathogens such as *M. cerebralis*.

Myxospore counts were correlated with PCR band strengths in both the free-ranging ($R^2 = 0.3393$) and hatchery ($R^2 = 0.3534$) fish samples (Table 2). The wide range of values observed in the myxospore counts and the qualitative estimation of PCR band strengths contributed to the less-than-perfect correlation. Nonetheless, a significant exponential increase was observed in average spore count with increasing PCR band strength for the free-ranging and hatchery samples ($p = 0.001$). Of the 22 samples from lakes or streams containing 'strong positive' or 'very strong positive' PCR results, 19 (86.4%) were identified as *M. cerebralis*-positive with the PTD method.

DISCUSSION

Single-round PCR testing for the detection of *Myxobolus cerebralis* resulted in many more fish identified as positive than would have otherwise been identified with PTD testing. In many cases, 'weak positive' PCR results were found in very high elevation and remote waters with no previous history of stocking with fish exposed to *M. cerebralis*. In addition, 'weak positive' results were identified in 5 (2.5%) individual fish from presumed-negative hatcheries. Positive PCR results have occurred in other situations where the subject population was previously considered *M. cere-*

Table 2. *Myxobolus cerebralis* myxospore counts per half-head, categorized by PCR band strength for free-ranging and hatchery-reared salmonids

PCR result	N	Myxospore count Mean	Range	SD
Free-ranging fish				
Negative	1333	203	0–79333	2891
Weak positive	113	958	0–31139	4757
Positive	87	1237	0–30300	4463
Strong positive	117	12617	0–256000	32075
Very strong positive	93	35070	0–291644	56770
Hatchery-reared fish				
Negative	230	467	0–47056	4111
Weak positive	10	0	0	0
Positive	24	8315	0–55561	15520
Strong positive	64	7848	0–102400	15288
Very strong positive	72	25392	0–157889	37901

bralis-negative, and subsequent testing with histology or PTD testing did not reveal evidence of the parasite. Walker et al. (1998) identified 5 of 92 (5.4 %) fish tested from a presumed-negative hatchery as 'weak positive' by the same single round PCR method. 'Weak positive' PCR results were found in 15 of 240 (6.25%) trout identified as negative with PTD from 4 streams in Colorado (D. Peterson, Colorado State University, pers. comm.). These results suggest that either single-round PCR is producing false positive results, or *M. cerebralis* has spread throughout the range of salmonids at levels too low for PTD to detect.

Explanations for false positive results include amplification of DNA from similar myxobolus species such as *Myxobolus kisutchi* that have not been tested for cross reactivity, nonspecific amplification, or the possibility of contamination in the samples. The single round PCR protocol has been tested, and shows no cross-reactivity, with DNA preparations from *M. insidiosus* and *M. squamalis*. These myxosporeans are the 2 known species most closely related to *M. cerebralis* (Andree et al. 1998). Therefore, false positive results due to the presence in the samples of cross-reacting DNA from a myxosporean species other than *M. cerebralis*, are considered less likely than the possibility of sample contamination. Upon the findings of the first non-concurrent results, measures were taken at both laboratories to reduce any opportunities for cross-contamination. However, the possibility of contamination cannot be completely ruled out. Non-specific PCR amplification of other non-related organisms is a concern expressed by other researchers (Hoie et al. 1997, Wilson 1997, Hiney & Smith 1998). While unlikely, nonspecific amplification is a possible factor that may affect the results of this PCR test as well.

A possible reason for the sample sets in which *Myxobolus cerebralis* was detected by PTD but not PCR might be non-symmetrical distribution of infection. Another explanation may lie in the fact that PTD tests all of the skeletal elements in a sample, whereas the PCR samples only a small, random fraction of macerated skeletal fragments. It may be possible in very lightly infected heads to randomly select uninfected material.

The PCR results in this study identified *Myxobolus cerebralis* significantly more often than did PTD testing, which would be expected when using a more highly sensitive technique. If the PCR results are flawless, one must conclude that *M. cerebralis* has become established virtually everywhere in the state of Colorado, and in several fish rearing units previously thought to be free of the parasite. However, the past disease and stocking histories of some of the locations identified with PCR as *M. cerebralis*-positive suggest that some false positive results may be produced with the procedure. If this test is to be used in a regulatory capacity, a confirmatory test such as histology should continue to be used until further validation of the technique is conducted.

In spite of the fact that the potential exists for occasional false-positive results, we feel that there is sufficient evidence that PCR testing can be used as a presumptive test for *Myxobolus cerebralis* in field research scenarios. In most cases where more than 1 'strong positive' or 'very strong positive' result was identified from a given sample of fish, *M. cerebralis* myxospores were found in great quantities. PCR band strength was also correlated with average spore count, allowing a semi-quantitative evaluation of infection severity. As long as the limitations of the procedure are recognized, the method provides us with a tool to rapidly assess the *M. cerebralis* status of a given water body.

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