5.4 *Tetracapsula bryosalmonae* (Proliferative Kidney Disease)

The myxozoan (PKX), known to cause proliferative kidney disease in salmonids, is difficult to detect because its life cycle is complex and requires a bryozoan to complete its development (Canning et al. 1999). Diagnosis of infections in fish is also complicated because the parasite does not develop completely in the fish, and resulting myxospores, if present, lack fully developed spore valves (Kent 1994). Two developmental stages occur in salmonid hosts: presporogonic stages found in blood and interstitial kidney tissue and sporogonic stages found within the lumen of kidney tubules. Although PKX-like cells have been identified from species other than in the family Salmonidae, their identity as *T. bryosalmonae* is unconfirmed.

A. Screening Tests

1. **Leishman-Giemsa Staining** (Klontz and Chacko 1983)

    a. Stain slides with tissue imprints using Leishman-Giemsa stain.

        i The fixed imprint is incubated with approximately 1 mL Leishman stain (Section 2, 5.6.L "Leishman Stain") for one minute.

        ii Giemsa stain (2 to 3 mL) (Section 2, 5.6.M "Giemsa Stain") is added to the Leishman stain and allowed to stand for an additional 10 to 15 minutes.

        iii Rinse the slide and examine.

    b. Whenever possible, a positive control slide should be examined prior to evaluating samples.

    c. Examine a minimum of 100 fields of the stained imprints at 400x using bright field microscopy for the presporogonic stages of the parasite.

    d. Presporogonic stages (Figure 5.1) are large (approximately 20 µm) with a prominent cell membrane. The primary cell (outer, surrounding cell) contains prominent granules, a nucleus with a large nucleolus, and one or more secondary or daughter cells. Macrophages are frequently adhered to the surface of the parasite.

    e. **The finding of presporogonic stages (Figure 5.1) is presumptive and warrants confirmation by histology**.

2. **Lectin-Based Staining** (Hedrick et al. 1992)

    a. Tissue imprints are stained with 50 µL biotinylated GS-1 lectin (L-3759, Sigma, St. Louis, Missouri) suspended in 0.01M phosphate buffer pH 6.8 (Section 2, 5.6.I "0.01 M Phosphate Buffer pH 6.8," pH adjusted).

b. To determine the appropriate concentration of lectin, dilutions of 25 to 0.5 µg mL^{-1} are tested on control tissue.

c. Slides are incubated in a moist chamber for 1 to 2 hours at 25˚C.

d. Rinse three times in PBS (Section 2, 5.6.D "Phosphate Buffered Saline (PBS)").

e. Apply 50 µL fluorescein avidin D (A-2001, Vector Laboratories, Inc., Burlingame, California) diluted to provide 10-30 µg mL^{-1} suspensions in PBS.

f. Incubate slides in moist chamber at 25˚C for 30 minutes.

g. Rinse three times, then carefully blot to near dry and mount with a coverslip using a drop of a mixture containing 1 part of 0.1M N-2-hydroxy-ethylpiperzine-N'-2-ethanesulfonic acid (HEPES) pH 8.0 and 9 parts glycerol.

h. Observe under a microscope with a UV light source equipped for fluorescein.

i. **The identification of fluorescing presporogonic stages (Figure 5.2) is presumptive and warrants corroboration by histology.**

B. Confirmatory Test

1. Histopathology (Hedrick et al. 1986)

a. Slides should be prepared from the kidney tissue that was placed in fixative at the time of collection using standard histological techniques.

b. Sections should be stained with hematoxylin and eosin (H&E) or Giemsa and examined for stages of *T. bryosalmonae*.

c. **The presence of presporogonic stages of *T. bryosalmonae* within the interstitial tissue of the kidney is corroboration of the infection (Figure 5.3).** A chronic inflammatory response is typically associated with the presence of the parasite (Figure 5.4) and a "whorled" appearance may be visualized corresponding to locations of the parasite. The presporogonic stage is approximately 20 µm in diameter and the primary cell has a lightly staining cytoplasm with a large, eosinophilic staining nucleolus (Figure 5.5). The primary cell may contain one to several spherical, dense, secondary (daughter) cells and macrophages are frequently seen adhered to the parasite. The inflammatory cell infiltrate is primarily composed of macrophages but numerous lymphocytes are also typically present. Sporogonic stages of *T. bryosalmonae* may be observed in the lumina of kidney tubules. Morphology of spores is poorly defined in fish and it appears that hardened valves typical of other myxosporidians do not form. Valveless myxospores are approximately 12 um X 7 um and have two spherical polar capsules 2 um in diameter (Figure 5.6).

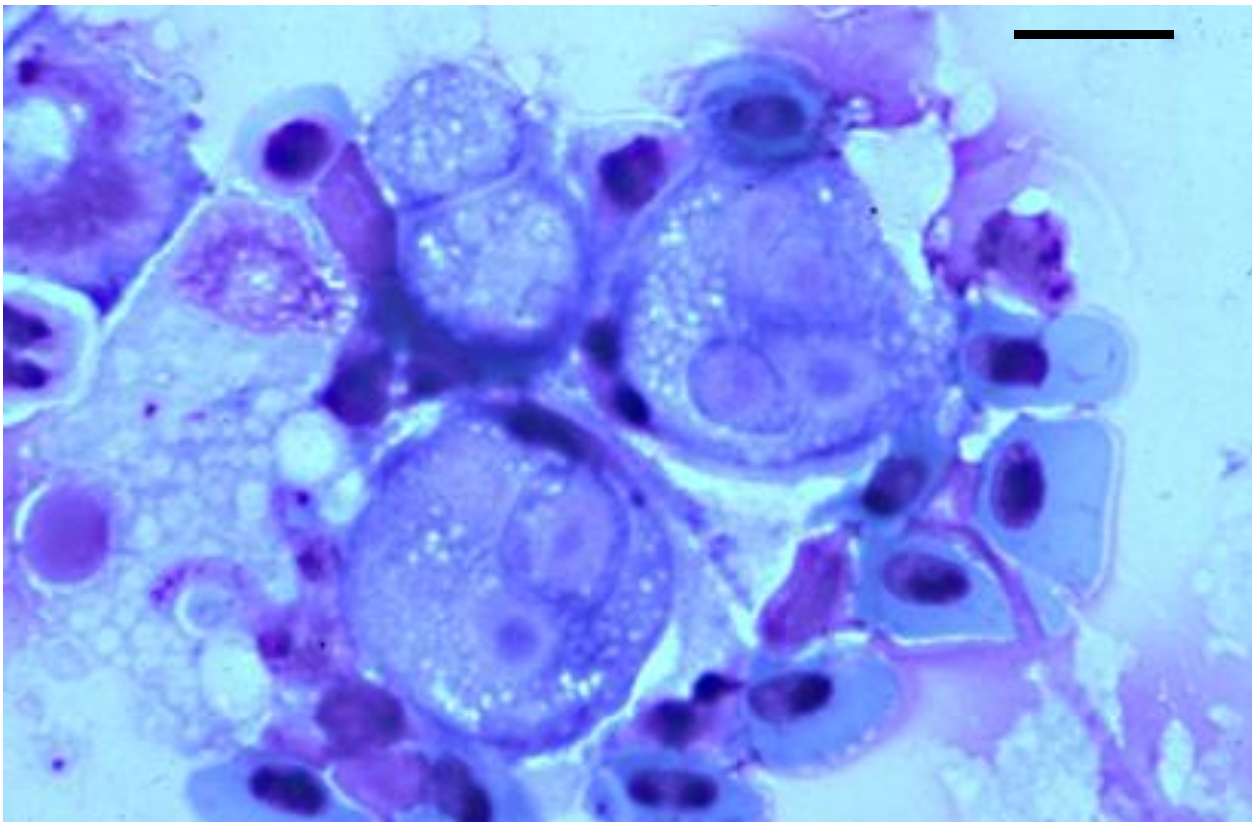

Figure 5.1. Multicellular *Tetracapsula bryosalmonae* in a Leishman-Giemsa stained imprint. Bar is 10 µm. Photograph courtesy of R. P. Hedrick.

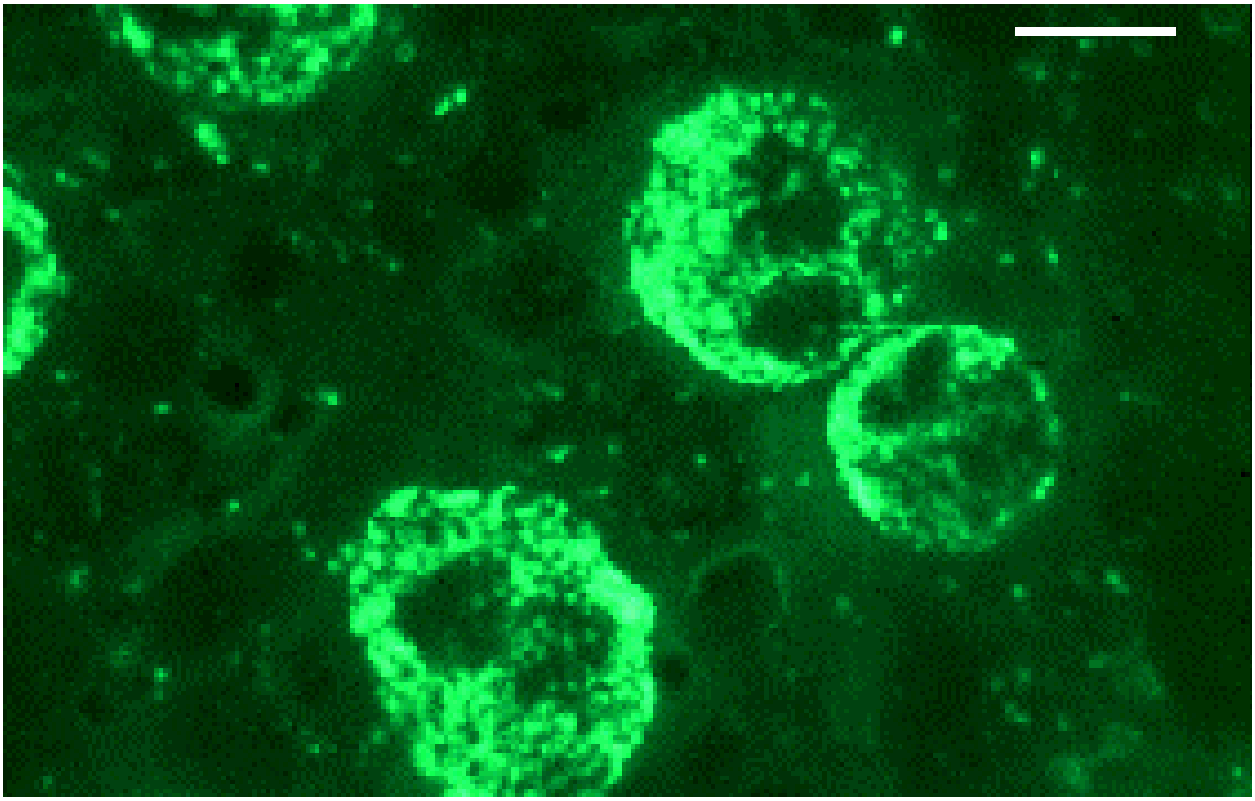

Figure 5.2. Multicellular *Tetracapsula bryosalmonae* in a lectin-stained imprint. Bar is 10 µm. Photograph courtesy of R. P. Hedrick.

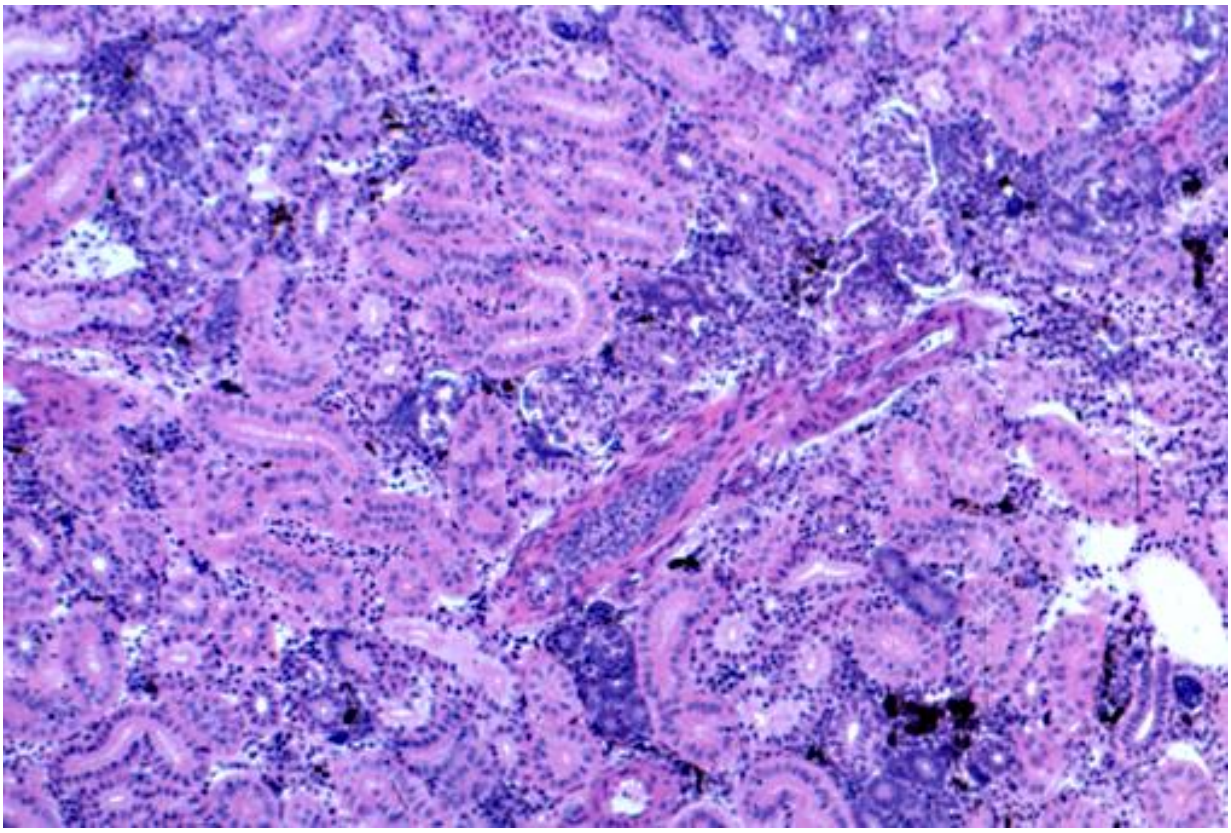

Figure 5.3. Histological sections of kidney tissue infected with *T. bryosalmonae* at low magnification of normal kidney. Photographs courtesy of R. P. Hedrick.

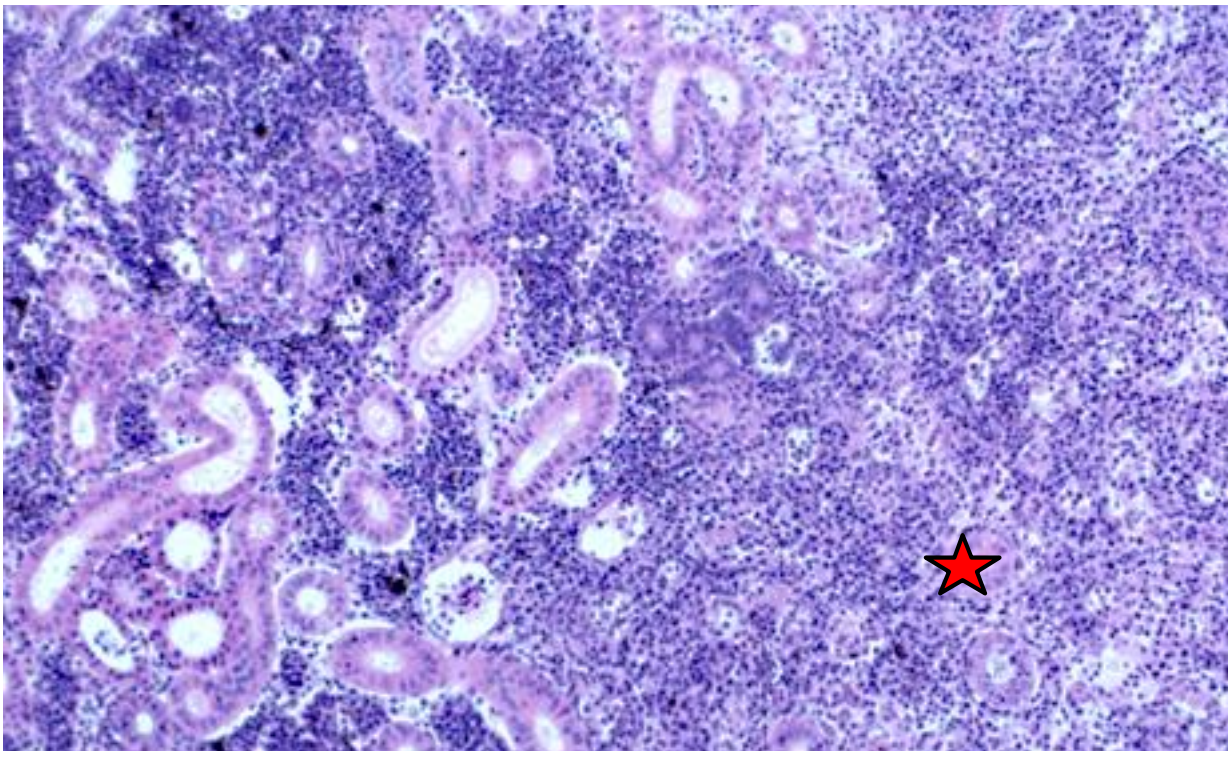

Figure 5.4. Histological sections of kidney tissue infected with *T. bryosalmonae*. at low magnification showing area of inflammatory cell infiltrate indicated by the star. Photographs courtesy of R. P. Hedrick.

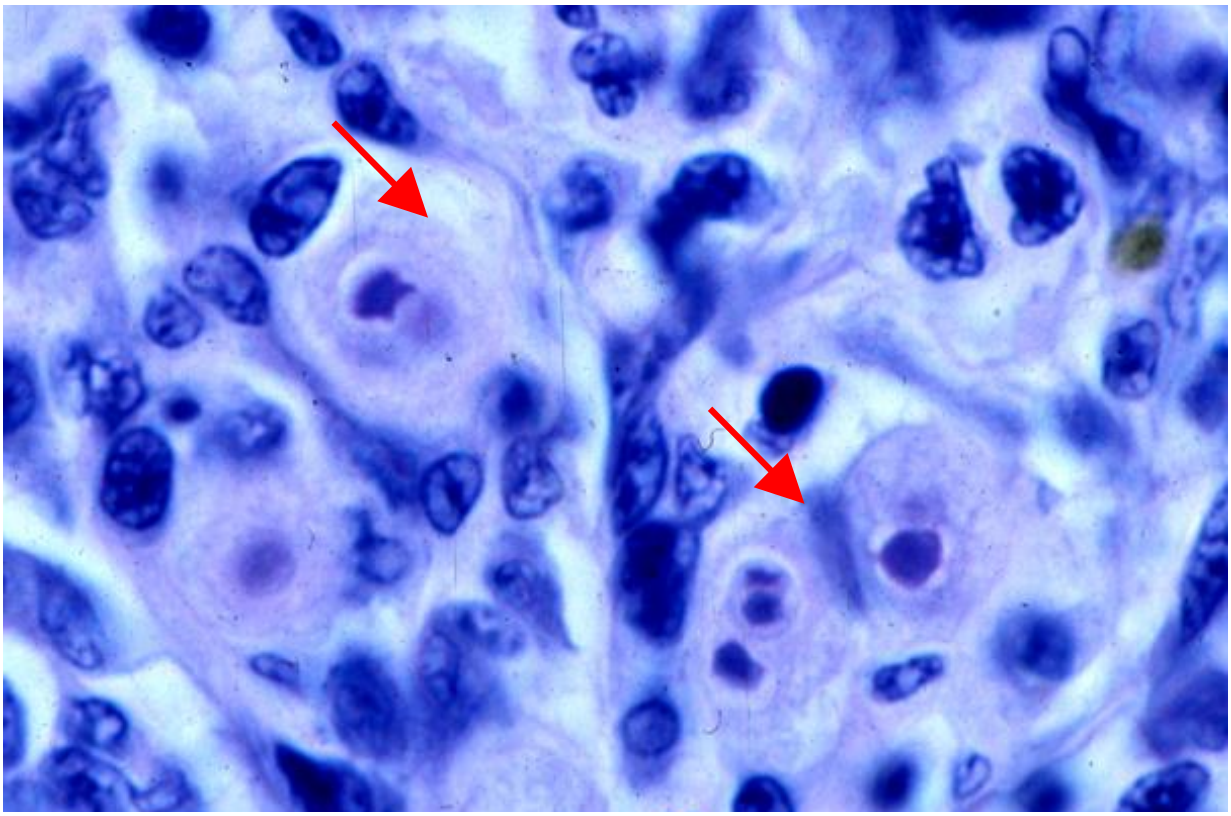

Figure 5.5. Histological sections of kidney tissue infected with *T. bryosalmonae*. at high (400X) magnification of parasites in tissue. Photographs courtesy of R. P. Hedrick.

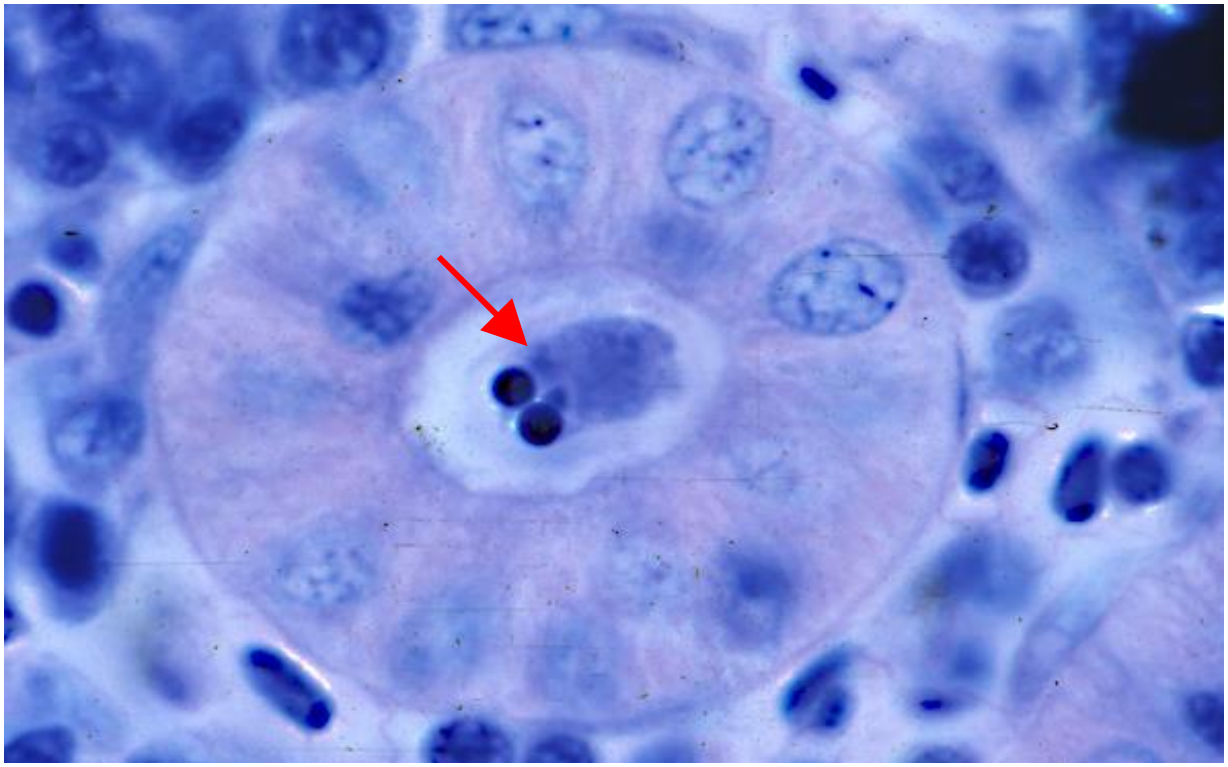

Figure 5.6. Histological sections of kidney tissue infected with *T. bryosalmonae*. sporogonic stage in kidney tubule (1000X). Photographs courtesy of R. P. Hedrick.

2. Polymerase Chain Reaction (Kent et al. 1998; Feist et al. 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
Note: This assay was optimized using phenol:chloroform extraction and if other extraction methods are substituted, these should be tested.

i. If the sample was fixed in ethanol, wash twice in TE buffer (Section 2, 6.4 Reagents), then soak in TE buffer for one hour. Lyse sample in 500 µL DNA extraction buffer (Section 2, 5.6.G "DNA Extraction Buffer") with 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) with agitation for at least one hour at 60˚C, or overnight at 37˚C.

ii. Extract twice with 400 µL phenol/chloroform/isoamy alcohol (50:50:1).

iii. Precipitate DNA in 800 µL absolute ethanol and 40 µL 3 µ sodium acetate, centrifuge at 13000 x g for 15 minutes and remove ethanol. Rinse with 70% ethanol, centrifuge at 13000 x g for 10 minutes and remove ethanol. Air dry pellet and resuspend in 20 µL molecular grade water. Sample can be stored at -20˚C.

c. Quantitation 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 "Extraction of DNA or RNA from Samples."

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 *T. bryosalmonae* DNA

i. 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 GIBCO (Gaithersburg, MD, USA) and if other buffer systems are substituted, these should be tested. The primers and reagents for this reaction are:

Master Mix: 50 µL total reaction volume

H_2O – sterile, molecular biology grade	33.4 µL
Mg CL_2 (25 mM)	5.0 µL (final concentration 2.5 mM)
10X PCR buffer	5.0 µL
dNTPs (25mM)	0.4 µL (final concentration 0.2 mM)
5-Forward primer (20 µM)	2.0 µL (final concentration 0.8 µM)

6-Reverse primer (20 µM)	2.0 µL (final concentration 0.8 µM)
Taq polymerase (5 units/µL)	0.2 µL (1U per reaction)

Primers (Kent et al. 1998):
Forward (PKX5f): 5' CCT ATT CAA TTG AGT AGG AGA 3'
Reverse (PKX6r): 5' GGA CCT TAC TCG TTT CCG ACC 3'

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

iii. Add 2 µL of each sample DNA to the appropriately labeled PCR tubes.

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

1. Cycle Parameters:
Denature at 94°C for three minutes.

2. 35 Cycles of:

94°C for one minute.
55°C for one minute.
72°C for one minute.
72°C for five minutes.
Hold to 4°C chill at end of program.

e. 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 "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. *T. bryosalmonae* positive reactions will have an amplicon of 435 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.