2006

Detection of Early Stages of Myxobolus Cerebralis in Fin Clips from Rainbow Trout (Onchorynchus mykiss)

Ramona T. Skirpstunas
Utah State University

J. Hergert

Thomas J. Baldwin
Utah State University

Recommended Citation
Skirpstunas RT, Hergert J and Baldwin TJ 2006. Detection of early stages of Myxobolus cerebralis in fin clips from Rainbow trout (Onchorynchus mykiss). Journal of Veterinary Diagnostic Investigation 18:3: 274-277.

J Vet Diagn Invest 18:274–277 (2006)

Detection of early stages of *Myxobolus cerebralis* in fin clips from rainbow trout (*Onchorynchus mykiss*)

Ramona T. Skirpstunas,[1] John M. Hergert, Thomas J. Baldwin

Abstract. A nested polymerase chain reaction (PCR) assay was used to detect early stages of *Myxobolus cerebralis* in caudal and adipose fin samples from rainbow trout (RT). To determine sensitivity, groups of 10 RT were exposed to 2,000 *M. cerebralis* triactinomyxons/fish for 1 hour at 15°C and subsequently moved to clean recirculating water. Fish were held for 2 and 6 hours and 1, 2, 3, 5, 7, 10, 30, and 60 days before sampling by nonlethal fin biopsy. Nested PCR performed on fin clips showed that *M. cerebralis* DNA was detected in caudal fin tissue in 100% of fish up to 5 days postexposure. At days 7 and 10 postexposure, 80% of fish were positive, and at 60 days postexposure, 60% of fish were positive using this technique. Conversely, testing on adipose fin clips proved less sensitive, as positive fish dropped from 80% at day 7 to below 20% at day 10 postinfection. Since detection of *M. cerebralis* infection using caudal fin samples coupled with nested PCR is an effective method for detection of early parasite stages, use of this technique provides for accurate, nonlethal testing.

Key words: Diagnostics; fin clips; *Myxobolus*; PCR; whirling disease.

Infection of both wild and farmed salmonid fish by *Myxobolus cerebralis*, the agent of whirling disease, can result in significant losses.[4,7] Mortality depends on species, exposure dose, age, and water temperature.[8,9,10,14,15,16,17] Given these variables and the inability to successfully treat infected fish, control relies on identification and culling of populations having infected fish, or withholding potentially infected stock from release into wild populations.[18]

Diagnostic methods for identification of *M. cerebralis* include mechanical homogenization and pepsin-trypsin

From the Utah Veterinary Diagnostic Laboratory, Utah State University, Logan, UT 84322.

[1] Corresponding Author: Ramona T. Skirpstunas, Utah Veterinary Diagnostic Laboratory, Logan, UT 84322-5700 (e-mail: rskirps@cc.usu.edu).

digestion (PTD) of cranial cartilages with subsequent microscopic examination of released myxospores,[13] histopathologic examination of cranial structures, or single-round, nested and real-time polymerase chain reaction (PCR)–based assays on infected cranial cartilage.[1,4,5,8,9,11] These methods vary in sensitivity, specificity, turnaround time, reagent cost, ability to handle large sample numbers, and parasite stage detected. Because PCR-based assays have high sensitivity and specificity, can identify *M. cerebralis* early developmental stages, and have rapid turnaround times, they have become assays of choice.[2,5] To date, all detection methods use samples obtained from sacrificed fish, a highly undesirable protocol in hatcheries, farmed fish, small populations (some endangered), valuable brood fish, or wild populations.

Since PCR assays can identify *M. cerebralis* early developmental stages, and *M. cerebralis* cell doublets and multinucleate plasmodia remain in trout skin for prolonged time periods, testing of skin samples biopsied from live fish has been attempted. Sandell (Proc. 6th Whirling Disease Symposium, Whirling Disease Foundation, 2000) identified the caudal fin as suitable for detection of early stages of *M. cerebralis* using a PCR-based assay. Additional tissues, including gill filaments and opercula, have also been evaluated (Vannest, Proc. 7th Whirling Disease Symposium, Whirling Disease Foundation, 2001) but failed to provide enhanced detection and tissue procurement was more invasive. Toner et al. (Proc. 10th Whirling Disease Symposium, Whirling Disease Foundation, 2004) evaluated the sensitivity of nested PCR assays on fin biopsies up to 11 months after triactinomyxon (TAM) exposure from fish infected under laboratory and natural conditions. In that study, sensitivity was below 80% after 1 month postexposure and below 50% after 3 months. In the author's laboratory, introductory experiments comparing caudal, adipose, and pectoral fin samples from rainbow trout exposed 1 time to various TAM numbers indicate that the use of fin tissue, particularly caudal fin, permits detection of *M. cerebralis* by nested PCR up to 5 months postexposure.

Since fish are sampled by population (usually 60 to 150 individuals at a time), determination of the infection status is possible even if testing methodologies used have sensitivities below 100%. Existing assays, such as PTD and histopathology, have sensitivities far below 100%[3,5] and yet provide useful information and continue to be used. Moreover, in wild populations where TAM exposure may be intermittent to nearly constant, assays that focus on detection of early *M. cerebralis* stages in skin samples may actually have higher sensitivities than traditional assays in current use.

Collectively, these results suggest that a nested PCR assay conducted on caudal fin samples from live fish is an excellent, rapid, sensitive, and nonlethal method for diagnosing whirling disease. However, the sensitivity of this method following exposure to *M. cerebralis* needs to be clearly defined, which constitutes the purpose of this study.

Fifteen-week-old rainbow trout were obtained from Mantua Fish Hatchery, Mantua, UT. The facility is certified *M. cerebralis*–free by annual inspection. Fish were maintained in plastic aquaria using dechlorinated, recirculating Logan, UT city water at 15°C to 17°C. Water quality was insured by weekly testing of standard parameters (temperature, nitrogen and ammonia levels, dissolved oxygen, and alkalinity). Trout were fed a commercial trout diet[a] at 2% body weight per day.

TAMs used in this study were harvested from an *M. cerebralis*–infected oligochaete culture (*Tubifex tubifex*) maintained by the Biology Department at Utah State University, Logan, UT. TAMs were enumerated using light microscopy (manual count in 20 µl, Standard Protocols for Whirling Disease Research, Whirling Disease Foundation 2001).[12]

One hundred twenty rainbow trout were infected by placing groups of 10 fish in 100 L of 15°C aerated water and adding TAM suspensions sufficient to create a 2,000 TAM per fish exposure. Fish were exposed to TAMs for 1 hour, after which grouped fish were transferred to individual aquaria and maintained until sampled.

Fish were euthanized by prolonged exposure to 250 mg/L tricaine methane sulfonate (MS-222)[b] in dechlorinated water. Ten fish were randomly sampled before infection (time 0), followed by groups of 10 fish sampled at 2 and 6 hours and 1, 2, 3, 5, 7, 10, 30, and 60 days postexposure. Clinical signs of whirling disease were not noted in any fish. The adipose fin (in entirety; weighing 10–15 mg) and a 10 to 15 mg sample of the anterior ventral portion of the caudal fin were obtained using scissors and forceps and placed in 1.5 ml microcentrifuge tubes. All instruments were disinfected between samples by immersion in 10% Nolvasan™ (chlorhexidine diacetate, 2%)[c] solution and rinsed in clean chlorinated water. Samples were placed directly in lysis buffer (see below) and either used immediately for DNA extraction or held at −20°C until further processing. Negative control tissues were collected randomly between exposed fish to ensure that cross contamination was absent and that cleaning of biopsy instruments was sufficient.

Total DNA was extracted from fin tissues using Qiagen's DNeasy DNA extraction kit[d] following the ''Protocol for Rodent Tails'' (DNeasy Tissue Handbook, Qiagen, 2002). PCR amplification was performed using primers and a modification of the procedure described by Andree et al.[1] Primers were supplied by Invitrogen[e]. The first-round primers are Tr 5–16 (5′-GCATTGGTTTACGCTG ATGTAGCGA-3′) and Tr 3–16 (GAATCGC CGAAACAATCATCGAGCTA-3′). Second-round primers are Tr 3–17 (5′-GGCACACTACTC CAACACTGAATTTG-3′) and Tr 5–17 (5′-GCCCTATTAACTAG TTGGTAGTATAGAAGC-3′). The standard reaction volume was 50 µl (48 µl of master mix and 2 µl of DNA template). The PCR master mix consisted of 5 µl 10× PCR buffer (50 mM KCl, 100 mM Tris-HCl pH 8.3, 15 mM $Mg(OAc)_2$), 1.5 mM $MgCl_2$, 400 µM dNTPs, 20 pmol of each primer, and 2U Taq polymerase.[f] Two µl of first-round amplified DNA product were used as template for the second round. All reagents were stored at −20°C and kept on ice after thawing. Amplifications were performed using an Eppendorf Mastercycler[g] gradient thermal cycler. A denaturation step in which the samples were held at 95°C for 5 minutes preceded the amplification cycles. Amplification cycles

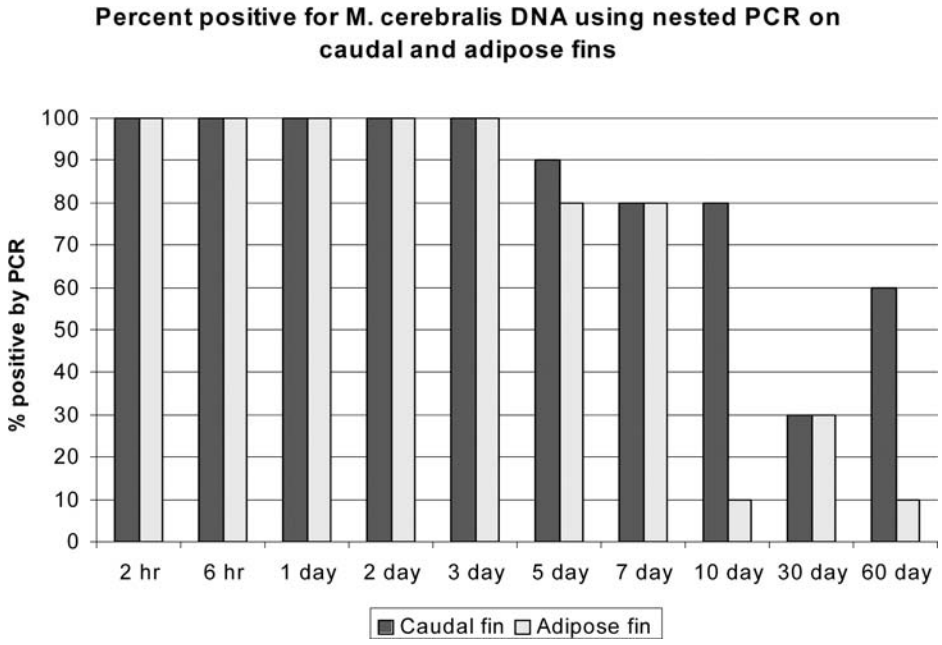

Figure 1. Caudal and adipose fin biopsy samples of 10 fish per sampling time after exposure to 2,000 TAMs for 1 hour at 15° C. Bars represent percent positive fish per group at each sampling time point identified as infected with *M. cerebralis* using nested PCR. Each sample was run in triplicate.

consisted of 1 minute 95°C, followed by 2.5 minutes at 65°C, followed by 1.5 minutes at 72°C. The cycle was repeated 35 times. An elongation step of 10 minutes at 72°C concluded the program.

Positive and negative controls were included with every PCR assay. The positive control consisted of 2 TAMs processed as described above in 10 mg of *M. cerebralis*–free rainbow trout caudal peduncle. The negative control consisted of 10 mg of *M. cerebralis*–free rainbow trout caudal peduncle.

Amplified PCR products were visualized by horizontal gel electrophoresis using 1.5% agarose containing 5 µl of 10 mg/ml ethidium bromide in TBE buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0). Ten µl of sample were added to 2 µl of loading dye, and 10 µl total volume was loaded into each well. A 100-bp ladder was used to estimate the size of PCR products. Gels were run at 120V for 1 hour. Amplified *M. cerebralis* DNA bands (415-bp fragments) were visualized and photographed under UV light.

Nested PCR assays on caudal fin samples demonstrated the presence of *M. cerebralis* DNA (415 bp fragment) in all fish sampled at 2 and 6 hours and 1, 2, 3, and 5 days postexposure (data not shown). At days 7 and 10 post-exposure, 80% of fish were identified as infected. At 30 and 60 days postexposure, 30% and 60% of fish were identified as infected, respectively (Fig. 1). Nested PCR assays on adipose fin samples demonstrated the presence of *M. cerebralis* DNA in all fish sampled at 2 and 6 hours and 1, 2, 3, and 5 days. At 7, 10, 30, and 60 days postinfection, 80%, 10%, 30%, and 10% of fish were identified as infected, respectively (Fig. 1). Samples collected from fish before TAM exposure (time 0; negative control) were all negative by PCR for *M. cerebralis* DNA.

Results indicate that nested PCR assays on caudal fin samples effectively identify *M. cerebralis* infection up to at least 60 days (last time period examined) after single exposure. As fish in natural or farmed waterways incur multiple sequential exposures from early spring through late fall, this procedure is likely to be effective at identifying populations infected with *M. cerebralis* at any given sampling time. Based on results of this study, sensitivity of this assay after the first 5 days postexposure varies depending on the length of time after a single exposure; however, fish in natural waterways are most certainly exposed numerous times throughout the year. Fin clips do need to be taken with clean sampling instruments, and sampling personnel should be trained to minimize cross contamination. Otherwise, training requirements are minimal for sample collection.

Caudal fin clips have multiple advantages over head and gill samples. Most importantly, clips can be obtained in a nonlethal fashion, sparing valuable fish within wild, endangered, or farmed populations. Fin clips are small and are easily stored, either refrigerated or frozen, before submission. Minimal training is required for sampling personnel. The nested PCR assay is rapid and not overly expensive.

Results do not support using adipose fin clips, as detection of early *M. cerebralis* stages is inconsistent and generally poor after 7 days postinfection. This may be due to tissue thickness or ease of movement of generative stages from this site, or may simply be reflection of a less popular site for TAM attachment. El-Matbouli et al.[6] theorized that the thin tissues composing the caudal fin dampen parasitic

dispersion. Conversely, thicker tissue, like that found in the adipose fin, permits more rapid movement of generative stages to deeper tissue leaving less parasite DNA available for detection.

Although previous work has indicated that TAMs bind preferentially near mucous cells of the gill and buccal cavity,[6] sampling at these sites would be stressful and potentially harmful/lethal for fish. Hence, nested PCR assays on samples of caudal fin are recommended for detection of *M. cerebralis* infection.

Acknowledgements. This work was supported by the Utah Agricultural Experiment Station. Triactinomyxons kindly provided by Dr. Don Roberts, Utah State University.

Sources and manufacturers

a. Rangen, Buhl, ID.
b. Tricaine methane sulfonate, Sigma, St. Louis, MO.
c. Nolvasan, Fort Dodge Animal Health, Fort Dodge, IO.
d. Qiagen Inc., Valencia, CA.
e. Invitrogen, Carlsbad, CA.
f. Eppendorf, Westbury, NY.
g. Eppendorf Mastercycler, Westbury, NY.

References

1. Andree KB, MacConnell TS, Hedrick RP: 1998, A nested polymerase chain reaction of the detection of genomic DNA of *Myxobolus cerebralis* in rainbow trout (*Oncorhynchus mykiss*). Dis Aquat Org. 34:145–154.
2. Andree KB, Hedrick RP, MacConnell E: 2002, A review of the approaches to detect *Myxobolus cerebralis*, the cause of salmonid whirling disease. Am Fish Soc Symp 29:197–211.
3. Antonio DB, Andree KB, McDowell TS, Hedrick RP: 1998, Detection of *Myxobolus cerebralis* in rainbow trout and oligochaete tissues by using a non-radioactive *in situ* hybridization (ISH) protocol. J Aquat Anim Health 10: 338–347.
4. Baldwin TJ, Vincent ER, Silflow RM, Stanek D: 2000, *Myxobolus cerebralis* infection in rainbow trout (*Oncorhynchus mykiss*) and brown trout (*Salmo trutta*) exposed under natural stream conditions. J Vet Diag Invest 12: 312–321.
5. Baldwin TJ, Mykelbust KA: 2002, Validation of a single round polymerase chain reaction assay for identification of *Myxobolus cerebralis* myxospores. Dis Aquat Org 49: 185–190.
6. El-Matbouli M, Hoffman RW, Mandok C: 1995, Light and electron microscopic observations on the route of the triactinomyxon-sporoplasm of Myxobolus cerebralis from epidermis into rainbow trout cartilage. J Fish Biol 46:919–935.
7. Hedrick RP, El-Matbouli M, Adkinson MA, MacConnell E: 1998, Whirling disease; re-emergence among wild trout. Immunol Rev 166:365–376.
8. Hedrick RP, McDowell TS, Gay M, et al.: 1999, Comparative susceptibility of rainbow trout *Oncorhynchus mykiss* and brown trout *Salmo trutta* to *Myxobolus cerebralis*, the case of salmonid whirling disease. Dis Aquat Org 37:173–183.
9. Hedrick RP, McDowell TS, Mukkatira K, et al.: 1999, Susceptibility of selected inland salmonids to experimentally induces infection with *Myxobolus cerebralis* the causative agent of whirling disease. J Aquat Anim Health 11:330–339.
10. Hedrick RP, McDowell TS, Mukkatira K, et al.: 2001, Susceptibility of three species of anadromous salmonids to experimentally induced infections with *Myxobolus cerebralis*, the causative agent of whirling disease. J Aquat Anim Health 13:43–50.
11. Kelley GO, Zagmutt-Vergara FJ, Leutenegger CM, et al.: 2004, Evaluation of five diagnostic methods for the detection and quantification of *Myxobolus cerebralis*. J Vet Diag Invest 16:202–211.
12. Lorz HV, Amandi A: 1994, VI. Whirling disease of salmonids. *In:* Suggested procedures for the detection and identification of certain finfish and shellfish pathogens, ed. Thoesen JC, 4th ed., pp. 1–7. Am Fish Soc, Fish Health Section, Bethesda, MD.
13. Markiw ME, Wolf K: 1974, Myxosoma cerebralis: isolation and concentration from fish skeletal elements—sequential enzymatic digestions and purification by differential centrifugation. J Fish Red Bd Can 32:561–564.
14. Markiw ME: 1989, Whirling disease: earliest susceptible age of rainbow trout to the triactinomyxid of *Myxobolus cerebralis*. Aquaculture 92:1–6.
15. Thompson KG, Nehring RB, Bowden DC, Wygant T: 1999, Field exposures of seven species or subspecies of salmonids to Myxobolus cerebralis in the Colorado River, Middle Park Colorado. J Aquat Anim Health. 11:312–329.
16. Thompson KG, Nehring RB, Bowden DC, Wygant T: 2002, Response of rainbow trout *Onchorynchus mykiss* to exposure to *Myxobolus cerebralis* above and below a point source of infectivity in the upper Colorado River. Dis Aquat Org 49:171–178.
17. Vincent ER: 2002, Relative susceptibility of various salmonids to whirling disease with emphasis on rainbow and cutthroat trout. Am Fish Soc Symp 29:109–115.
18. Wagner EJ: 2002, Whirling disease prevention, control, and management: a review. Am Fish Soc Symp 29:217–225.