

# Whirling Disease: Diagnostic Techniques and Potential for Range Expansion

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## ABSTRACT

Whirling Disease (WD) is caused by the exotic parasite *Myxobolus cerebralis* (Mc) which was introduced into North America from Europe in 1956. WD has had extremely detrimental effects on native trout populations in the intermountain west. Mc has not yet been reported from Wisconsin and Minnesota. This study was initiated to assess the potential for WD becoming established in Wisconsin trout. We also began the process of evaluating the specificity of a new polymerase chain reaction assay designed for early detection of Mc. Two-hundred and six fish representing 27 species from 11 sites were examined for parasites using standard necropsy procedures. No Mc were recovered. The infection rates for myxosporeans related to Mc, however, ranged from 0 to 66%, and no trout species examined (brown, brook) were infected with myxosporeans. Many white suckers and redhorse which inhabit the same trout streams, as well as spotted suckers and carp from the Mississippi River, harbored myxosporeans whose spores were saved for use in testing the PCR assay specificity. We began the process of identifying these myxosporeans using classical morphometrics, and work is being continued at the La Crosse Fish Health Center, where it appears that this assay is indeed specific for Mc. We have also conducted surveys of benthos from the trout streams and established several hopefully pure laboratory cultures of oligochaetes. These benthic samples are being examined for oligochaete community structure. Many specimens of *Tubifex tubifex*, the only known intermediate host for Mc, have been identified from these streams, and their presence as well as that of susceptible salmonid species, suggests that conditions are appropriate for WD to establish in the Coulee region.

## INTRODUCTION

Whirling disease (WD) is caused by the exotic parasite *Myxobolus cerebralis* (Mc), which was described in Europe in 1903 (Hofer, 1903). WD exists as myxospores located in the head and gill cartilage of salmonid fish, or alternatively as actinospores in its secondary host, the oligochaete worm *Tubifex tubifex* (Tt) (Hedrick et al., 1996). Since 1903, Mc has experienced significant transcontinental spread due to the increased importance of salmonid fish farming throughout the world (El-Matbouli et al., 1992). Since its introduction into the United States in the mid-1950's, WD has been reported mainly in fish hatcheries. In 1994, however, a severe outbreak of WD was reported in the rainbow trout (*Oncorhynchus mykiss*) population of the Madison River of Montana (MacConnell and Lere, 1996). It soon became evident that similar losses were occurring in other rivers in the intermountain west, and that careful control measures

should be enacted and enforced. Relatively rapid methods of identification of both Mc and Tt were needed to control the spread of this harmful pathogen.

Since the tubificid oligochaete Tt was first described as being the secondary host for Mc in 1986 (Wolf, Markiw, and Hiltunen, 1986), much has been learned about the culture and identification of these worms. Any study on the potential for spread of WD must take into consideration the role of *Tubifex tubifex* in the Mc life cycle. Mason (1994) reviewed the common culture methods for five species of oligochaetes, including Tt. In his review, he described the methods used to culture Tt as fish food, but his methods remain sound for use in the laboratory as well. In 1997, an aquatic oligochaete workshop was held in Logan, UT, that focused primarily on the collection of Tt from rivers and streams and the subsequent culture and identification of these worms. It not only expanded on Mason's methods, but also included a key for the microscopic identification of Tt (Standard Operating Procedures, Aquatic Oligochaete Workshop, 1997).

The identification of Mc is time and labor intensive. First, the characteristic signs of WD must be observed in a population of fish. These include tail-chasing, a blackening of the caudal peduncle, and severe deformation of the axial skeleton. Once these signs are observed, the potentially infected fish are sacrificed and their heads are removed and halved, with one half ground and directly examined for microscopic myxospores and the other half used for confirmatory diagnosis (Blue Book, Thoesen, 1994.) Confirmatory diagnosis can involve direct fluorescent antibody testing, plankton centrifuge, or a pepsin-trypsin digest, all of which are time and labor intensive (Markiw and Wolf, 1978, Mitchell et al., 1983, Landolt, 1973).

A better method for detecting and identifying Mc in salmonids and oligochaetes involves the use of the polymerase chain reaction to specifically amplify Mc DNA (Andree et al., 1998). Use of this new method could result in rapid, early detection of WD, even in sub-clinical infections. Much work, however, still needs to be done on testing the specificity of this relatively new assay.

The specific goals of our research were to determine the overall myxosporean community structure in streams in western Wisconsin, and in the process, to determine the potential for Mc to invade Coulee Region streams and rivers. We did this by looking for Mc and myxosporeans related to Mc, and by determining oligochaete worm community structure, specifically that of Tt. In addition to this, all myxosporeans found were used to help determine the specificity of Andree's PCR assay with the help of personnel at the La Crosse Fish Health Center.

## **MATERIALS AND METHODS**

Fish sampling was conducted using back pack electroshockers, seines and dipnets in the small trout streams, and larger boat electroshockers were used in the Mississippi River. Fish were placed in holding tanks containing stream water for transport to the laboratory. Live fish were maintained in raceways containing aerated continuous flow well water until necropsy.

Prior to necropsy, fish were immersed in Finquel (MS-222) then measured to length (standard and total), weighed, and identified by taxonomic keys (Becker 1983, Pflieger 1975). The necropsies were performed using scissors, scalpels, and dissecting microscopes. All host tissues were examined for parasites including skin, fins, gills,

muscles, gastrointestinal tract, urinary bladder, gall bladder, and other viscera. Parasites were stored in finger bowls containing tap water. Myxosporeans were frozen in microcentrifuge tubes at  $-80^{\circ}\text{C}$  and used for PCR testing. Representative parasites were stained and mounted on slides as permanent preparations.

Oligochaete sampling was accomplished using various tools. Streams were sampled using Surber samplers (quantitative method) and kick nets (semi-quantitative method); pools and lake bottoms were sampled using Ekman dredges (quantitative method). Once collected, the samples were analyzed for the presence of oligochaetes by sieving mud through a 250 micrometer mesh screen and examining the sieve with a dissecting microscope. Oligochaetes were removed using a camel's hair brush. Some were placed in 400ml glass jars for culturing. Prior to culturing, fine grained mud had to be autoclaved to ensure pure cultures. Representative oligochaetes from eleven sites were fixed in formalin and mounted on slides using CMCP-10 mounting media. Oligochaetes were identified using the taxonomic key of Brinkhurst (1986).

The PCR assay of Hedrick (Andree et al. 1997) was used at the La Crosse Fish Health Center (LFHC), U.S. Fish and Wildlife Service, Onalaska, WI to determine if myxosporeans collected from local fish would cross react with Mc (See Appendix I for protocol). The LFHC has previously performed the plankton centrifuge test as its presumptive diagnosis of WD (O'Grodnick, 1975). The plankton centrifuge cooking method of half of the fish heads in a microwave, then removing all skin, brain tissue, and muscles from the head. Next the skull bones and cartilage are homogenized in a blender, filtered through cheese cloth and spun in a plankton centrifuge where a milk-like supernatant is gathered. This supernatant is examined for the presence of myxosporean spores. If spores are present, the other half of the fish head is examined to determine histologically that the spores did originate in the cartilaginous portions of the fish skull.

## RESULTS

The myxosporeans recovered from Coulee Region trout streams are summarized in tables 1 and 2.

Table 1. Prevalence of infection by species in Coulee Region Streams.

Species	# of fish sampled	Prevalence of myxosporean infection	Prevalence of fish with parasitic load
White Sucker	35	25.7%	60.0%
Spotted Sucker	3	66.0%	100.0%
Slimy Sculpin	9	44.9%	44.9%
Central Mudminnow	13	15.4%	84.6%
Brook Stickleback	20	0.0%	65.0%
Blacknose Dace	1	0.0%	0.0%
Fantail Darter	1	0.0%	0.0%
Banded Darter	5	0.0%	0.0%
Johnny Darter	4	0.0%	75.0%
Channel Catfish	3	0.0%	66.0%
Freshwater Drum	3	0.0%	100.0%
Bowfin	1	0.0%	100.0%
Creek Chub	21	9.5%	47.6%
Carp	5	0.0%	80.0%
Bluegill	1	0.0%	100.0%
Black Crappie	1	0.0%	100.0%

*Chart continued on next page.*

Species	# of fish sampled	Prevalence of myxosporean infection	Prevalence of fish with parasitic load
Sauger	2	0.0%	50.0%
Walleye	3	0.0%	66.0%
Silver Redhorse	4	50.0%	75.0%
Golden Redhorse	4	50.0%	50.0%
Shorthead Redhorse	3	33.3%	100.0%
Smallmouth Bass	3	0.0%	100.0%
Largemouth Bass	2	0.0%	100.0%
Rock Bass	2	0.0%	100.0%
White Bass	3	0.0%	100.0%
Brook Trout	25	0.0%	84.0%
Brown Trout	29	0.0%	13.8%

Table 2. Prevalence of all parasite species infection by sample areas.

Sample Area	# of fish sampled	Prevalence of infection
Timber Coulee	20	55.5%
Goose Island	4	50.0%
Spring Coulee	42	35.7%
Burns Creek	25	84.0%
Dutch Creek	8	12.5%
Trempealeau		
National Wildlife Refuge	10	100.0%
Mississippi River	42	83.3%
Midway Creek	27	55.6%
Halfway Creek	7	85.7%
Bohemian Coulee	10	40.0%
North Fork Chipmunk	11	45.5%

**Summary of Nested PCR.** The PCR results indicate that the nested PCR protocol is specific for Mc. There was one nonspecific cross reaction with the first primers for a myxosporean that inhabits the meninges of a bluegill.

## DISCUSSION

To date, no Mc have been isolated from brown and brook trout in Coulee Region trout streams. Considering the devastation that has occurred in the Western United States, it is surprising that the Wisconsin Department of Natural Resources has only recently begun surveys of salmonids within the state for Mc. The implications of this disease becoming established include the loss of a multi-billion dollar a year Great Lakes sport fishery and a multi-million dollar a year inland trout fishery. Many Great Lakes fish are at great risk of devastation due to WD. Steelhead trout (*Oncorhynchus mykiss*) is an anadromous strain of rainbow trout; rainbow trout are considered to be the species of salmonid that is most susceptible to development of clinical signs of WD and represent the most seriously affected species of trout in the intermountain west (Matthews 1995). The widespread distribution of brown trout in Wisconsin is also of concern since brown trout co-evolved with Mc in Eurasia, brown trout and Mc are relatively tolerant of each other and clinical signs of WD rarely occur in Mc infected brown trout. As a result, brown trout could serve as a reservoir population for Mc should the

parasite be introduced into Wisconsin. The mixing of a carrier species such as brown trout with sensitive species such as rainbow and brook trout in the same stream may not be the preferred management strategy.

Currently no myxosporeans have been isolated from Coulee Region trout. The absence of Mc, or other myxosporeans in local trout streams will make it easier to identify Mc should it be introduced into Wisconsin. The presence of other myxosporeans in trout populations in the western states confound the accurate determination of Mc in fish from that area. For instance, *Myxobolus neurobius* is a central nervous system parasite of brown trout and differentiation of this species from Mc is not easy based on morphology alone (Markiw 1992). The fact that myxosporeans (*M. bibullatum* in white suckers and *Thelohanellus* sp in slimy sculpins) occur in the Coulee Region streams suggests that Mc would be able to establish itself here. Since all myxosporeans for which the life cycles have been worked out utilize an oligochaete intermediate host (El-Matbouli and Hoffmann 1989, 1991, 1993; Kent, Whitaker and Margolis 1993; Yokoyama, Ogawa and Wakabayashi 1995) then substantial oligochaete populations must exist in these streams. Preliminary studies indicate that the only known intermediate host for Mc, *T. tubifex* (Markiw and Wolf 1983, Wolf and Markiw 1984), has been found in abundance in Coulee Region trout streams. *Limnodrilus hoffmeisteri*, a key intermediate host for several other myxosporeans, is also common in local streams.

The myxosporeans collected from local waterways have been analyzed via the Hedrick PCR assay, and currently there have been no cross reactions. This indicates the WDP-PCR assay is specific for Mc. This assay can be used with more confidence now that the work done on the assay indicates that they should not have false positive results. The PCR assay should replace the plankton centrifuge method (O'Grodnick 1975). The plankton centrifuge method is time consuming and labor intensive, and the fish must have a heavy load of myxosporeans for a positive determination. The improvements brought by the PCR include the ability to determine infection in lightly infected fish, as well as increased specificity, and savings in time and cost. Current diagnostic procedures involve sacrificing the host, and the PCR assay may allow for the development of a non-lethal sampling procedure using a small biopsy specimen.

In conclusion, the findings of this study indicate the likelihood that WD can become established in Wisconsin. The intermediate host (*T. tubifex*), carrier host (brown trout) and highly sensitive salmonid species (rainbow, steelhead and brook trout) are widespread and abundant in the state's waterways.

## APPENDIX I

### Sample Preparation for Polymerase Chain Reaction (PCR)

#### A. From fish

Samples of *M. cerebralis* will be obtained from fish using the protocols currently being developed by Hedrick. These protocols are still in laboratory development and Hedrick (personal communication with Dr. Sutherland) recommends the following procedures for archiving specimens for future utilization with PCR. If Hedrick's procedures do not become available, then it will be necessary to develop our own protocols. Most likely, some modification of the plankton centrifuge method of O'Grodnick (1975) would yield *M. cerebralis* spores for testing specificity of the PCR assay.

#### B. From oligochaetes and myxosporeans easily accessible from fish

Samples will be obtained from oligochaetes and fish using standard procedures. Tissue will be ground in 0.1 ml of Tissue Digestion Buffer (20 mM tris, 20 mM KCl, 0.5% Tween 20, 0.5% Nonidet P-40, 5% Chelex). Proteinase K will be added to a concentration of 0.2 mg/ml. Samples will be incubated at 56° C for four hours, boiled for ten minutes, vortexed and microcentrifuged at 6500 rpm for two minutes. The supernatant will be removed for PCR amplification.

#### Nested PCR Protocol

Samples will be subjected to nested PCR as outlined by Hedrick (personal communication with Dr. Sutherland). The final reaction conditions will be used: 1X PCR buffer, 1.5 mM MgCl<sub>2</sub>, 1.5 U Taq polymerase, 5 pmol of each primer (Tr 3-16 and Tr 5-16), 0.4 mM dNTPs and 7 microliters tissue digestion supernatant. Each sample will be subjected to 34 cycles of amplification at 94° C for 1 minute, 65° C for 9 minutes and 73° C for 1.5 minutes. The amplification will be initiated with a five minute hold at 94° C, and finished with a five minute hold at 72° C and stored at either 4° C or -20° C. A second round of PCR will then be performed using the PCR product from the first reaction as template, and the nested primers (Tr 3-17 and Tr 5-17).

To prevent contamination of samples with PCR products from previous reactions, we will use dUTP in place of dTTP in the reactions. The first round of amplification will be performed in the presence of the enzyme uracil n-glycosidase which will degrade any DNA containing uracil. Each sample will also be spiked with an internal control. Amplification of this positive control ensures that a negative result for *M. cerebralis* will not be due to sample inhibition of the PCR amplification.

#### Analysis of PCR products

PCR products will be analyzed by agarose, gel electrophoresis (1.0% agarose in 0.5X TBE) and followed by ethidium bromide staining. Samples testing positive for *M. cerebralis* will show a 800 bp fragment. Samples that do not contain *M. cerebralis* will not show an 800 bp band after PCR amplification.

#### PCR Troubleshooting

Hedrick has checked his PCR assay on eight different species of *Myxobolus* from the west coast, and saw no cross-reactivity. We do not expect extensive cross-reactivity using these PCR primers on *Myxobolus* species indigenous to the midwest. If cross-reactivity with pure preparations of indigenous *Myxobolus* is encountered then several options are available. Amplified regions from indigenous species could be cloned and sequenced, and the sequences compared with that of *M. cerebralis*. Regions of DNA sequence unique to *M. cerebralis* could then be chosen to generate more specific primers that would not produce cross-reactions with indigenous species of *Myxobolus*. A second approach would be to examine the DNA sequence of *M. cerebralis* for unique restriction sites that would allow one to distinguish between species. This could easily be accomplished using computer programs available at UW-La Crosse. Alternatively, a DNA hybridization procedure could be established that would serve as a second criterion to be met for a more specific identification.

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