5.2 *Myxobolus cerebralis* (Whirling Disease)

Myxobolus cerebralis may be difficult to detect because the life cycle of the parasite includes two alternate hosts: salmonids and the aquatic oligochaete worm, *Tubifex tubifex* (Wolf and Markiw 1984); and the extended time required for sporogenesis in the salmonid host. For an *M. cerebralis* inspection, sampling should target the most appropriate species, age, and rearing units that are most likely to reflect the presence of the parasite. The species most susceptible are rainbow trout, sockeye salmon, and steelhead. Brook trout, cutthroat trout, Atlantic salmon, and chinook salmon are moderately susceptible; brown and bull trout, coho salmon, and splake are partially resistant. Conflicting data are present for Arctic grayling and lake trout but in general, these species may be considered resistant or partially resistant to *M. cerebralis.* Fish are most susceptible if exposed when young; however, older fish may become infected and act as carriers of spores. Fish that have a high degree of resistance should not be selected for sampling unless they are the only species present (O'Grodnick 1979; Hedrick et al. 1999a, b; Hedrick et al. 2001; MacConnell and Vincent 2002).

Development of myxospores is temperature dependent, requiring a minimum of 90 days at 16 to 17˚C, and 120 days at 12 to 13˚C (El-Matbouli et al. 1992). When temperature data is available, sample fish that have been on the water supply for a minimum of 1800 degree-days. If continuous temperature monitoring data is not available, select fish that have been on the water supply for a minimum of six months. Select fish that reside in locations on an aquaculture facility most likely to result in exposure to the parasite such as earthen rearing containers and from locations receiving untreated surface water.

Screening for *M. cerebralis* is by examination for spores in cranial cartilage processed by pepsin-trypsin digest. Up to five fish may be pooled for screening; confirmation is on individual fish. Subsampling from large fish by using a halved head or a core sample is accepted; however, detection sensitivity may be decreased if the number of cartilaginous areas containing the parasite are few or if parasite numbers are low. Another difficulty is parasite affinity for different tissue sites may vary between salmonid species (in rainbow trout the parasite prefers the ventral calvarium; and brown trout the parasite is detected in gill arches). Confirmation of *M. cerebralis* is by identification of parasite stages in histological sections of cartilage tissue, or by amplification of parasite DNA by the polymerase chain reaction (PCR).

A. Screening Test

1. **Pepsin-Trypsin Digestion (PTD)** (Markiw and Wolf 1974; Lorz and Amandi 1994)
   This method is recommended for fresh samples; if the sample has been frozen, follow the procedure below with modifications noted in A.1.c.1 and A.1.d.

   a. Defleshing

      i. Samples should be placed in an appropriate container and heated at 45°C in a water bath until flesh is soft and eyes are opaque.

      ii. Deflesh the samples and retain all cartilage/bone including that from the gill arches and opercula.

   b. Pepsin Digestion

i. Weigh cartilage and add 0.5% pepsin solution (Section 2, 5.6.A "0.5% Pepsin Solution") at a ratio of 20 mL/g cartilage.

ii. Large heads (greater than 20 g after defleshing) may be homogenized in the pepsin solution with an electric blender.

iii. Stir at 37°C and monitor pH of pepsin solution. If pH increases above 4.0, centrifuge samples at 1200 x g for 10 minutes, decant pepsin, and add fresh pepsin to the sample.

iv. Process samples until all cartilage/bone is reduced to a consistently small, granular size (e.g. the size of beach sand; one hour is generally sufficient for small fish; two hours to overnight may be required for larger fish).

v. When digestion is complete, centrifuge at 1200 x g and decant pepsin into a solution of 1:4 bleach/water (5,000 ppm chlorine) for disposal.

c. Trypsin Digestion

i. Add 0.5% trypsin (Section 2, 5.6.B "0.5% Trypsin Solution or 0.5% Trypsin with Phenol Red") at a ratio of 20:1 (volume/g) to the pellet.

Note: If samples have been frozen prior to processing, reduce the concentration of trypsin to 0.05%

ii. Adjust to pH 8.5 with 1 N sodium hydroxide (Section 2, 5.6.C "1N Sodium Hydroxide (NaOH)").

iii. Stir at room temperature for 30 minutes.

iv. If samples are to be refrigerated at this stage, inactivate trypsin by adding serum to a final concentration of 20%, or powdered bovine serum albumin to a final concentration of 1%.

v. Pour digested sample through a non-absorbent disposable filter [e.g. paint filters (any paint store), urinary calculi filters, or synthetic material such as nylon screen] into a new tube. Mesh sizes should approximate 200 µm. Reusable filters are not recommended because of difficulties in decontamination. Autoclave filters before disposal.

vi. Centrifuge at 1200 x g for 10 minutes.

vii. Discard supernatant into a solution of 1:4 bleach/water (5,000 ppm chlorine) while retaining pelleted material.

d. Dextrose Centrifugation (Markiw and Wolf 1974)
This concentration step is recommended if spores are not found on initial examination or if a large amount of tissue debris makes microscopic examination difficult; it is not recommended if samples have been frozen because it causes distortion and decreased recovery of the spores.

i. Add 1 mL of phosphate buffered saline (PBS) (Section 2, 5.6.D "Phosphate Buffered Saline (PBS)") to the sample and vortex (this volume may need to be adjusted depending on pellet size, but should be sufficient to suspended all material).

ii. A 55% solution of dextrose is used to provide a 5 cm deep gradient in a 15 mL centrifuge tube. For larger samples, a 50 mL centrifuge tube may be required.

iii. Carefully layer the suspended sample onto the dextrose solution.

iv. Centrifuge at 1200 x g for 30 minutes.

v. Carefully decant the supernatant into a solution of 1:4 bleach/water (5,000 ppm chlorine) while retaining all pelleted material.

vi. If PTD sample will be used for PCR confirmation, the pellet should either be suspended in 0.5 to 1.0 mL of PBS and examined immediately (a 10 fold dilution of the pellet should be sufficient to disperse material enough to see clearly under the microscope). The sample may then frozen until assay or preserved using 70% ETOH.

e. Analyzing Samples

i. Vortex samples prior to preparing slide.

ii. Samples may be examined stained or unstained by wet mount or using a hemocytometer. A number of simple staining methods are suitable for staining spores; e.g. add 60 µL of a saturated solution of crystal violet biological stain to 1 mL subsample, mix and examine.

iii. A minimum of 150 fields should be examined at 200x magnification (20x objective with 10x ocular eyepiece). Measure ten representative myxospores and record the average size and size range. Identification of myxospores with the appropriate size and morphology (8 to 10 µm, rounded, two polar capsules; Figure 5.1) results in a sample designation of **PRESUMPTIVE positive.** If the size is appropriate but internal morphology is not clear, the sample should also be identified as **PRESUMPTIVE positive.** A single myxospore of appropriate size is sufficient to declare a **PRESUMPTIVE positive**. **All other samples are reported as negative.**

Note: Other myxobolid species of salmon have similar morphologies that may be confused with *M. cerebralis*. Photographs and measurements of some of these species are provided in Figure 5.2 for comparison.

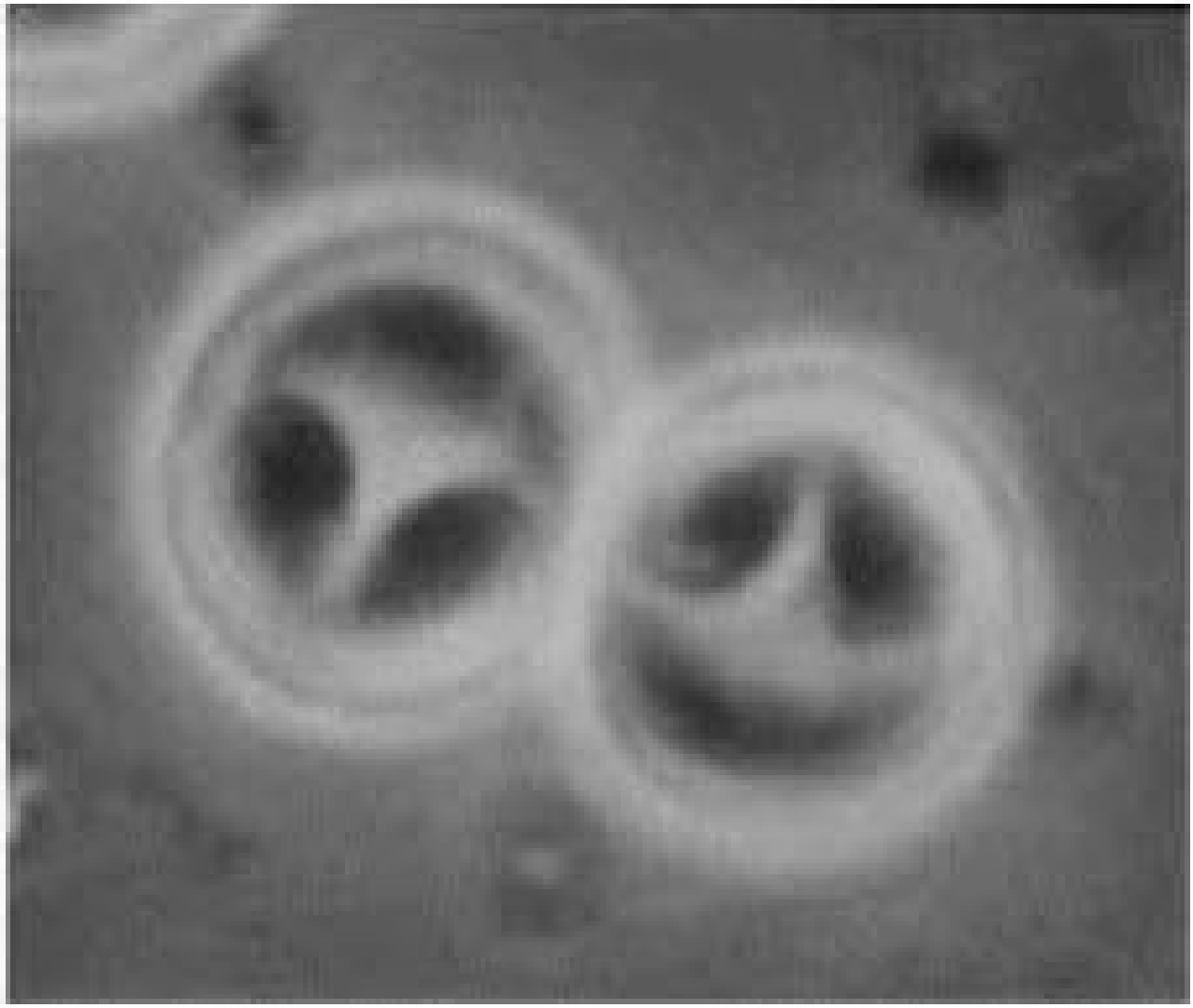
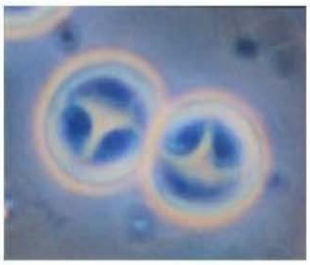


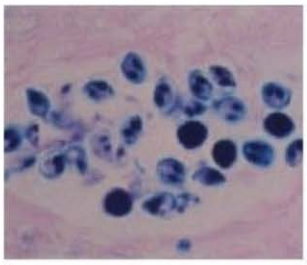
Figure 5.1. Spores of *Myxobolus cerebralis*. Photograph courtesy of R. P. Hedrick.

Figure 5.2: Myxozoans Common To Salmonid Fish



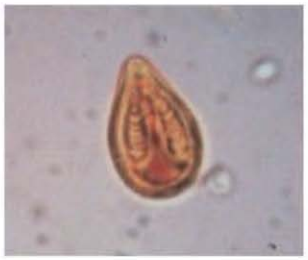
Myxobolus cerebralis

L 7.4 - 9.7 x W 7 - 10 x T 6.2 x 7.4 µm



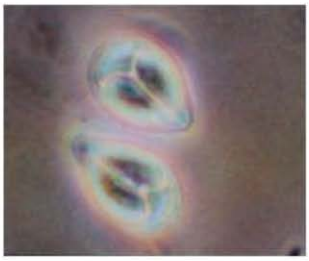
Myxobolus kisutchi

L 8.5 x W 7 µm



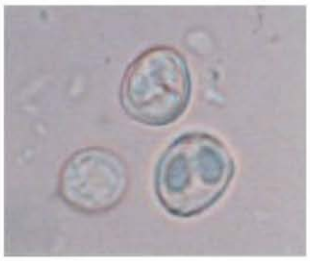
Myxobolus insidiosus

L 12.8 - 17.1 x W 8 - 11.9 µm



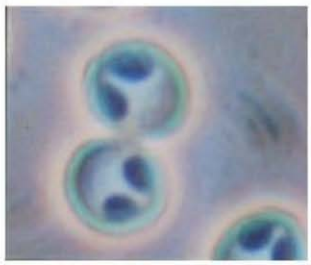
Myxobolus arcticus

L 14.3 - 16.5 µm



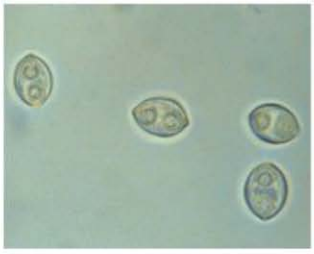
Myxobolus neurobius

L 10 - 14 x W 8 - 9.2 µm



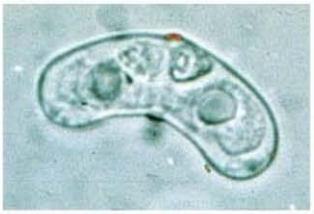
Myxobolus squamalis

L 8.4 - 9.9 x W 7.7 - 9.9 µm



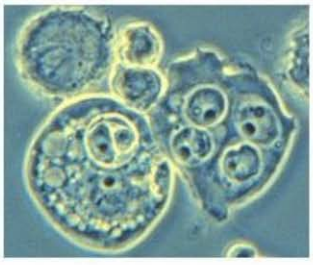
Myxidium minteri

L 9.3 - 12.6 x W 4.5 - 5.3 µm

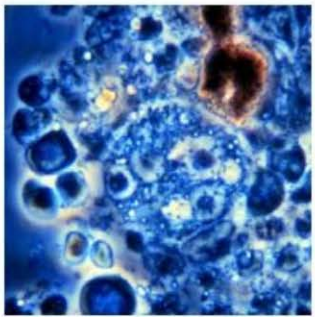


Ceratomyxa shasta

L 14 - 23 x W 6 - 8 µm



Ceratomyxa shasta trophozo



PKX organism

B. Confirmatory Tests

Confirmation of *M. cerebralis* should rely on either histopathology or the nested version of the polymerase chain reaction (PCR) (Kelly et al. 2004).

1. Histopathology

a. Confirmation of *M. cerebralis* may be done using standard histological techniques. Detection of spores with the morphological characteristics of those identified in the digest preparation in tissue other than the cartilage (e.g. brain, nervous tissue, skin, or muscle) is sufficient to report the sample as negative for *M. cerebralis*. However, failure to detect spores of the correct morphology in any tissue is not sufficient to report the sample as negative. Half heads, wedges, or core samples, which were placed in 10% neutral buffered formalin or Davidson's fixative at the time of collection (Section 2, 2.2.E.4 "Collection of Specimens for the Detection of *Myxobolus cerebralis* Evaluation") will be used for corroboration. Process tissues corresponding to a sample number in which a presumptive positive was found.

b. Small samples (from fish <15 cm) fixed in Davidson's can be transferred to 70% ethanol after 24 to 48 hours. Larger samples should remain in Davidson's for 48 hours and may require an additional decalcification step (as specified below).

c. Samples fixed in 10% formalin should be transferred after 24 to 48 hours to a commercially available chemical decalcification solution (e.g. Lerner-D-Calcifier, Decal-Stat Solution, Cal-Ex, and others) or acid decalcifying solution (e.g. 10% Nitric Acid, 5% Formic Acid).

d. Samples requiring decalcification are placed into embedding cassettes in a beaker with a magnetic stir bar and sufficient decalcifying solution (20:1 solution to gram of head tissue). Covered and place on a stirring plate for 4 to 16 hours at room temperature. After three hours, and periodically thereafter, use physical and/or chemical tests to determine if the process is complete (Hauck and Landin 1997).

   i. Physical
   Probe using a dissecting needle to determine softness of bone and/or cartilage (avoid puncturing the tissues). Soft tissues are adequately decalcified.

   ii. Chemical
   Remove 5 mL of decalcifying solution from beaker and neutralize (pH 7.0) with 0.1 N sodium hydroxide (Section 2, 5.6.C "1N Sodium Hydroxide (NaOH)" stock solution). Add 1 mL of saturated ammonium oxalate solution and mix. Allow to stand for 15 minutes and determine if a precipitate (slight cloudiness) of calcium oxalate forms. If so, the decalcification is incomplete. If incomplete, replace decalcifying solution and continue decalcification process until no calcium oxalate precipitate can be detected.

   iii. After decalcification, rinse specimens in distilled water and transfer via graded ethanol series to 70% ethanol.

e. Embed and section tissues at approximately 4 to 5 µm using standard methods. Two sections, taken at 100 µm apart, should be mounted from each sample (or each tissue block if more than one block per fish) and stained with Giemsa (May-Grunwald Giemsa works well, Luna 1968) or Hematoxylin and Eosin (H&E). The sections should target gill arches

(especially from brown trout) and the ventral calverium (floor of the brain case). Examine slide at 200X for cartilage lesions, spores and developing stages of *M. cerebralis* (Figures 5.3 and 5.4).

f. At least two additional sections (100 µm apart) should be taken, mounted and stained if histological lesions and/or spores are not observed.

Observation of any presporogonic or sporogonic stage of *M. cerebralis* in cartilage tissue is reported as positive. Observation of spores with the same morphology as those identified in the digest preparation in tissues other than cartilage are reported as negative. Confirmation cannot be reported if no spores are detected.

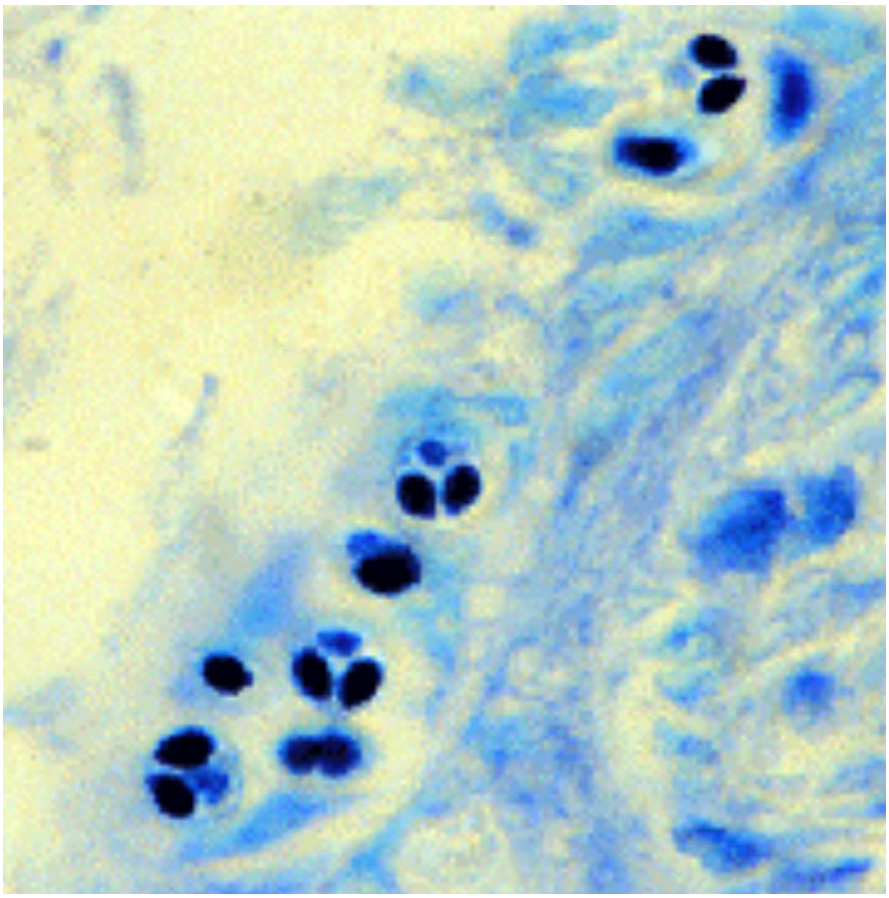


Figure 5.3. Histological sections from a fish infected with *Myxobolus cerebralis*. High magnification showing stained spores (note darkly stained polar capsules). Photograph courtesy of H. V. Lorz.

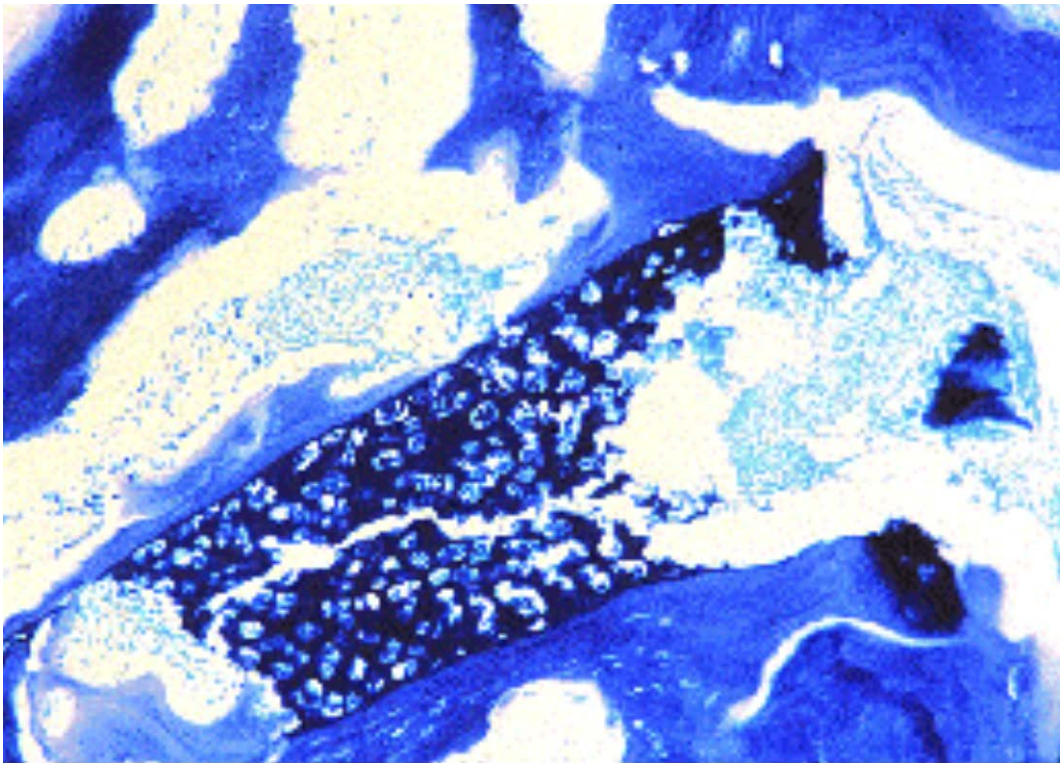


Figure 5.4. Histological sections from a fish infected with *Myxobolus cerebralis*. Low magnification showing degradation of cartilage (Leishman-Giemsa stain).

2. Polymerase Chain Reaction (PCR) (Andree et al. 1998; Schisler et. al. 2001)

PCR should be utilized as a confirmatory test *only* on tissue collected as described under specimen collection for PCR at the time of inspection. The assay may be performed directly on the remaining half head or core sample, allowing detection of target DNA from immature parasites as well as from spores (Section 2, 5.2.B.2.a "Extraction of DNA from a Half Head or Core for PCR Confirmation of *Myxobolus cerebralis*"). An alternative method (Baldwin and Myklebust,2002) identifies *M. cerebralis* DNA from spores concentrated by PTD (Section 2, 5.2.B.2.b "Extraction of DNA of the PTD Product for PCR Confirmation of *Myxobolus cerebralis*"). Use of this method allows the other half head to be preserved for histology. However, by applying PCR methods to a digested preparation DNA, presporogonic life stages of the parasite are destroyed, with a potential loss of detection sensitivity. The University of California has awarded an exclusive license to Biogenetic Services, Inc., for the use of the DNA-based diagnostic test for detecting the *Myxobolus cerebralis* organism described by Andree et al. (1998). Contact Biogenetic Services, Inc. for additional information on the availability and limitations to the use of this PCR-based technology. (Phone 605/697-8500; Fax 605/697-8507; Email: biogene@brookings.net; Website: www.biogeneticservices.com)

a. Extraction of DNA from a Half Head or Core for PCR Confirmation of *Myxobolus cerebralis*

Note: The following protocol recommends use of the Qiagen DNeasy Tissue Kit (Qiagen Inc, Valencia, CA; Qiagen #69506). Although other kits may work similarly, they have not been tested for this application. Use the kit as per the handbook, with the following modifications:

i. See Section 2, 6.2 PCR – Quality Assurance/Quality Control for specific QA/QC considerations for PCR.

ii. Place half-head or core sample in screw capped tubes appropriate for the sample size. Add tap water to cover the tissue and heat in a water bath at 95°C for 15 minutes. For heads ≥ 5 centimeters (2 inches), use 8 ounce sample cup or small beaker and heat for 20 minutes.

iii. Pour water into a solution of 1:4 bleach/water (5,000 ppm chlorine) for 30 minutes to disinfect and place fish sample on polypropylene cutting mat or other surface that can be disinfected or disposed of (autoclave).

iv. Deflesh head using a clean scalpel and forceps; place bone and cartilage in a 1.7 mL micro centrifuge tube or a 50 mL polypropylene screw cap centrifuge tube for larger samples.

v. Add a sufficient volume of ATL buffer, such that it is mixed with the bone material approximately 1:1 (v:w) and proteinase K solution equivalent to 1/10 volume of the buffer. If glass beads are used, extra beads can be added for the larger heads.

Tissue lysis buffer	ATL buffer (Qiagen #69504)
Proteinase K solution (See Section 2, 5.6.E "Proteinase K.")	(17.86 mg/mL)
150 mg small glass beads (optional)	710 – 1180 μm, acid washed
3 large glass beads (optional)	3 mm, acid washed

vi. Vortex until the sample is broken up.

vii. Incubate at 55°C on a rotating platform (or with occasional vortexing) for at least 3 to 4 hours to overnight.

viii. Centrifuge at 16,000 x g for five minutes.

ix. Add 200 μL *aqueous* supernatant to clean micro centrifuge tube.

Note: Occasional clear layer above the white lipid layer is oil; aqueous layer is between lipid layer and debris/glass bead pellet and may be turbid. Dark particulates do not affect DNA yield but can increase column spin times required.

x. Add 20 μL RNase A (Section 2, 5.6.F "RNAse A") (20 mg/mL; Qiagen #19101), vortex until mixed thoroughly, and incubate at room temperature for two minutes.

xi. Follow kit instructions for elution of DNA using buffers and spin column supplied in the kit (DNeasy Tissue Kit; Qiagen #69506), with the following precautions:

Note: "Dirty" preps may require longer spins. DNA preps from positive samples give consistent results when using spin columns. When expecting very small amounts of DNA, the volume of elution buffer can be reduced to 50 μL.

b. Extraction of DNA of the PTD Product for PCR Confirmation of *Myxobolus cerebralis*

i. Pellet the myxospores of the PTD product by micro centrifugation at maximum speed (at least 10,000 rpm) for one minute.

ii. Decant the supernatant and air dry for five minutes

iii. Microwave for one minute in an 800-watt microwave oven at full power.

iv. Re-suspend the pellet in 500 μL of Proteinase K lysis buffer (10 mM Tris-Cl, pH 8.0, 2 mM EDTA, 0.1 % sodium dodecylsulfate and 0.5 mg mL^{-1} Proteinase K) and incubate at $55^{o}C$ for four hours frequently vortexing gently.

v. Remove sample to a clean laboratory hood and add 500 μL of phenol:chloroform:isoamyl alcohol (25:24:1) to the lysed sample.

Note: Procedures are being developed to substitute the phenol:chloroform extraction (steps v–x) with extraction using Qiagen Kits (Qiagen Inc, Valencia, CA, Qiagen # 69506). Currently this work remains unpublished, but the procedure is as above, with the modification that after microwave treatment, 180 μL of Qiagen Buffer ATL and 20 μL of Proteinase K are added to the sample. The sample is vortexed and incubated at $55^{o}C$ for 6

to 8 hours for complete cellular lysis. The DNA then can be selectively bonded to the Dneasy membrane columns, washed to reduce contaminates and inhibitors, then eluted using the Dneasy protocol for rodent tails. As will any procedure modifications, this should be thoroughly tested and documented in the laboratory.

vi. Gently invert sample several times to form an emulsion, then centrifuge at 1700 x g at room temperature for 10 minutes to separate the aqueous and organic phases.

vii. Transfer the upper aqueous phase (with the nucleic acid) to a clean micro centrifuge tube and add 500 µL of chloroform.

viii. Mix by inverting the sample several times and centrifuge at 1700 x g.

ix. Add 800 µL of ice cold ethanol and incubate at -20°C for one hour to precipitate the DNA.

x. Centrifuge at 12,000 x g for 10 minutes at 4°C, decant the supernate, and resuspend the pellet in 50 µL of TE buffer (10mM Tris, 0.1 mM EDTA, pH 8.0).

xi. Incubate sample overnight at room temperature.

c. Quantification of DNA
It is advisable that extracted products be measured using a spectrophotometer to ensure that enough DNA was successfully extracted. Quantification guidelines are in Section 2, 6.2.C.4 "Quantification of DNA."

Note: Quantify DNA of a representative sample (5% or 6%) from each group of a particular size range and assume all those within that size range have a similar concentration. If they do not range too widely, average the values and determine the DNA concentration. Then add an appropriate volume to each PCR assay such that the amount per reaction is between 100 and 300 ng.

d. Amplification of *M. cerebralis* DNA

i. Following general PCR protocols (Section 2, 6.2 PCR Quality Assurance/Quality Control). The reagents and primers for this reaction are:

1° Master Mix: 50 µL total reaction volume

H_2O – sterile, molecular biology grade	32.1 µL
Mg Cl_2 (50 mM)(5.6.K)	1.5 µL (final concentration 1.5 mM)
10X Taq Polymerase Buffer	5 µL
dNTPs (5 mM stock)	4 µL (final concentration 0.4mM)
Tr5-16 Primer 1 (20 µM)	2 µL (final concentration 0.8 µM)
Tr3-16 Primer 2 (20 µM)	2 µL (final concentration 0.8 µM)
Taq Polymerase (5 units/µL)	0.4 µL (2 units per reaction)

Primers (Andree et al. 1998)
Tr5-16 = 5'-GCA TTG GTT TAC GCT GAT GTA GCG A-3'
Tr3-16 = 5' -GAA TCG CCG AAA CAA TCA TCG AGC TA-3'

ii. Add PCR reagents **except for sample DNA** to the MM tube, adding water first and Taq last. Keep all reagents cold in frozen cryo-rack during mixing, and return them to freezer immediately after use.

iii. Place 48 μL of MM into each 0.5 mL PCR tube. Close caps tightly. Move PCR tubes to sample loading area.

iv. Load 2 μL of each sample DNA to the appropriately labeled PCR tubes.

v. Thermocycler should be programmed for 35 cycles of the following regime:

1. Cycle Parameters:

a. Denature at 95 °C for five minutes.

b. Then, 35 cycles at:
95 °C for one minute.
65 °C for 2.5 minutes.
72 °C for 1.5 minutes.

c. Finally, 1 cycle at 72 °C for ten minutes.

Note: For maximum sensitivity with weak positive samples these conditions are critical.

vi. For the nested amplification prepare the 2nd MM as before, but substitute primers Tr5-17 and Tr3-17. Transfer 1 μL of the amplification product from the first amplification to the appropriately labeled reaction tube containing the 2nd MM and load the thermal cycler using the same program as for the first amplification.

Primers (Andree et al. 1998)
Tr5-17 = 5' -GCC CTA TTA ACT AGT TGG TAG TAT AGA AGC-3'
Tr3-17 = 5'-GGC ACA CTA CTC CAA CAC TGA ATT TG-3'

e. Visualization of PCR Product by Electrophoresis

i. Prepare agarose gel as indicated in Section 2, 6.3.C "Detection of Product" and load 6.0 μL of amplified product + 1.5 μL loading buffer into sample wells.

ii. After electrophoresis of products (Section 2, 6.3.D "Electrophoresis"), stain and photograph gel as described in Section 2, 6.3.E "Staining the Gel" through 6.3.G "Visualize the DNA."

iii. Carefully record locations of bands on positive control samples in relation to DNA ladder bands. **Detection of the expected 415 bp (base pair) amplicon in a sample amplified using the nested PCR protocol will result in corroboration of the presence of *M. cerebralis* in the sample. Any samples not yielding this band with no apparent assay problems is reported as negative.**

iv. If any unusual bands are present in the samples or if bands are present in negative controls, re-run PCR reaction from template DNA tube.

v. **Photo document all gels** and attach the photo to the case history information.