

3.6 *Piscirickettsia salmonis*

Piscirickettsia salmonis has primarily been reported as an emerging marine pathogen of salmonids (Bravo and Campos 1989; Fryer et al. 1990; Cvitanich et al. 1991; Brocklebank et al. 1992; Rodger and Drinan 1993; Grant et al. 1996; Cusack et al. 1997; Olsen et al. 1997), although there are reports of isolations in other species (Chen et al. 2000b) and in juvenile salmonids in freshwater (Bravo 1994; Gaggero et al. 1995). Additionally, there are an increasing number of reports on isolations of *P. salmonis*-like organisms from other fish species (Chern and Chao 1994; Chen et al. 1994; Chen et al. 2000a; Mauel et al. 2003). Clinical signs of disease and mortality have been reported in fish in marine net pens (Cvitanich et al. 1991; Branson and Diaz- Munoz 1991; Brocklebank et al. 1992; Olsen et al. 1997; Kent and Poppe 1998). The first evidence of disease may be the appearance of small white lesions or shallow hemorrhagic ulcers on the skin. Affected fish appear dark and lethargic. The major gross pathological changes are gill pallor, peritonitis, ascites, enlarged spleen, swollen gray kidney, and in some cases, a liver with large pale necrotic lesions.

A. Summary of Screening Tests

These techniques represent the best methods available for isolating and identifying *Piscirickettsia salmonis*, but they have not been demonstrated to be sensitive enough to detect a covert infection or subclinical infections. These methods are most effective in fish with clinical cases of infection.

1. Culture of Obligate Intracellular Bacteria in Cell Culture (Lannan and Fryer 1991)

Piscirickettsia salmonis is a Gram-negative, non-motile, highly fastidious intracellular bacterial pathogen. *Piscirickettsia salmonis* does not replicate on bacteriological media, but instead it must be grown in cell culture, and therefore escapes detection by routine techniques used for bacterial isolation. Additionally, *in vitro*, this organism is sensitive to many antibiotics used in routine virus isolations, and will not grow even if inoculated onto suitable host cells if such compounds are included in the culture medium.

- a. Samples of kidney, spleen, liver and blood suitable for virology testing are aseptically collected from diseased fish during either overt or covert infections as described in Section 2, 2.2.E.3 "Collection of Tissues for the Detection of Viral Agents." DUE TO SENSITIVITY OF *P. SALMONIS* TO ANTIBIOTICS *IN VITRO*, NONE SHOULD BE USED IN MEDIA DURING COLLECTION OF TISSUE OR THE CULTURE OF CELLS and because no antibiotics will be used, stringent aseptic techniques should be used to collect tissues. Musculature underlying skin lesions may also be a suitable tissue if collected aseptically by removing the contaminated outer skin layer.
- b. Tissue should be homogenized at 1/20 (w/v) in sterile antibiotic-free HBSS. Do not centrifuge. *Piscirickettsia salmonis* cells are bound in membranes and centrifugation will remove the bacteria from the supernatant. The 1:20 homogenate should then be further diluted 1/5 and 1/50 in antibiotic-free HBSS. These final two dilutions of the homogenate are the inocula for cell cultures, and are 1:100 and 1:1,000 dilutions of the original tissue.

- c. The diluted homogenate can be inoculated directly (0.1 mL/culture per well of a 24-well plate) into the antibiotic-free culture medium overlaying the CHSE-214 cell monolayer (refer to Section 2, 4.3 Cell Culture).
- d. The cell cultures must be incubated at 15 to 18°C for 28 days and observed for the appearance of cytopathic effect (CPE). The piscirickettsial CPE consists of plaque-like clusters or rounded cells (Figure 3.1). With time, the CPE progresses until the entire cell sheet is destroyed. A drop of the suspect cell culture supernatant can be dried on a glass slide, fixed with methanol and stained with Giemsa stain (described below). Examine for presence of the bacteria.
- e. If CPE does not occur (except in positive control) cultures should be incubated at 15 to 18°C for an additional 14 days.
- f. If CPE is absent after the 42 day combined incubation period, samples are reported as negative and may be discarded using the proper decontamination procedures. If CPE associated with the bacteria occurs at any time during this assay, it is considered a PRESUMPTIVE positive and the identification of *P. salmonis* should be confirmed by the appropriate method.

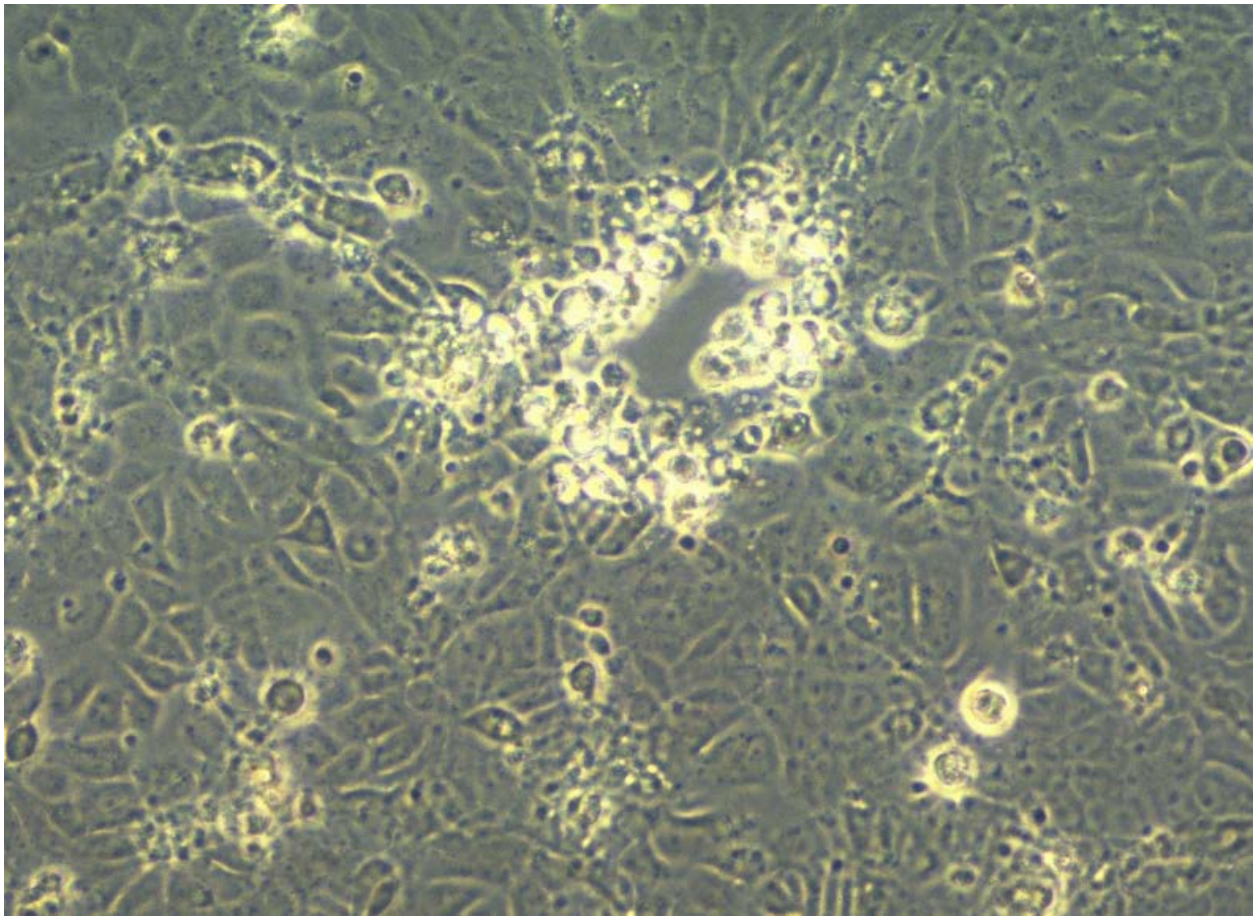


Figure 3.1. Cytopathic effect typical of *Piscirickettsia salmonis* on a monolayer of CHSE-214 cells. (Photo courtesy of Marcia House, NWIFC.)

2. Tissue Impressions of Kidney Stained with Giemsa Stain

Piscirickettsia salmonis can also be diagnosed based on clinical signs and detection in Giemsa stained tissue impressions, or sections (refer to **Note** below). It is pleomorphic, ranging from 0.5 to 1.5 μm in diameter and frequently is seen in pairs (Figure 3.2). Positive identification is confirmed by IFAT using *P. salmonis*-specific antibodies (Section 2, 3.8.E.3 “Indirect FAT (IFAT) Staining”).

- a. Preparations of tissue culture supernatant, smears or impressions of the kidney, liver, and spleen should be prepared, air-dried, and fixed for five minutes in absolute methanol.
- b. Immerse slides in working solution of Giemsa stain for 30 minutes. Stock solution: 0.4 w/v in buffered methanol solution, pH 6.9 (commercially available). Working solution: diluted 1:10 in phosphate buffer pH 6.0 (0.074M NaH_2PO_4 , 0.009M Na_2HPO_4).
- c. Destain with tap water.
- d. Observe slides under oil immersion. Examine a minimum of 50 fields. Tissue smears from infected organs show darkly stained pleomorphic organisms occurring in coccoid or ring forms, frequently in pairs, with a diameter of 0.5-1.5 μm (Figure 3.2). Visualization of organisms in smears is considered a PRESUMPTIVE positive result and the identification of *P. salmonis* should be confirmed by the appropriate confirmatory method.

Note: Every effort should be made to culture *P. salmonis* because it is the most sensitive screening method.

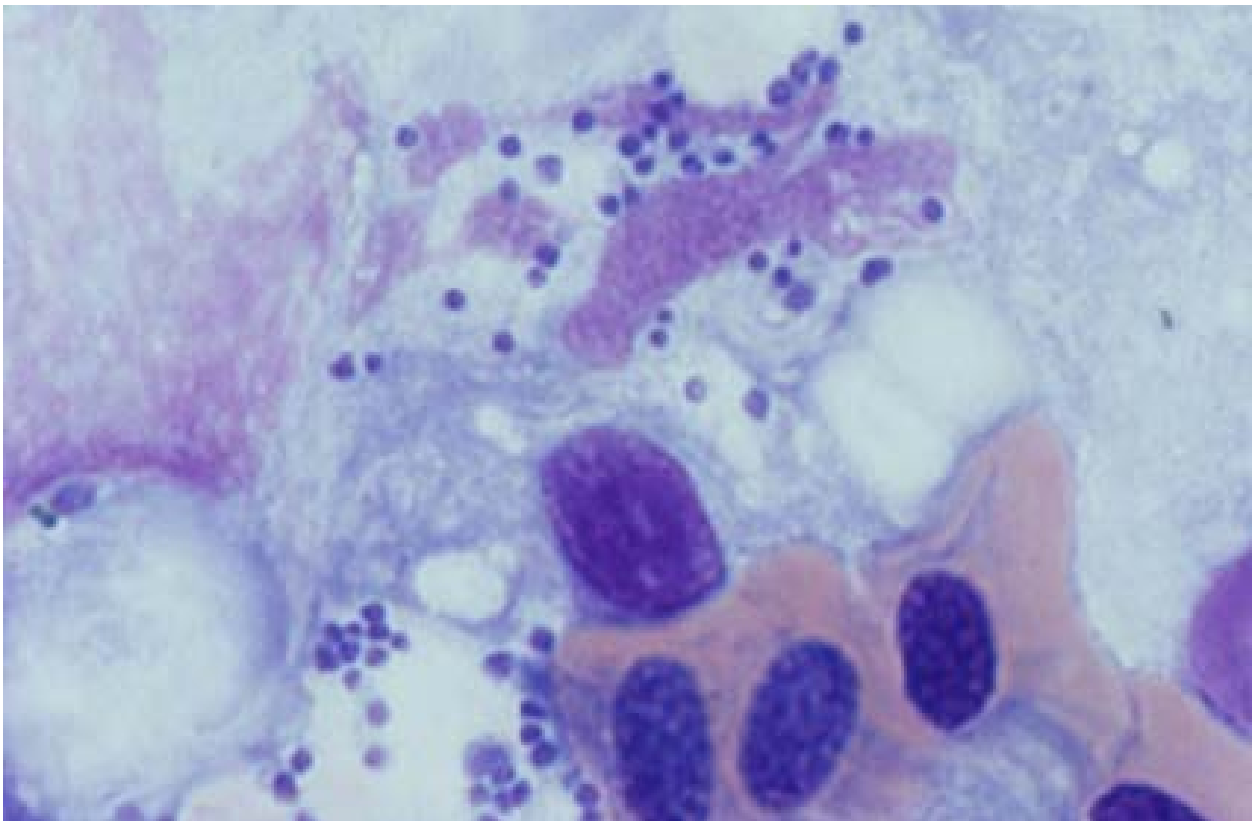


Figure 3.2. Giemsa stained liver impression from *Piscirickettsia salmonis* infected coho salmon (1000X magnification). Photo courtesy of Marcia House, NWIFC.

B. Confirmatory Tests

1. Serological Methods

The positive identity of *P. salmonis* isolated in cell culture or observed in Giemsa stained smears may be determined by serological methods, including IFAT or immunocytochemistry of histological sections.

- a. Indirect Fluorescent Antibody Test (IFAT) (refer to Section 2, 3.8.E.3 “Indirect FAT (IFAT) Staining”) (Lannan et al. 1991)
 - i. Smears or impressions of the kidney, liver, and spleen should be prepared, air-dried, and fixed for five minutes in absolute methanol.
 - ii. Tissues smears to be examined by IFAT must be freshly prepared or stored at -20°C.
 - iii. The sample is first incubated with anti-*P. salmonis* polyclonal or monoclonal antibody, then washed and incubated with a secondary antibody conjugated with fluorescein isothiocyanate (FITC).
 - iv. Following washing, apply glycerol-based mounting media and coverslip, then examine with a microscope equipped with epi-fluorescence.
- b. Immunohistochemistry of Tissue Sections (Alday-Sanz et al. 1994)
 - i. Sections (5 µm) of formalin fixed, paraffin-embedded tissues are deparaffinized and treated to eliminate endogenous peroxidase activity.
 - ii. The tissue is first incubated with anti-*P. salmonis* polyclonal or monoclonal antibody, then washed and incubated with a secondary antibody conjugated with horse-raddish peroxidase.
 - iii. Following washing, the tissue is exposed to a chromagen (hydrogen peroxide and 3,3-diaminobenzidine), counterstained with hematoxylin, dehydrated and prepared for examination under a light microscope.

2. Polymerase Chain Reaction (PCR)

A nested polymerase chain reaction (PCR) has been developed to detect genomic DNA of *P. salmonis* using general bacterial 16S rDNA primers in the first amplification and *P. salmonis*-specific primers in a second reaction. A direct (single amplification) PCR can be run using the *P. salmonis* specific primers.

- a. Preparation of Infected Cell Culture Supernatant or Tissue

Use of a commercially available spin column to purify DNA from cell culture supernatant or tissue is recommended for PCR sample preparation. In addition to following the manufacturer’s instructions on use of the columns, initial digestion of the sample in lysis buffer (20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100, 4 mg/mL lysozyme) at 37°C for 30 minutes is suggested (refer to Section 2, 3.5.B.2 “Nested Polymerase Chain Reaction (PCR) for Confirmation of *R. salmoninarum* DNA”).

 - i. The cell culture supernatant preparation:

1. Triturate the overlying cell culture media to disrupt the suspect cell culture, as if you were preparing a re-inoculation in a virology assay.
 2. Transfer 0.2 mL of the suspension to a clean sterile 1.5 mL microcentrifuge tube and centrifuge at 4°C.
 3. Discard the supernatant and treat the pellet as a tissue sample in the extraction procedure.
- b. Nested Polymerase Chain Reaction (Mauel et al. 1996)
- i. Initial amplification using eubacterial primers:
 1. General QA/QC considerations must be reviewed before performing PCR (see Section 2, Chapter 6 Polymerase Chain Reaction (PCR) for more specific QA/QC considerations for PCR).
 2. Procedures for the initial round:
 - a. Using Section 2, 3.A3.A Worksheet A – PCR Sample Data/Log Sheet, record appropriate data for each sample to be tested by PCR.
 - b. Using Section 2, 3.A3.D Worksheet D – Initial Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis*, record the date of assay and then calculate the amount of each reagent to go into the “Master Mix” (MM) according to the number of samples to be processed and the amount of MM needed per reaction (45 µL). Add four to the number of samples so that there is enough to run controls.
 - c. Under a UV cabinet, add PCR reagents except for sample DNA to the MM tube in the order listed in Section 2, 3.A3.D Worksheet D – Initial Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis*, adding water first and Taq last. Keep all reagents cold during mixing, and return them to the freezer immediately after use. Do not expose enzymes, primers, or dNTP's to UV light.
 - i. Water to make a 45 µL total volume per reaction.
 - ii. 1X PCR buffer (10 mM Tris HCl, pH 9, 50 mM KCl, and 0.1% Triton X100).
 - iii. 1.5 mM MgCl₂.
 - iv. dNTP mix (0.2 mM).
 - v. Primers (1 mM each)
 - 1) EubA 5'-AAG-GAG-GTG-ATC-CAN-CCR-CA-3'
 - 2) EubB 5'-AGA-GTT-TGA-TCM-TGG-CTC-AG-3'

- vi. Taq polymerase (2.5 units per reaction).
- d. Place 45 μ L of MM into each 0.2 mL PCR tube and close the caps tightly. Move PCR tubes to sample loading area.
- e. In sample loading area, load 5 μ L of each sample DNA to the appropriately labeled PCR tubes. Close caps tightly. Remove sample tubes from UV cabinet to thermocycler.
- f. Load the sample tubes into the thermocycler wells.
- g. The thermocycler should be programmed for the following regime:
 - i. Denature the mixture at 94°C for two minutes.
 - ii. Amplify by 35 cycles of:
 - 1) Denaturing at 94°C for one minute.
 - 2) Annealing at 50°C for two minutes.
 - 3) Extending at 72°C for three minutes.
 - 4) Post-dwell at 4 to 15°C after cycling is complete.
- ii. “Nested” PCR-secondary amplification using *P. salmonis* specific primers.
 - 1. Materials, methods, and general QA/QC considerations of this subchapter and Section 2, Chapter 6 Polymerase Chain Reaction (PCR) also apply to the nested PCR process.
 - 2. Procedures for the second round:
 - a. Using Section 2, 3.A3.E Worksheet E – Nested Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis*, record date of assay and then calculate the amount of each reagent to go into the “Master Mix” (MM) according to the number of samples and controls to be processed and the amount of MM needed for each reaction (48 μ L).
 - b. Add PCR reagents except the first round amplified DNA to the Master Mix (MM) tube. Return reagents to the freezer. The reaction mixture is the same as the initial with the exception of the primers, which are:
 - i. PS2S (223F) 5'-CTA-GGA-GAT-GAG- CCC-GCG-TTG-3'
 - ii. PS2AS (690R) 5'-GCT-ACA-CCT-GAA-ATT-CCA-CTT-3'
 - c. Place 48 μ L of the MM into each PCR tube (0.2 mL) and close caps tightly. Remove the tubes from UV cabinet to amplified DNA area.

- d. Load 2 μ L of amplified sample DNA into the appropriate PCR tubes.
 - e. Load the tubes into the thermocycler wells.
 - f. Thermocycler should be programmed for the following regime:
 - i. Denature the mixture at 94°C for two minutes.
 - ii. Amplify by 35 cycles of:
 - 1) Denaturing at 94°C for one minute.
 - 2) Annealing at 65°C for two minutes.
 - 3) Extending at 72°C for three minutes.
 - 4) Post-dwell at 4 to 15°C after cycling is complete.
- c. Direct (single round) polymerase chain reaction.
- i. Materials, methods and general QA/QC considerations of this subchapter and Section 2, Chapter 6 Polymerase Chain Reaction (PCR) also apply to the direct PCR process.
 - 1. Using Section 2, 3.A3.E Worksheet E – Nested Amplification of Nucleic Acid by PCR for the Confirmation of *Piscirickettsia salmonis*, record date of assay and then calculate the amount of each reagent to go into the “Master Mix” (MM) according to the number of samples and controls to be processed and the amount of MM needed for each reaction (45 μ L). Add four to the number of samples so that there is enough to run controls.
 - a. Add PCR reagents except the first round amplified DNA into the Master Mix (MM) tube. Return reagents to the freezer. The reaction mixture is the same as the nested with the exceptions of the volume of water and primers. The primers are:
 - i. PS2S (223F) 5'-CTA-GGA-GAT-GAG- CCC-GCG-TTG-3'
 - ii. PS2AS (690R) 5'-GCT-ACA-CCT-GAA-ATT-CCA-CTT-3'
 - b. Place 45 μ L of the MM into each PCR tube (0.2 mL) and close caps tightly. Remove the tubes from UV cabinet to amplified DNA area.
 - c. Load 5 μ L of sample DNA into the appropriate PCR tubes.
 - d. Load the tubes into the thermocycler wells.
 - e. The thermocycler should be programmed for the following regime:
 - i. Denature the mixture at 94°C for two minutes.

- ii. Amplify by 35 cycles of:
 - 1) Denaturing at 94°C for one minute.
 - 2) Annealing at 65°C for two minutes.
 - 3) Extending at 72°C for three minutes.
 - 4) Post-dwell at 4 to 15 °C after cycling is complete.
- d. Visualization of PCR product by electrophoresis (see Section 2, Chapter 6 Polymerase Chain Reaction (PCR) for general procedures).
 - i. Visualization of amplified products resulting from PCR for detection of *P. salmonis* DNA is best accomplished after electrophoresis on a 2% agarose gel (Section 2, 6.3C “Detection of Product”).
 - ii. Using Section 2, 3.A3.G Worksheet G – Photodocumentation of the PCR Product Gel, record location of each sample on the agarose gel at the time samples are loaded.
 - iii. After electrophoresis, stain gel with ethidium bromide and visualize on an UV transilluminator.
 - iv. Carefully record locations of bands on positive control samples in relation to DNA ladder bands.
 - 1. Band locations of positive controls should be at anticipated locations according to primers used in both the first (EubA/ EubB) and second (PS2S/ PS2AS) round PCR assays (first round (EubA/ EubB) band = 1540 bp, nested and direct PCR (PS2S/ PS2AS) = 476 bp). **Bands occurring at the PS2S/ PS2AS product locations are confirmatory of *P. salmonis* and are reported as POSITIVE.**
 - 2. Note any unusual band occurrences. Negative controls should not have any bands. Suspicion of contamination indicates that PCR should be re-run on samples from the extracted DNA tube.
 - v. Document the electrophoresis results (Section 2, 6.3.G “Visualize the DNA”). Photograph all gels and attach the photo to Section 2, 3.A3.G Photodocumentation of the PCR Product Gel. Attach to case history information.

Note: Other PCR assays have been developed to detect *P. salmonis* (Marshall et al. 1998). These primer sequences and reaction conditions would also be suitable for confirmation of the presence of *P. salmonis*. Furthermore, additional diagnostic tests, such as transmission electron microscopy (TEM) and enzyme-linked immunosorbent assay (ELISA) (Aguayo 2001) may also aid in the detection of *P. salmonis*.