Evaluation of Five Diagnostic Methods for the Detection and Quantification of *Myxobolus Cerebralis*

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Evaluation of five diagnostic methods for the detection and quantification of *Myxobolus cerebralis*

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Abstract. Diagnostic methods were used to identify and quantify *Myxobolus cerebralis,* a myxozoan parasite of salmonid fish. In this study, 7-week-old, pathogen-free rainbow trout (*Oncorhynchus mykiss*) were experimentally infected with *M. cerebralis* and at 7 months postinfection were evaluated with 5 diagnostic assays: 1) pepsin–trypsin digest (PTD) to detect and enumerate spores found in cranial cartilage, 2) 2 different histopathology grading scales that provide a numerical score for severity of microscopic lesions in the head, 3) a conventional single-round polymerase chain reaction (PCR), 4) a nested PCR assay, and 5) a newly developed quantitative real-time TaqMan PCR. There were no significant differences ($P > 0.05$) among the 5 diagnostic assays in distinguishing between experimentally infected and uninfected control fish. The 2 histopathology grading scales were highly correlated ($P < 0.001$) for assessment of microscopic lesion severity. Quantification of parasite levels in cranial tissues using PTD and real-time TaqMan PCR was significantly correlated $r = 0.540$ ($P < 0.001$). Lastly, 104 copies of the 18S rDNA gene are present in the *M. cerebralis* genome, a feature that makes this gene an excellent target for PCR-based diagnostic assays. Also, 2 copies of the insulin growth factor–I gene are found in the rainbow trout genome, whose detection can serve both as an internal quality control for amplifiable DNA and as a basis to quantify pathogen genome equivalents present in quantitative PCR assays.

The phylum Myxozoa represents a diverse group of multicellular parasites with more than 1,300 species found in marine and freshwater environments.[25] *Myxobolus cerebralis* is the most-studied myxozoan and the causative agent of salmonid whirling disease. Whirling disease contributes to severe population declines in both wild and farmed salmonid fish.[6,15,34,44] The development of *M. cerebralis* involves the alternation between 2 spore forms, an actinosporean (also referred to as a triactinomyxon or actinospore) that develops in the oligochaete worm *Tubifex tubifex* and a myxosporean (myxospore) in the salmonid host.[10,11,33,48] Fish with signs of *M. cerebralis* infection exhibit darkened coloration, skeletal deformities, and undergo periods of rapid erratic swimming behavior (whirling).[13,38] Microscopic lesions associated with the developmental stages of the parasite in young salmonid fish include focal to diffuse granulomatous inflammation associated with cartilage lysis.[16,17,19] Previous laboratory[16–18,20,30,31] and field studies[36,42,43] have demonstrated that susceptibility to infection and severity of disease are dependent on several factors including fish species, dose of parasite, age and size of the salmonid host at the time of first exposure to the parasite, and water temperature.[13,27,45]

The control of whirling disease in farmed salmonids has focused on parasite detection and then elimination of infected salmonid and oligochaete hosts. Detection and slaughter approaches have been effective when the water supply to the farm or hatchery is free of either host (e.g., well or spring water). Attempts to control infections by drug therapy have only been partly successful because fish with no clinical signs may still harbor spore stages of the parasite that may infect susceptible oligochaetes.[9] The control of whirling disease among wild salmonid fish has been more problematic, with major efforts aimed at preventing stocking with fish from infected farms and reducing environmental conditions that may contribute to the abundance of susceptible oligochaete populations.[7,14,46] These control programs, whether in farmed or wild salmonid fish, rely on sensitive and specific detection of *M. cerebralis*.

Currently, a presumptive diagnosis of *M. cerebralis* infection is dependent on the identification of the 8- to 10-μm myxospore stage isolated from fish skeletal tissues by the plankton centrifuge assay or more com-

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monly the pepsin–trypsin digest (PTD) assay.[32,35] Enumeration of the myxospores by the plankton centrifuge and PTD assays of individual fish has been accepted as 1 estimate of the severity of infection by the Fish Health Section of the American Fisheries Society.[26] Quantification in both assays depends on the presence of mature myxospores that survive the extraction procedure and their proper identification as *M. cerebralis* rather than as related myxozoans that may be found in salmonid tissues.[19] A confirmatory diagnosis of *M. cerebralis* infection therefore relies on observing the parasite associated with lesions in cartilage in hematoxylin and eosin (HE)–stained tissue sections, most often of the head of the fish. However, if fish are lightly infected, detection of the parasite and microscopic lesions becomes problematic. In addition, during early stages of infection, no spores would have formed, and the parasite may be found in more cryptic stages in the skin or nervous tissues thus yielding false negatives.

Molecular detection methods such as conventional polymerase chain reaction (PCR) and in situ hybridization assays that target the 18S rDNA gene of *M. cerebralis* have been highly effective in confirming diagnoses of parasite infections detected by the PTD and histopathology in stained tissue sections.[2,4,5,40] Conventional PCR assays overcome the sensitivity limitations of traditional diagnostic tests by exponential amplification of minute amounts of 18S rDNA gene from all developmental stages of the parasite. Attempts to subjectively quantify *M. cerebralis* infections by visually scoring PCR amplicon intensities in ethidium bromide–stained agarose gels has been reported.[40]

Quantitative real-time TaqMan PCR offers a highly sensitive assay that is based on the 5′→3′ exonuclease activity of DNA polymerase and the use of dual-labeled fluorogenic probe (reporter and quencher dyes).[21] Genomic DNA quantification is determined by measuring the PCR product accumulation through reporter dye fluorescence whose intensity is directly correlated to the amount of target DNA in each reaction. Real-time TaqMan PCR uses a standardized 96-well plate format that enables large numbers of samples to be screened within a few hours using an automated fluorometer. Quantitative real-time TaqMan PCR has several advantages over the current conventional PCR systems such as increased analytical sensitivity incurred through the probe-based detection method, elimination of postamplification steps, elimination of PCR product–related contamination, and quantification of target genes during the logarithmic phase of amplification, instead of endpoint PCR or time-consuming quantitative conventional PCR.[22–24,37]

Despite the growing number of diagnostic methods used to detect *M. cerebralis,* including newly developed TaqMan PCR assays, there have been no direct comparisons of their relative accuracy. Therefore, 5 diagnostic assays, including traditional and newly developed approaches to detect *M. cerebralis,* were evaluated using experimentally infected rainbow trout.

Materials and methods

Parasites. A laboratory population of known susceptible aquatic oligochaetes was maintained in aquaria for the production of the triactinomyxon stage of *M. cerebralis*. The oligochaetes were exposed to myxospores freshly isolated from experimentally infected rainbow trout as described previously.[3] Beginning at 90 days postexposure, triactinomyxons released in the aquarium water were harvested and enumerated.[3,29]

Fish exposure. Pathogen-free rainbow trout (Mt. Lassen strain; $n = 200$; 7-wk posthatch) were exposed in 450 ml of 15 C water without aeration for 30 min to 2,000 triactinomyxons per fish. A control group ($n = 100$) from the same rainbow trout population was maintained under the same conditions but with no exposure to the parasite. Experimentally infected and unexposed control fish were maintained in 20 liter aquaria receiving 15 C water at 2 liters/min. All fish were fed once daily with a commercial trout diet.

Collection of samples for DNA extraction and histopathologic evaluations. At 7 mo after initiating experimental infections, all exposed and control fish were euthanized with a 500-mg/liter benzocaine solution. Groups A through D were generated by combining fish from known parasite-exposed and unexposed fish as follows: Group A = 38 exposed and 12 unexposed fish; Group B = 32 exposed and 18 unexposed fish; Group C = 50 unexposed fish; and Group D = 25 exposed fish. Fish heads from all groups were collected by making a midsagittal cut using a new, sterile razor blade, resulting in two halves for DNA and histopathologic processing. For DNA preparations, one half-head from each fish in groups A, B, and C was sampled with a disposable sterile 2-mm tissue punch[a] by removing tissue including a portion of the ventral calvarium. Individually removed tissues were placed in sterile 1.5-ml microcentrifuge tubes. Deoxyribonucleic acid was extracted[b] from tissues collected with the 2-mm tissue punch from fish in groups A, B, and C. The final elution volume of 270 μl from the DNA extraction was aliquoted equally for conventional PCR, real-time TaqMan PCR, and an archival sample stored at −20 C. Individual half-heads from group D were not sampled with a 2-mm tissue punch.

The other half-head from each fish in groups A, B, and C (not used for DNA extraction) and one half-head from each fish from group D were preserved in Davidson's fixative for 48–72 hr. Tissues were transferred to 70% ethanol and then processed for standard paraffin embedding, sectioning (5–6 μm), and staining with HE. Tissues in each section were examined for *M. cerebralis* and associated lesions. Two different histopathology grading scales,[6,16] designated grading scale 1 and 2, were used to evaluate the severity of microscopic lesions. Scores for scales 1 and 2 were assigned independently by different authors (KAM and GDM).

Spore enumeration. Pepsin–trypsin digestion[32] with mod-

ifications was performed on individual half-heads sampled with (groups A, B, and C) and without (group D) the 2-mm tissue punch. After digestion, 1 ml of bovine calf serum was added to each sample to neutralize trypsin activity. The digest solution was passed through a calculus filter[c] to remove large debris, and the filter was rinsed with sterile water. The filtered digest solution and rinses were centrifuged for 10 min at 1,300 × *g* to pellet the spores. Spores were resuspended in 1 ml of 10% neutral-buffered formalin. Aliquots of the sample were placed on both sides of a hemocytometer counting chamber and viewed at a magnification of 400×. Only intact spores of correct size (8–10 μm diameter) and shape were counted.[18]

Conventional PCR (single-round and nested). Deoxyribonucleic acid samples from groups A, B, and C were used in conventional single-round and nested PCR tests. As described previously, appropriate primers for single-round and nested PCR were used in a 50-μl PCR cocktail.[1,3] All reactions were performed by an independent laboratory.[d]

Quantitatve real-time TaqMan PCR systems. Using the 18S rDNA (*M. cerebralis*) and the insulin growth factor–I (IGF-I) gene of rainbow trout, 2 primers and an internal fluorescent-labeled probe (Table 1) (5′ end, reporter dye 6-carboxyfluorescein FAM [3′ end, quencher dye 6-carboxy-tetramethylrhodamine] TAMRA) were designed.[e] The real-time TaqMan PCR assay is based on a unique 18S rDNA sequence for *M. cerebralis*. To confirm that the assay was specific for *M. cerebralis,* the following related and unrelated myxozoans were tested: *M. arcticus* (AF085176); *M. squamalis* (U96495); *M. insidiosis* (U96494); *M. muelleri* (AY129314); *M. spinacurvatura* (AF378341); *M. episquamalis* (AY129312); *M. exiguus* (AY129317); *M. bizerti* (AY129318); *M. ichkeulensis* (AY129315); *M. neurobius* (AF085180); *M. sandrae* (AF085181); *Ceratomyxa shasta* (AF031579); and *Kudoa thyrsites* (AF031412). To enumerate the number of cells present in each reaction, a real-time TaqMan PCR system targeting an exon 3 of the trout IGF-I was designed. The PCR products were very short (between 67 and 88 bp) to enable high amplification efficiencies. Primer and probe sequences and relevant information to the real-time TaqMan PCR assay are listed in Table 1.

Each PCR reaction contained 400 nM of each primer, 80 nM of the TaqMan probe, and commercially available PCR mastermix[f] containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction, and 5 μl of the extracted DNA samples in a final volume of 25 μl. The samples were placed in standard 96-well plates and amplified in an automated fluorometer.[g] Amplification conditions were 2 min at 50 C, 10 min at 95 C, 40 cycles of 15 sec at 95 C and 60 sec at 60 C.

Amplification efficiencies and linearity of the *M. cerebralis* 18S rDNA and trout IGF-I TaqMan PCR systems were tested by running standard curves on 18S rDNA plasmids or DNA extracted from infected trout tissues (or both) in triplicate. Efficiencies of amplification were calculated using the formula: $E = 10^{1/-s} - 1$, where E(100) is the efficiency in percentage, and s is the slope of the standard curve.

Copy numbers were determined with real-time TaqMan

Table 1. Sequence of PCR primers and TaqMan probes.

Target	Primer	Sequence (5′ → 3′)	Product length	Probe	Sequence (5′ → 3′)	Accession no.
IGF-I	tgIGF1-231f	CAGTTCACGGCGGTCACAT	67	tgIGF1-252p	CCGTGGTATTGTGGACGAGTGCTGC	M95183
	tgIGF1-297r	CCGTAGCTCGCAACTCTGG				
Myx18S	Myx18-909f	CTTTGACTGAATGTTATTCAGTTACAGCA	88	Myx18-953p	ACCGGCCAAGGACTAACGAATGCG	AF115253
	Myx18-996r	GCGGTCTGGGCAAATGC				

Table 2. Number of rainbow trout (*Oncorhynchus mykiss*) from group A (n = 50) and group B (n = 50) positive for *Myxobolus cerebralis* using conventional single-round and nested PCR, real-time TaqMan PCR, PTD, and histopathology.*

	Single-round PCR	Nested PCR	Real-time TaqMan PCR	PTD	Histopathology scale 1†	Histopathology scale 2†
Group A	36	40	38	39	38	38
Group B	32	34	33	33	32	32

* PCR = polymerase chain reaction; PTD = pepsin–trypsin digest.
† Histopathology grading scale 1[6] and 2[16].

PCR according to a previously described method.[8] Briefly, plasmids carrying the 18S rDNA and the trout IGF-I TaqMan PCR product as inserts were used to generate standard curves. In parallel, standard curves were prepared with DNA extracted from known numbers of triactinomyxon stages and known numbers of rainbow trout cells. Differences between the 2 standard curves were used to extrapolate from known plasmid copy numbers to unknown gene copy numbers in the *M. cerebralis* genome for 18S rDNA and in the rainbow trout genome for the IGF-I gene copy number. Dilutions were made in water containing 20 ng/ml of calf thymus DNA[h] to prevent the loss of target nucleic acids in high dilutions. All standard curves were run in triplicate. All analyses were carried out using the same settings: a threshold of 0.04 and baseline values of 3–15.[i] For strong IGF-I signals, baseline values were changed to 3–9.

The final quantification was done using a modification of the comparative threshold cycle (CT value) method.[j] The real-time PCR system targeting the trout IGF-I gene was used to quantify the genome equivalents leading to cell numbers from a known trout cell line.[49] In parallel, the parasite load was extrapolated to a standard curve generated with a cloned 18S rDNA gene[1] of *M. cerebralis*. To quantify the number of 18S rDNA copies per *M. cerebralis* cell, a standard curve was generated with known numbers of *M. cerebralis* cells (1 triactinomyxon = 70 cells). These values were compared and extrapolated to the standard curve obtained with the cloned 18S rDNA fragment to quantify the 18S rDNA gene copies per *M. cerebralis* cell. The final quantification of the parasite as found in fish tissues is reported as the absolute number of *M. cerebralis* genome equivalents (18S rDNA copies) per 10^6 rainbow trout cells.

Statistical analysis. Myxospore counts by PTD were compared between half-heads sampled with (group A) or without (group D) 2-mm tissue punch, and analyses were conditional on the fish being exposed to the triactinomyxon stage. Therefore, all unexposed fish were excluded from the statistical analyses. Myxospore count results for individual half-heads sampled with or without the 2-mm punch necropsy were compared using the Mann–Whitney U-test. Results from the 2 histopathological grading scales were compared using a Spearman rank correlation coefficient. The specificity and sensitivity of all the diagnostic tests were estimated by standard methods, and 95% confidence intervals for these values were computed.[k] The dichotomized results of the diagnostic assays were compared using McNemar's chi-square test for paired data. All infected fish from groups A and B were used for quantification comparisons between real-time TaqMan PCR and PTD. Results for real-time TaqMan PCR and PTD were log transformed and visually compared with a scatter plot, and their correlation was tested using a Spearman rank correlation coefficient. For all the statistical analyses, $P < 0.05$ was considered significant. All the statistical analyses were performed using SPSS 10.0.[l]

Results

Diagnostic comparison. Detection results for *M. cerebralis* using single-round and nested PCR, real-time TaqMan PCR, PTD, and microscopic lesions for groups A and B are shown in Table 2. Group C was negative for *M. cerebralis,* as indicated by all methods. Quantification results from PTD with and without 2-mm tissue punch (groups A and D) were not significantly different ($P > 0.05$): group A $\bar{x} = 4.2 \times 10^5$ (range, 2.5×10^3 to 1.2×10^6); group D $\bar{x} = 4.7 \times 10^5$ (range 5.1×10^4 to 1.7×10^6). The comparison of histopathology grading scales 1 and 2 was used on fish (n = 150) from groups A, B, and C. Group C had no lesions associated with *M. cerebralis* using either grading scale. Correlation of histopathology grading scales 1 (relative mean score = 3.8; range = 2–5) and 2 (relative mean score = 3.1; range = 2–4) was high ($r = 0.909$, $P < 0.001$). None of the diagnostic tests significantly differed in sensitivity or specificity ($P > 0.05$, Table 3). Infected fish from groups A and B were used for *M. cerebralis* quantification using real-time TaqMan PCR and PTD. *Myxobolus cerebralis* 18S rDNA quantification by real-time TaqMan PCR for groups A and B was on average 2.9×10^6 (range, 8.3×10^1 to 1.0×10^7) and 3.5×10^6 (range, 9.0×10^1 to 2.1×10^7) genome equivalents per 10^6 trout cells, respectively. Pepsin–trypsin digest quantification results from group A (see above) were used for comparison, and in addition, myxospore counts from group B were on average 4.3×10^5 (range, 7.5×10^3 to 1.1×10^6). Real-time TaqMan PCR and PTD quantification assays yielded similar results (Fig. 1) that were highly correlated ($r = 0.540$, $P < 0.001$).

Real-time TaqMan linearity and amplification efficiencies. Quantitation of rainbow trout IGF-I plasmid and cell line showed linearity over 7 orders of magnitude. In addition, quantitation of *M. cerebralis* 18S rDNA plasmid and of serially diluted triactinomyxons showed linearity from 7 to 8 orders of magnitude, re-

Table 3. Sensitivity and specificity with corresponding 95% confidence intervals for the diagnosis of *Myxobolus cerebralis* using conventional single-round and nested PCR, real-time TaqMan PCR, PTD, and 2 histopathology grading scales.* Sensitivity was calculated for 70 exposed fish from groups A and B. Specificity was determined using 30 unexposed fish randomly added to groups A and B.

	Single-round PCR	Nested PCR	Real-time TaqMan PCR	PTD	Histopathology scale 1†	Histopathology scale 2†
Sensitivity (95% CI)	97.1% (90.1–99.7)	100.0% (94.9–100.0)	100.0% (94.9–100.0)	100.0% (94.9–100.0)	100.0% (94.9–100.0)	100.0% (94.9–100.0)
Specificity (95% CI)	100.0% (88.4–100.0)	86.7% (69.3–96.2)	96.7% (82.8–99.9)	93.3% (77.9–99.2)	100.0% (88.4–100.0)	100.0% (88.4–100.0)

* PCR = polymerase chain reaction; PTD = pepsin–trypsin digest.
† Histopathology grading scale [16] and [216].

spectively (Fig. 2). Efficiencies were as follows—for *M. cerebralis* 18S rDNA TaqMan PCR system on the plasmid carrying the 18S rDNA gene: 95.7%, on DNA extracted from the triactinomyxon stages: 102.0%, for IGF-I TaqMan PCR system on the plasmid carrying the IGF-I TaqMan PCR product: 100.3%, and on DNA extracted from rainbow trout cell line: 99.0%.

18S rDNA gene copy number of M. cerebralis cells and IGF-I gene copy number of rainbow trout cells. Comparison of the standard curves allowed the estimation of copy numbers of the 18S rDNA gene in the triactinomyxon stages and of the IGF-I gene copy number in rainbow trout genomic DNA (Fig. 2). These comparisons showed 104 copies of the 18S rDNA gene per *M. cerebralis* cell. The IGF-I copy number per diploid rainbow trout cell was 2, confirming that this gene is a single-copy gene in rainbow trout as described in humans and rats.[39]

Cross-reactivity of TaqMan probes and primers. On the basis of their location within the 18S rDNA gene, the current primers and probes for *M. cerebralis* (Table 1) revealed no cross-reactivity with DNA derived from the other myxozoans used in this study.

Discussion

The evaluation of the 5 diagnostic assays for *M. cerebralis* indicated that when rainbow trout were heavily or moderately infected, as in the current study, any of the 5 diagnostic approaches was effective in identifying infected fish. Under the conditions tested, all 5 assays were capable of distinguishing between experimentally infected and uninfected control fish. Under the conditions tested, all 5 assays also had high sensitivity and specificity.

Previous studies[3–5,40] have examined improved detection procedures for *M. cerebralis,* but this is the first published study comparing the sensitivity and specificity for PTD, histopathological grading of lesions, and conventional PCR with the new diagnostic approach of real-time TaqMan PCR. PTD sensitivity was found to be 100%. However, PTD specificity (93.7%) indicated that several unexposed fish within groups A and B were identified as false positives for myxospore infection, whereas all other diagnostic tests were negative. The false-positive samples were reanalyzed, and myxospore presence was reaffirmed. Previous reports suggested that PTD false positives resulted from erroneous myxospore identification from samples with no previous history of *M. cerebralis* exposure.[40] These prior false-positive results may be from mixed infections with other myxozoans morphologically similar to *M. cerebralis*.[19] Because the rainbow trout were raised from eggs in well water in a containment laboratory, it is unlikely that they became infected with non–*M. cerebralis* myxozoans. Instead, the source of the false

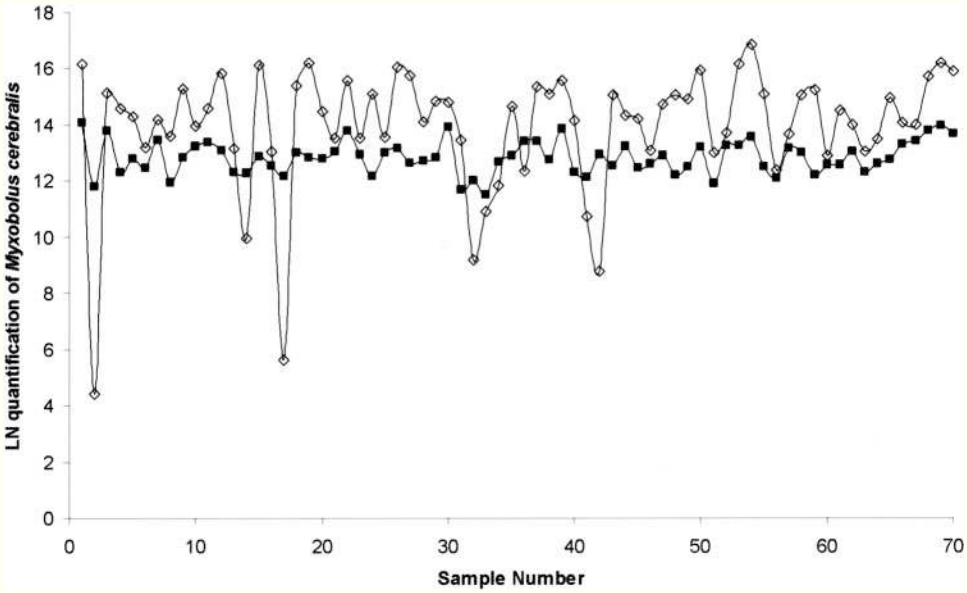

Figure 1. *Myxobolus cerebralis* quantification (LN = natural log-transformed) as determined for rainbow trout (*Oncorhynchus mykiss*) tissue samples with real-time TaqMan PCR and PTD digest. Real-time TaqMan PCR (◊) is measured as copies of the 18S rDNA gene per 10^6 rainbow trout cells. PTD (■) is numerated by the number of myxospores per half-head. Quantification by real-time PCR and PTD digest were correlated ($P < 0.001$).

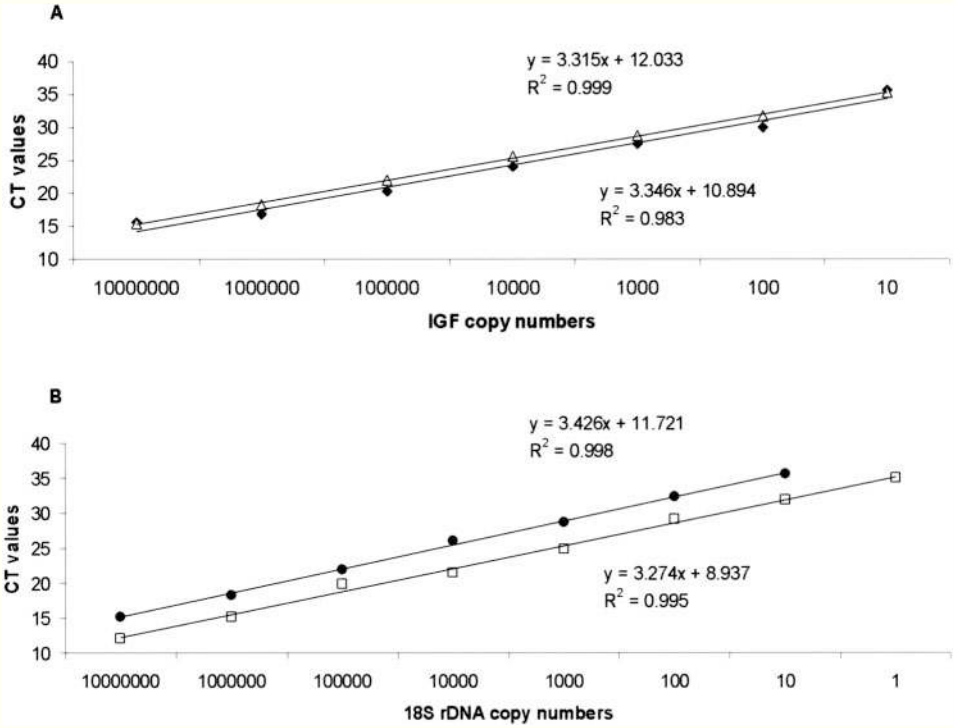

Figure 2. **A**, amplification efficiency of target DNA and linear range from 10^7 to 10 copies for rainbow trout (*Oncorhynchus mykiss*) insulin growth factor–I (IGF-I) plasmid (△) and rainbow trout cell line (♦) standards. **B**, Amplification efficiency determination for the 18S rDNA gene of *Myxobolus cerebralis*. Linear range from 10^7 to 1 copy of the 18S rDNA gene and analytical sensitivity for 18S rDNA plasmid (●) and *M. cerebralis* cell (□) standards. Amplification efficiencies are equivalent if the difference in the slopes is <0.1. Standard deviations are not shown. Threshold cycle (CT value) is the PCR cycle at which the fluorescent intensity exceeds the threshold.

positives was most likely a result of myxospore carryover from positive fish through equipment used in the PTD assay. Because of this possibility, it is critical that proper disinfection of equipment and aseptic tissue preparation are carried out before PTD analyses.

The development of single-round and nested PCR assays has allowed detection of all known stages (prespore and spore) of *M. cerebralis* with relatively minute amounts of fish tissues.[3–5,40] The results reported in this study support previous work that found single-round PCR to be highly specific (reduced numbers of false positives) but with less sensitivity than the nested PCR.[3,5] In addition, both the real-time TaqMan and the single-round PCR specificity (96.7% and 100.0%, respectively) results were greater than nested PCR specificity (86.7%). The high specificity found for real-time TaqMan and single-round PCR suggests that additional amplification steps in heavy to moderately infected fish were not essential and with their elimination, contamination problems with PCR products may be reduced. In addition, real-time TaqMan PCR uses an internal probe for the detection of the PCR products during amplification, leading to increased PCR specificity. Specificity results from the real-time TaqMan PCR indicate the potential risk of introducing *M. cerebralis* cross-contamination during batch sampling, a problem also encountered with nested PCR assays. Under the conditions tested, real-time PCR sensitivity (100.0%) was greater than that of single-round PCR (97.1%). Direct comparisons of analytical sensitivity between real-time TaqMan PCR and nested PCR protocols have shown TaqMan PCR protocols to be about 10-fold more sensitive than conventional gel-based PCR.[12] The use of real-time TaqMan PCR as a confirmatory test instead of microscopic pathology or conventional PCR may be preferred because of its high sensitivity, high throughput, reduced risk of cross-contamination, and elimination of the need for a hazardous chemical such as ethidium bromide for staining agarose gels.

The results for the 2 independent histopathological grading scales were essentially identical when evaluating disease severity. This was a welcomed result because these pathology assays have been widely used to assess disease severity in field[6,41] and laboratory studies.[16–18,44] The exposure dose of 2,000 triactinomyxon per rainbow trout used to induce experimental infections with *M. cerebralis* in this study was sufficient to induce severe gross and microscopic lesions in all exposed fish (100.0% sensitivity). Although grading scale 1 averages lesion scores and grading scale 2 recognizes the highest lesion score observed across all tissues, both are based on the presence and number of microscopic lesions associated with extrasporogonic (prespore) and sporogonic (spore) stages of development. The values obtained with both grading scales (scale 1 and 2 mean lesion scores = 3.8 and 3.1, respectively) were similar to those found among severely impacted wild rainbow trout populations and among fish after field or laboratory exposures to the parasite.[6,16–18,41,43] Both the prevalence and severity of infections with *M. cerebralis* can be substantially less in hatchery and wild trout populations than those observed in the experimentally infected rainbow trout studied here. Host and environmental factors and lower challenge doses of the parasite can result in infected populations that may show few or no gross signs of infection and that may go undetected by histopathological assays.[17,44] When fish are more heavily infected, as in the current study, both histopathologic grading scales provide similar disease assessments, but studies with lightly infected trout are also needed, particularly to evaluate detection by PCR and real-time TaqMan PCR methods. The current presumption is that the 2 microscopic grading scales would be similar, regardless of the prevalence or severity of infection in the fish because each test relies on measurements of the same criteria differing mostly in the range of numeric values assigned.

In rainbow trout, myxospores are commonly found in cranial cartilage during later stages of infection. The ventral calvarium is one prominent area where myxospores and associated lesions are observed in rainbow trout.[6] Pepsin–trypsin digest results for half-heads sampled with a 2-mm tissue punch (group A) in the ventral calvarium region were not significantly different ($P > 0.05$) when compared with myxospore counts from half-heads from which tissue punches were not taken (group D). Similar concentrations of myxospores were observed from fish sampled with or without a 2-mm tissue punch (Group A: 2.5×10^3 to 1.2×10^6; Group D: 5.1×10^4 to 1.7×10^6). The concentrations of myxospores found in trout from Groups A and D were within the range expected from naturally and experimentally infected rainbow trout, as found by previous investigators[16–18,41–43] evaluating experimentally and naturally infected fish. These myxospore concentrations would indicate moderate to severe infection. The results from this study suggest that removal of a 2-mm tissue punch for PCR analysis does not compromise the subsequent recovery of myxospores recovered by PTD analyses. This result might be expected because PTD analysis includes additional skeletal elements found in the head and the gills[6,16] that contain myxospores not found in the 2-mm tissue punch. Hence, tissue punches can be taken from the same half-head used for DNA-based assays (conventional and real-time TaqMan PCR), traditional spore detection, and enumerations by PTD. The other half-head can be retained for histopathological assays thus

allowing the use of new and traditional diagnostic assays with the same fish collected in field samples. Further studies, including direct comparisons of myxospore concentrations by PTD and parasite DNA detection with tissue punches of whole or half-heads from more lightly infected fish are warranted.

The quantitative real-time PCR results revealed that an individual *M. cerebralis* cell contains approximately 104 copies of the 18S rDNA gene. The gene quantification results reported here are similar to previous copy numbers of 18S rDNA found in a variety of copepods (140–33,500 copies)[47] and in *Zygomycete mucor miehei* (Fungi) (100 copies).[28] Testing of the real-time TaqMan PCR assay with other related *Myxobolus* spp. and unrelated myxozoans indicated that the assay specifically detected *M. cerebralis*. The real-time TaqMan PCR assay also had a high analytical sensitivity for the 18S rDNA gene of *M. cerebralis,* detecting as few as 10 copies of the target sequence (Fig. 2). This analytical sensitivity was similar to that reported previously for the nested PCR protocol.[3]

The significant correlation between the PTD and the real-time TaqMan PCR for quantification of *M. cerebralis* infections indicates that real-time TaqMan PCR might be used as an alternative to PTD and manual counting of myxospores. Also, because the real-time TaqMan PCR was capable of detecting late stages of parasite development in fish tissues, it provides the first molecular-based technique to objectively quantify *M. cerebralis*. In contrast, myxospore counts from the PTD method represent only the final developmental stages of the parasite or the mature myxospores that can withstand the chemical and physical treatments inherent to the extraction procedure. The real-time TaqMan PCR thus allows quantification of the parasite throughout development and under varying host-, environmental-, or parasite-exposure conditions encountered in the laboratory or natural aquatic habitats. Quantification of *M. cerebralis* also is improved with real-time TaqMan PCR because certain steps used in conventional PCR are obviated. This includes replacement of spectrophotometric determinations of the genomic DNA concentration in conventional PCR by the enumeration of the genome equivalents by targeting a single-copy gene of the susceptible host species. Spectrophotometry has poor reproducibility, particularly between laboratories, and provides little information about DNA integrity and its use for PCR. Alternatively, the quantitative real-time TaqMan PCR assay recognizes the trout IGF-I gene on exon 3 of rainbow trout and therefore calculations of host cell number and suitability of the DNA for PCR amplification can be made.

Rapid and accurate detection of *M. cerebralis* infection is integral to controlling the spread of whirling disease. The results from this study confirm that currently used procedures and a newly developed TaqMan PCR are effective tools in detecting and evaluating the severity of *M. cerebralis* infections in moderately to heavily infected rainbow trout. Also, the tissue punch samples can be used for real-time TaqMan PCR assays providing both reliable detection and accurate quantification of *M. cerebralis* in infected rainbow trout. Using the genome equivalents with real-time TaqMan PCR avoids the subjective and semiquantitative estimations of amplicon intensity as found on agarose gels. Another advantage of real-time TaqMan PCR relative to current gel-based conventional nested PCR assays is the closed tube reaction technique that eliminates postamplification steps and thereby reduces the likelihood of false positives by contamination with PCR products. In addition, the AmpErase UNG system integrated into the commercially available PCR mastermix eliminates the residual risk of PCR product carryover. Real-time TaqMan PCR is a highly standardized protocol that can be reproduced easily in different laboratories. The suitability of the real-time TaqMan PCR assay is also currently being tested for quantification of triactinomyxons, as found in water samples. Lastly, the TaqMan PCR will be used to evaluate infections among rainbow trout that receive low-dose exposures to triactinomyxons to better evaluate the performance of the assay on lightly infected fish.

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Sources and manufacturers

a. Miltex Instrument Company Inc., Bethpage, NY.
b. QIAamp® DNA Mini Kit, Qiagen, Valencia, CA.
c. Gerson Healthcare, Middleboro, MA.
d. Biogenetic Services Inc., Brookings, SD.
e. Primer Express software, Applied Biosystems, Foster City, CA.
f. TaqMan Universal PCR Mastermix, Applied Biosystems, Foster City, CA.
g. ABI PRISM 7700 Sequence Detection System, Applied Biosystems, Foster City, CA.
h. Sigma Chemical Co., St. Louis, MO.
i. SDS Analysis Software, Applied Biosystems, Foster City, CA.
j. User Bulletin #2, Applied Biosystems, Foster City, CA.
k. EpiInfo version 6 (Centers for Disease Control and Prevention), Atlanta, GA.
l. SPSS Inc., Chicago, IL.

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