3.2.4.1 Salmonid Ceratomyxosis Methods Appendix 1

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Molecular Detection of *Ceratomyxa shasta* Using the Polymerase Chain Reaction Assay (Palenzuela et al. 1999)

A. Sample Collection

Because of the specificity of the polymerase chain reaction (PCR) assay, contamination of the samples by bacteria or other contaminants is not a problem and sterility or aseptic techniques are not strictly necessary. However, the risk of cross contamination and carry-over of DNA from other samples is high and sampling must be done with this in mind.

Samples should be collected as follows:

1. Use disposable material for each fish or alternatively (see notes below) destroy the DNA from the dissecting tools between fish. Process one fish at a time.

2. Remove the intestine (or a piece) from the fish and place it on a clean, disposable surface (a piece of aluminum foil works just fine).

3. Cut a small piece of the gut (25 to 100 mg, about 2 to 5 mm), preferably from the lower tract. We use disposable, nonsterile razor blades to cut this small piece, and a simple toothpick to transfer it to the screw-cap vial with 500 µL DNA extraction buffer (see next section for recipe). Conventional dissecting tools can be used but only if they are completely decontaminated between samples.

4. Dispose of the working surface, change gloves if they came in contact with the tissues, and proceed to the next fish.

   Notes on decontamination of material: Conventional methods for disinfecting dissecting tools, such as spraying with ethanol and flaming, not only **DO NOT** completely destroy the DNA, but

they can actually fix it in the material. Procedures which are used to clean the material between samples include:

5. Use of the commercial products DNA AWAY or RNAse AWAY. Both destroy DNA quickly and safely. It is sufficient to wipe the material with a paper towel wetted with the product and then rinse it with fresh, distilled water from a lab bottle. These products are not recommended for use with metallic material, but we have used them liberally on forceps and scissors and have not encountered problems.

6. First wipe off any pieces of tissue, blood, or mucus from the tools, then immerse them in 10% bleach (Clorox) solution for ***at least*** one minute. Rinse before use with distilled water from a wash bottle. This concentration of bleach is corrosive for metals after long exposures, but this is not a problem if the exposure is limited to the sampling and tools are rinsed well in distilled water before storage. Use two containers (per person) with bleach during sampling, each containing a set of dissecting tools so that tools can soak between uses. After use, the tool is wiped clean with a piece of paper and put back in the bleach solution.

B. Protocol for the Diagnosis of *Ceratomyxa shasta* in Fish Intestines by PCR

1. DNA Extraction

a. Reagents:

i. DNA Extraction Buffer
The buffer is NaCl 100 mM, Tris-HCl 10 mM, EDTA 25 mM, SDS 1%.

To prepare 1 Liter of Extraction Buffer:
into 500 mL HPLC H_2O
20 mL 5M NaCl
5 mL 1M Tris-HCl , pH 7.8
50 mL 0.5M EDTA, pH 8.0
50 mL 20% SDS
pH solution to 8.2 then add HPLC H_2O to a final volume of 1 Liter

Aliquot extraction buffer into 50 mL tubes and seal with parafilm. The aliquots are stable at room temperature for 2+ years.

ii. Proteinase K
Obtain commercially as a stable liquid solution (store at 4°C) or make at a concentration of 20 mg/mL and freeze at -20°C in 1 mL aliquots

iii. RNAse A
100mg/mL stock commercially available. Dilute for use as needed.

b. Methods:
When processing tissue, special care must be taken as carry-over of DNA between samples occurs easily. Use new blades and/or tools when handling the tissues. Samples can be fresh or frozen. There is no need to clean the tissues of fat or adherent tissue.

i. Dissect intestine on a clean surface as described above. Cut a small piece (25 to 75 mg) with a new razor blade and transfer the tissue to a 1.5 or 2 mL nuclease-free microtube. The first few times it may be helpful to weigh the tissue, to get an idea of the size of tissue that should be cut. Samples should be frozen.

ii. Add 500 µL of DNA extraction buffer. Once the extraction buffer is added to the tissue the sample can be stored at room temperature or refrigerated for up to a few weeks.

iii. To complete extraction, add 5 µL of 20mg/mL proteinase K (final concentration of 200 µg/mL). Incubate at 37°C, horizontally on a slow rocking platform or with frequent inversion of the tubes until the tissue is completely digested (this takes about 4-5 hours, but overnight incubation does not affect the quality of the DNA and is recommended for larger samples).

iv. Following proteinase K incubation, add 5 µL of 10 mg/mL RNase A to the samples and incubate at 37°C for one hour.

NOTE: Centrifuging the tubes briefly before opening them should be done on a routine basis. Be very careful when opening the tubes: the samples are viscous and stick to the walls, cap and lid. Cross contamination easily occurs, either by aerosol when opening the tubes or just with the fingers when touching lids and caps.

v. Place sample at 85°C for 15min.

vi. Dilute the DNA samples 1:100 with ultrapure, nuclease-free water. Samples need to be diluted greater than 1:10, especially at low infection levels, as the crude DNA preparation contains PCR inhibitors. Dilution of samples to 1:100 works for all the samples tested to date. Very low infection levels have tested positive when diluted 1:1,000.

vii. Store at -20°C until needed.

2. PCR Reagents

a. Primers

The sequences for the forward and reverse *C. shasta*-specific PCR primers are as follows. Primers can be synthesized by a variety of companies.

CS-1 (forward)	5'- GGG CCT TAA AAC CCA GTA G -3'
CS-3 (reverse)	5'- CCG TTT CAG GTT AGT TAC TTG -3'

b. PCR Master Mix

The concentrations of the reagents are calculated for 20 µL reactions with 1 µL of sample and 0.2 µL of Taq polymerase per reaction. The Taq polymerase is added to the master mix immediately prior to aliquoting into individual PCR tubes/wells and template (sample DNA) is then added to each reaction.

For **10 mL** of master mix:

Sterile molecular grade water	5.9 mL
5x colorless Taq PCR Buffer (Promega)	2 mL
$MgCl_2$ 25 mM (Promega)	800 µL
dNTPs (at 100mM; Promega)	80 µL (20 µL each A,T,G,C)
Primer F (CS1 at 100 µM)	50 µL
Primer R (CS3 at 100 µM)	50 µL
Rediload (Invitrogen)	500 µL

Make the mix on ice and under nuclease-free conditions. Aliquot to avoid freeze-thaw cycles and store at -20°C.

3. PCR Protocol

Thaw an aliquot of master mix (18.8 µL per sample) and add 1 U Taq polymerase per sample (usually 0.2 µL, depending on supplier). Assemble the reaction mixture on ice in 20 µL reactions as follows:

19 µL master mix
1 µL diluted template (sample DNA)

Briefly mix and zip-spin reaction mixture.

PCR program for amplification of *C. shasta*:

95°C/3 min + 34 cycles x {94°C/1 min + 56°C/30 sec + 72°C/1 min} +72°C/10 min.

Analyze 10 µL of each reaction in a 1% agarose gel stained with SYBR Safe DNA stain (Invitrogen) or ethidium bromide. Positive samples will have an amplicon of 640 bp.