5.3 *Ceratomyxa shasta* (Ceratomyxosis)

Ceratomyxa shasta is difficult to detect because the life cycle of the parasite includes two alternate hosts: salmonids and the freshwater polychaete worm, *Manayunkia speciosa* (Bartholomew et al. 1997). Salmonids are the only known fish hosts for *C. shasta*. Although susceptibility may vary between species and strain, all salmonids should be considered susceptible. The parasite is enzootic in a number of waters in California, Oregon, Washington, Idaho, Alaska, and B.C., Canada (Bartholomew et al. 1989a), but has not been reported outside of that region. Diagnosis is complicated by the long period required for development of mature myxospores in the fish and by the pleomorphic appearance of the presporogonic stages. Parasite development is temperature dependent, but in general, at 12ºC, a minimum of 40 days is required for spore development in rainbow trout in the laboratory. Confirmation of *C. shasta* is based on identification of myxospores with the appropriate morphology or by PCR confirmation of presumptive presporogonic life stages.

A. Screening Test

1. **Examination of Wet Mounts** (Bartholomew et al. 1989a, Bartholomew 2001)

   a. To prepare a wet mount, the intestine is removed, placed on a disposable surface and opened longitudinally with a clean scalpel or scissors. Scrape the posterior 1/3 of the intestinal mucosa and mix in a drop of water on a microscope slide. Prepare wet mounts from any areas of hemorrhage as well.

   b. Scan wet mount in a systematic manner under phase contrast or bright field microscopy at 200 to 440X magnification. Examine the entire smear or an area equal to that under a 22 mm^2 coverslip.

   c. Presumptive diagnosis is based on identification of multicellular myxosporidian pre-sporogonic stages (trophozoites) (Figure 5.4A). Visualization of prespore stages is not sufficient for definitive diagnosis. Any samples in which the organisms are not seen may be discarded and reported as negative.

B. Confirmatory Test

Confirmation of *C. shasta* should rely on either detection of mature spores or on amplification of parasite DNA using the polymerase chain reaction (PCR).

1. **Morphology** (Bartholomew 2001)
   Confirmatory diagnosis of *C. shasta* is based on identification of the characteristic myxospore (Figure 5.2). Myxospores of *C. shasta* measure 14 to 23 μm long and 6 to 8 μm wide at the suture line. The ends of the spores are rounded and reflected posteriorly and the suture line is distinct.

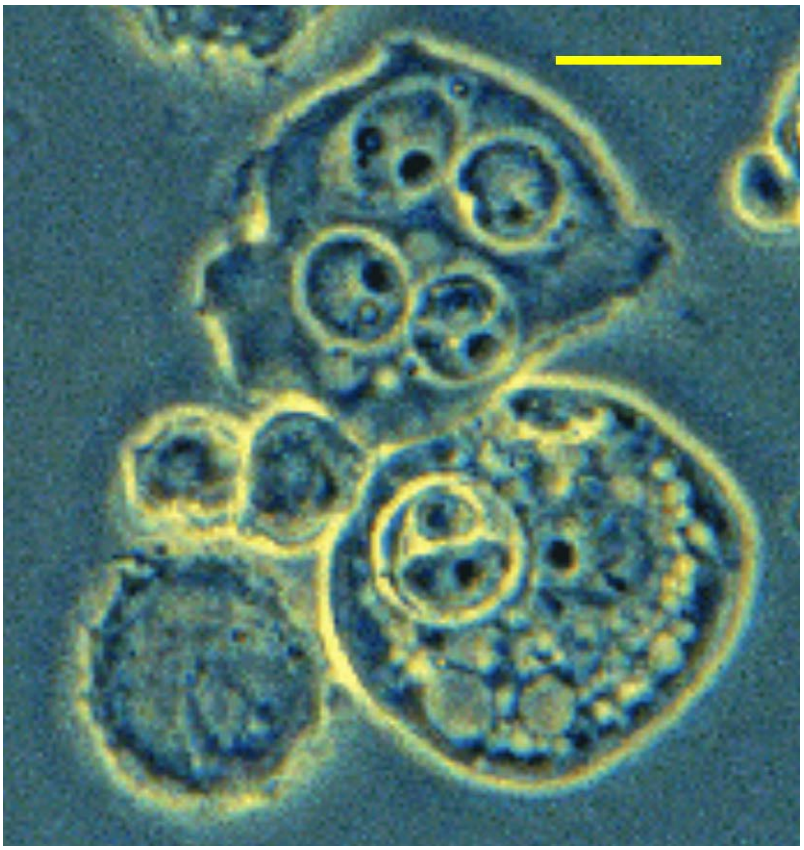

Figure 5.1. Wet mount of presporogonic stages of *Ceratomyxa shasta*. Bar equals 10 µm. Photograph courtesy of J. L. Bartholomew.

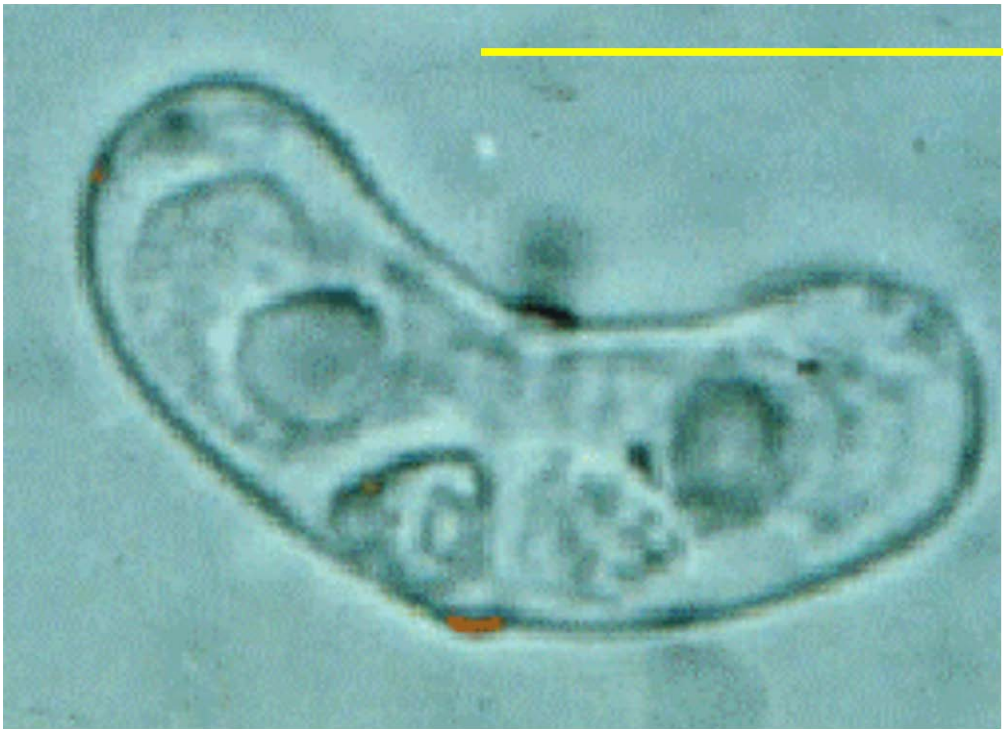

Figure 5.2. Wet mount of myxospore stage of *C. shasta***.** Bar equals 10 µm. Photograph courtesy of J. L. Bartholomew.

2. Polymerase Chain Reaction (Palenzuela et. al. 1999; Bartholomew 2001; Palenzuela and Bartholomew 2001)
PCR should be utilized as a confirmatory test *only* on tissue collected at the time of inspection, using methods described under specimen collection.

a. General Protocols and QA/QC Considerations (See Section 2, 6.2 PCR Quality Assurance/Quality Control for specific QA/QC considerations and general protocols for PCR.)

b. Extraction of DNA

i. If the sample was fixed in ethanol, transfer to micro centrifuge tube with 500 µL DNA extraction buffer (Section 2, 5.6.G "DNA Extraction Buffer"); if frozen, add 500 µL DNA extraction buffer.

ii. Add Proteinase K solution (Section 2, 5.6.E "Proteinase K") to a final concentration of 200 µg/mL (example: if stock solution is 20 mg/mL, add 5 µL).

iii. Incubate micro centrifuge tubes at 37°C in a horizontal position on a slow platform rocker or with frequent inversion by hand. Digestion of sample will require about 4 to 5 hours, but overnight incubation does not affect the quality of the DNA and is recommended.

iv. After samples are completely digested, add 5 µL of RNAse A (10 mg/mL stock) (Section 2, 5.6.F "RNAse A") to each micro centrifuge tube and incubate one hour at 37°C with rocking.

v. Heat samples at 100°C for five minutes (in a water bath, heat block, or thermocycler).

vi. Remove rack and cool at room temperature. Upon cooling, samples are ready for dilution and PCR. Samples at this stage may be stored at -20°C.

c. Amplification of *C. shasta* DNA

i. Dilute the DNA template (sample) 1:10 with ultra pure (molecular grade) sterile water.

Note: Because this assay utilizes crude DNA, quantitation by UV spectrophometer does not provide useful information. A 1:10 dilution is generally sufficient unless the tissue size is larger than recommended (if this is suspected, include a 1:100 dilution in addition)

ii. Following general PCR protocols (Section 2, 6.3 PCR Protocols), record appropriate data for PCR and calculate reagent volumes that go into the Master Mix (MM). This assay was optimized using reagents from Promega (Madison, WI, USA) and if other buffer systems are substituted, these should be tested. The primers and reagents for this reaction are:

Master Mix: 20 µL total reaction volume

H_2O – sterile, molecular biology grade	14.6 µL
Mg CL_2 (25 mM; Promega #A351B)	1.6 µL
10X PCR Poly Buffer (Promega #M190A)	2.0 µL

dNTPs (at 10mM each)	0.4 µL (final concentration 0.2 mM)
Forward Primer (100 µM)	0.1 µL (final concentration 0.5 µM)
Reverse Primer (100 µM)	0.1 µL (final concentration 0.5 µM)
Taq Polymerase (5 units/µL)	0.2 µL (1U per reaction)

Primers (Palenzuela and Bartholomew 2001):
Forward: 5' GGGCCTTAAAACCCAGTAG 3'
Reverse: 5' CCGTTTCAGGTTAGTTACTTG 3'

iii. Place 19 µL of MM into each 0.2 mL PCR tube. Close caps tightly. Move PCR tubes to sample loading area.

iv. Add 1 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 three minutes.

b. 35 Cycles of:
94°C for one minute.
58°C for 30 seconds.
72°C for one minute.
72°C for 10 minutes.
Hold to 4°C chill at end of program.

d. Detection

i. Prepare agarose gel as indicated in Section 2, 6.3.C "Detection of Product" and load 10.0 µL of each PCR reaction + 1.5 µL loading buffer (Section 2, 5.6.H "Loading Buffer") into sample wells.

ii. After electrophoresis of products (Section 2, 6.3.D "Detection of Product"), 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. ***C. shasta* positive reactions will have an amplicon of 640 bp. Any samples not yielding this band with no apparent assay problems is reported as negative and the samples are discarded.**

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** (Section 2, 6.3.G "Visualize the DNA") and attach the photo to the case history information.