1.3.1 Bacterial Kidney Disease

Diane G. Elliott

U. S. Geological Survey, Western Fisheries Research Center
6505 NE 65th Street
Seattle, WA 98115
206/526-6282
dgelliott@usgs.gov

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A. Name of the Disease and Etiological Agent

Bacterial kidney disease (BKD) is caused by *Renibacterium salmoninarum.*

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

This disease is found in North America, Chile, Europe (including Iceland), and Japan.

2. Host Species

All fishes in the family Salmonidae are considered susceptible (Elliott et al. 2014). Among non-salmonids, clinical BKD has been reported in cultured ayu *Plecoglossus altivelis* (family Plecoglossidae) (Nagai and Iida 2002), and the bacterium also has been isolated from kidneys, but not blood, of sea lampreys *Petromyzon marinus* (Petromyzontidae) (Eissa et al. 2006). In addition, experimental infections have been established in sablefish *Anoplopoma fimbria* (Bell et al. 1990), and Pacific herring *Clupea pallasii* (Traxler and Bell 1988; Evelyn 1993).

C. Epizootiology

Renibacterium salmoninarum can be transmitted both vertically and horizontally, and the bacterium has been detected in both wild fish and hatchery populations (see reviews in Evelyn 1993; Fryer and Lannan 1993; Pascho et al. 2002). Infections can occur at any life stage in a salmonid population, but clinical signs of disease are uncommon in fish less than six months old (Evelyn 1993). Bacterial kidney disease occurs over a wide range of temperatures. Mortality has been reported in experimentally infected salmonids at temperatures between 4°C and 20.5°C, with the disease progressing most rapidly at the higher temperatures tested (e.g. 15.0 to 20.5°C) (Sanders et al. 1978). However, the highest mortality is often

observed at cooler water temperatures (e.g. 7 to 12°C), depending on fish species and temperature regime (Sanders et al. 1978; Jones et al. 2007; Purcell et al. 2016). Fish cultural practices may influence the progress of the disease in a hatchery (reviewed by Elliott et al. 1989; Pascho et al. 2002). For example, there are indications that certain components of diets fed to hatchery salmonids may decrease their susceptibility to BKD, but further research is needed to define precisely the relation between diet and BKD susceptibility. Anadromous salmonids that are infected with *R. salmoninarum* in fresh water may continue to die of BKD after entry into seawater (Fryer and Sanders 1981; Elliott et al. 1995). Infected salmonid populations are believed to be the principal reservoir of infection (Wiens 2011).

D. Disease Signs

1. **Behavioral Changes**
   Acute and subacute forms of BKD occur only sporadically; more typically the course of the disease is chronic. Diseased fish may show no behavioral changes or may exhibit loss of appetite (Pirhonen et al. 2000) and lethargy.

2. **External Gross Signs**
   External and internal disease signs and histopathological changes in fish affected by BKD have been reviewed by several authors (e.g. Ferguson 2006; Bruno et al. 2013; Elliott 2017). The external clinical signs of BKD are not pathognomonic. Fish may appear normal or they may show one or more of the following signs: exophthalmos (Figure 1), abdominal distension (Figure 2), pale gills associated with anemia, skin petechiation or hemorrhages at the bases of fins or near the vent, skin vesicles filled with clear, bloody or turbid fluid, shallow skin ulcers, large abscesses or cystic cavities that extend into the skeletal muscle. Although BKD is generally considered to be a systemic infection, more localized forms may occur, such as postorbital infections in Pacific salmon (Figure 2), and the superficial pustulous dermatitis known as “spawning rash” in rainbow trout (Figure 3).

Figure 1. Exophthalmos and swollen narial tissues in juvenile Chinook salmon *Oncorhynchus tshawytscha* affected by BKD. Many fish in this population had focal BKD lesions associated with coded-wire tags injected into the snouts. Photo credit Ron Pascho.

Figure 2. Abdominal distension associated with ascites in a juvenile Chinook salmon affected by BKD. Photo credit Ron Pascho.

Figure 3. Lateral view (top) and ventral view (bottom) of an adult Chinook salmon with dermatitis ("spawning rash") caused by *R. salmoninarum*.

3. Internal Gross Signs

Bacterial kidney disease is frequently characterized internally by a large edematous kidney that may appear gray and corrugated. The kidney often exhibits off-white nodular lesions that vary in size and number (Figure 4). Similar lesions also can occur in other organs such as the liver, spleen and heart, and the spleen may be markedly swollen. Hemorrhages may occur in visceral organs and the abdominal wall. Turbid or serosanguinous fluid is often present in the abdominal and pericardial cavities, and a viscous yellow or blood-tinged fluid may be present in the intestine. An opaque diffuse membranous layer (pseudomembrane) may cover the kidney, liver, spleen, or gonads.

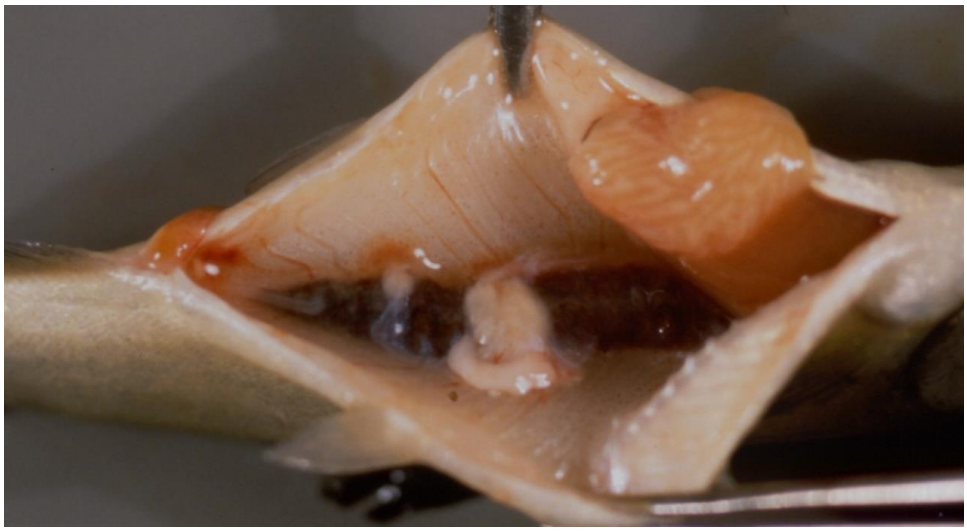

Figure 4. Kidney lesions in a juvenile Chinook affected by BKD. Photo courtesy of Dr. Caroline O'Farrell.

4. Histopathological Changes

Histologically, typical BKD is classified as a systemic, chronic granulomatous inflammatory disease, characterized by a proliferation of macrophages and fibroblasts in areas of infection. The granulomas may be discrete or diffuse (Figure 5); granulomatous lesions in Pacific salmon often have poorly defined borders, whereas those in Atlantic salmon may be more encapsulated by fibrotic tissue (Evelyn 1993). Central areas of necrosis, surrounded by epithelioid macrophages, fibrosis, and infiltrating lymphocytes, are common in older granulomas. Pseudomembranes formed over the capsules of organs are comprised of thin layers of fibrin and collagen with trapped phagocytes and bacteria. A common lesion in the renal kidney is membranous glomerulopathy / glomerulonephritis, hyaline thickening of the glomerular basement membrane linked to subendothelial deposition of antigen-antibody complexes in the glomeruli. Localized *R. salmoninarum* infections, restricted to tissues such as the eye or postorbital tissues, central nervous system, or skin and fins, are also characterized by granulomatous or pyogranulomatous lesions in affected tissues.

Giemsa or Gram staining of infected tissue sections (Figure 6 and Figure 7) reveals both extracellular bacteria and bacteria within macrophages. Disruption and lysis of melanomacrophages in kidney and spleen tissues, resulting in dispersal of pigment in tissues, is a common histopathological characteristic of BKD. In Figure 5, for example, intact melanomacrophages with dense brown-black cytoplasmic pigment are prominent around the edges of the focal BKD granuloma shown in the upper photo, whereas dispersed, lighter brown pigment is more evident in some areas of the diffuse granulomatous lesion shown in the lower photo

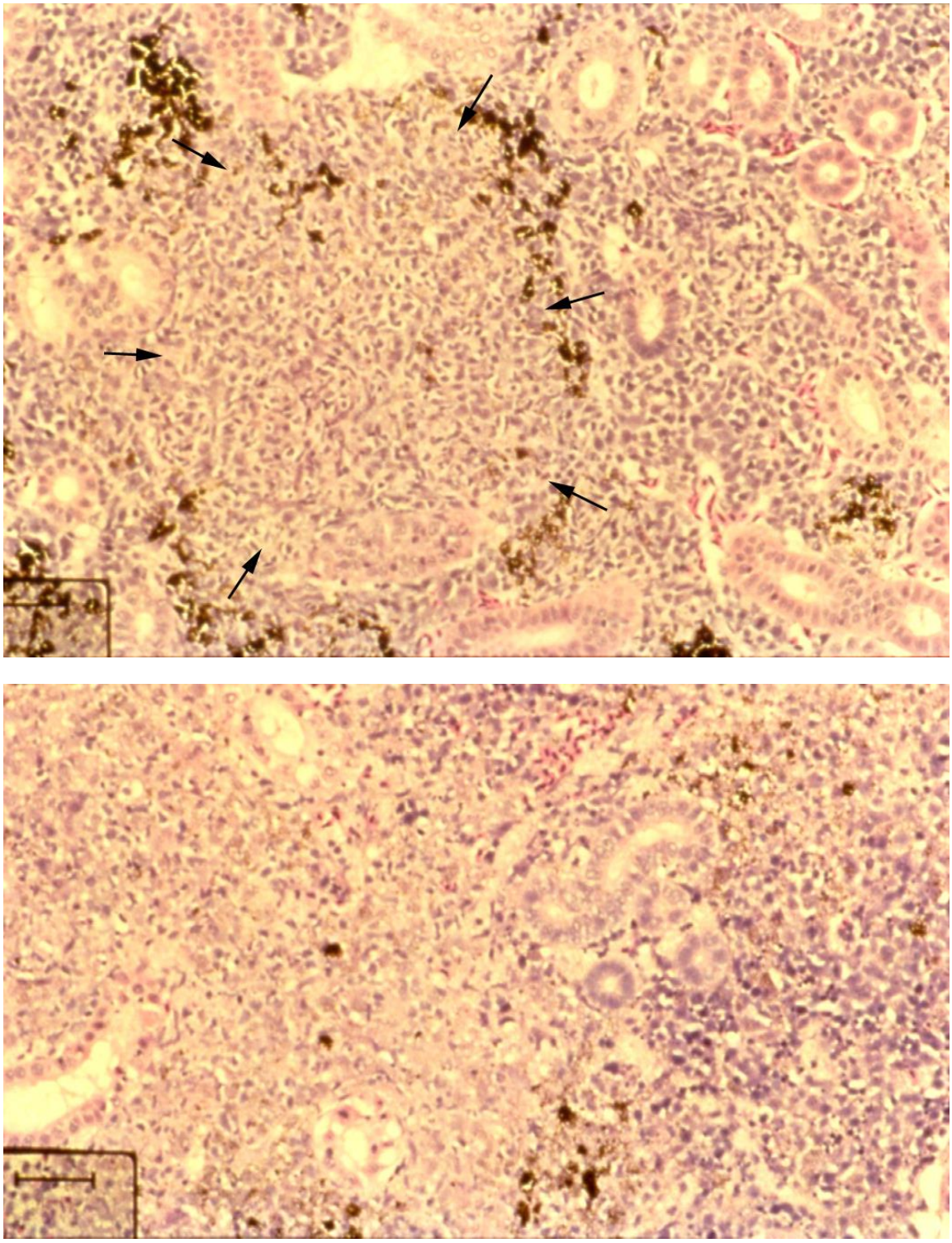

Figure 5. Focal (top photo, arrows) and diffuse (bottom photo) granulomas in the posterior kidney of juvenile Chinook salmon with BKD. Hematoxylin and eosin stain; scale bars = 50 µm. Photos courtesy of Dr. Caroline O'Farrell.

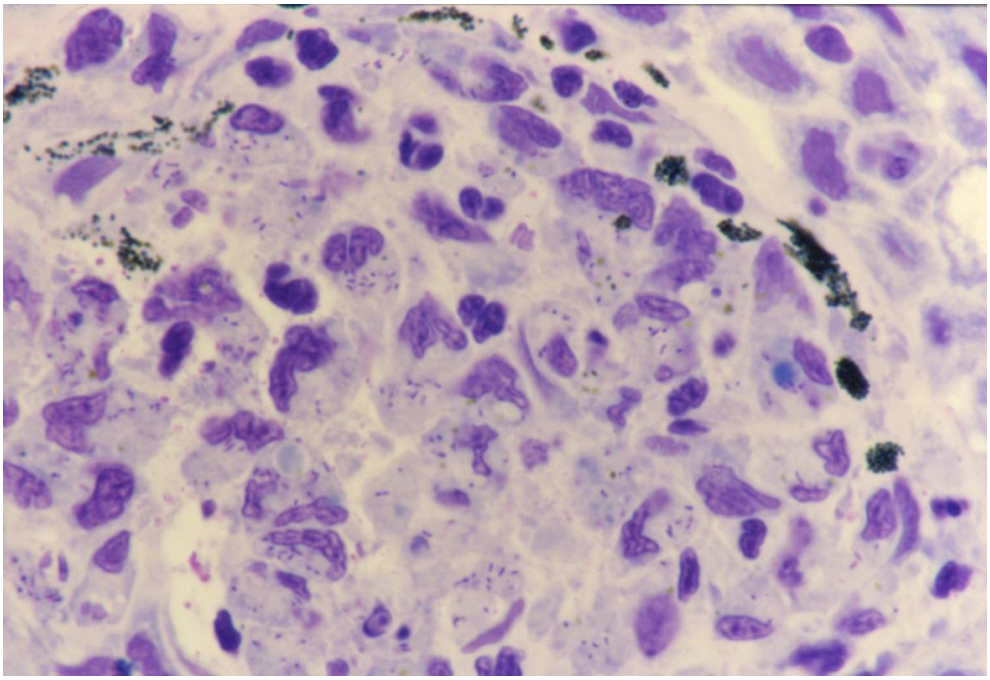

Figure 6. Histological section of a skin lesion of a juvenile Chinook salmon infected with *R. salmoninarum*. Most of the small rod-shaped *R. salmoninarum* are visible within the cytoplasm of macrophages. The bacteria are purple-blue in this Giemsa-stained preparation, in contrast to the black melanin granules.

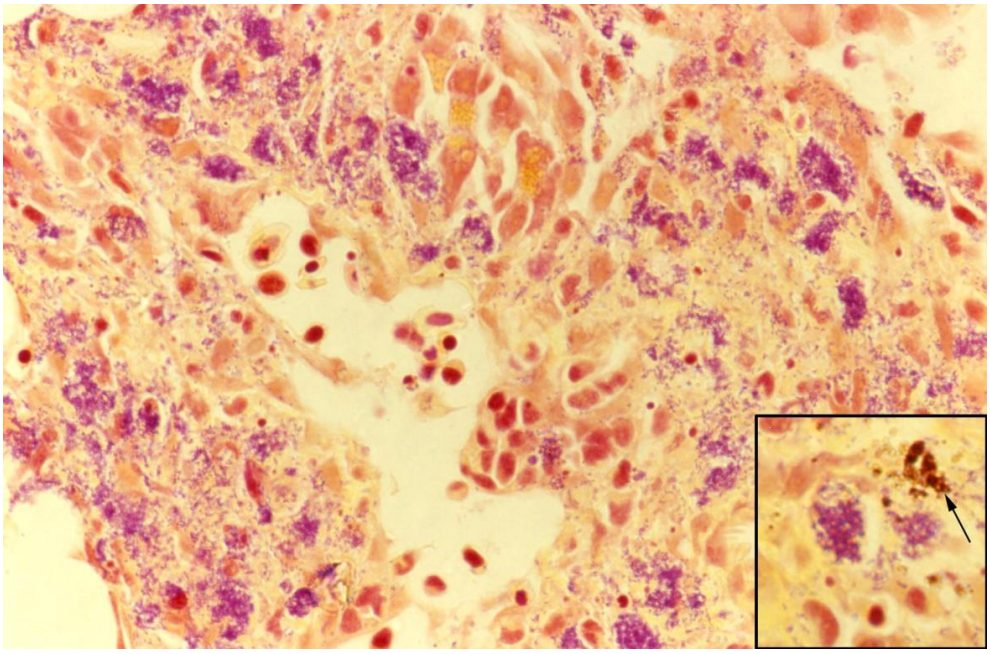

Figure 7. Gram-stained histological section of pancreatic tissue of a juvenile Chinook salmon with systemic BKD. *R. salmoninarum* cells are present extracellularly and intracellularly within macrophages. Note the color difference between the gram-positive (purple-blue) bacteria and the brown-black melanin granules (arrow, inset). Procedures are available for bleaching of melanin in tissue sections to allow easier observation of bacteria (Bruno and Poppe 1996).

E. Disease Diagnostic Procedures

1. Presumptive Diagnosis

The diagnostic procedures described in this section are for use with samples from clinically diseased fish. For screening and confirmatory tests to be used for fish health inspections, please refer to Chapter 3.5. Smears or histological sections of infected tissue should contain numerous small, gram-positive, non-acid-fast diplobacilli that occur both intracellularly and extracellularly (Figure 7). The organism should fail to grow on TSA at 20°C, even when extended incubation periods (e.g. 2 weeks) are used.

2. Confirmatory diagnosis

Bacteriological culture can confirm the presence of live bacteria in a sample, but must followed by further biochemical, immunological or molecular testing as described below to establish bacterial identity. Several immunological tests to confirm the presence of cellular or soluble antigens of *R. salmoninarum* have been reported, including immunodiffusion (Chen et al. 1974), the fluorescent antibody test (FAT; Bullock and Stuckey 1975; Bullock et al. 1980), staphylococcal coagglutination (Kimura and Yoshimizu 1981), counterimmunoelectrophoresis (Cipriano et al. 1985), immunoblot (Western blot; Turaga et al. 1987; Rockey et al. 1991), the semi-quantitative enzyme-linked immunosorbent assay (ELISA; Pascho and Mulcahy 1987; Pascho et al. 1987; Turaga et al. 1987; Pascho et al. 1991) and several qualitative enzyme immunoassays (Sakai et al. 1987a and b, 1989). One is encouraged to review several of the published reports describing comparative abilities of these tests to confirm the presence of BKD (e.g. Kimura and Yoshimizu 1981; Cipriano et al. 1985; Dixon 1987; Pascho et al. 1987, Sakai et al. 1987a and b, 1989; Pascho et al. 2002; Elliott et al. 2013). More recently, polymerase chain reaction (PCR) techniques have been developed to confirm the presence of unique *R. salmoninarum* nucleic acid sequences. The strengths and weaknesses of conventional (non-quantitative) PCR compared with other *R. salmoninarum* diagnostic techniques are reviewed by Pascho et al. (2002), and Elliott et al. (2013) compared both conventional and quantitative PCR to several other diagnostic methods. The most widely used procedures for confirming the presence of *R. salmoninarum* in clinically diseased fish are those described below.

a. Bacterial kidney disease can be diagnosed by a direct FAT (DFAT, Bullock et al. 1980) or indirect FAT (IFAT, Bullock and Stuckey 1975) applied to fresh, formalin-fixed, or frozen infected tissues. Procedures for FAT staining of tissue smears are described in Section 2, 3.8.E "Fluorescent Antibody Test (FAT)." The quality of the preparations and consequent sensitivity of the test may be enhanced by homogenization of kidney tissue samples prior to smear preparation and treating smears with a solvent (xylene or acetone for 1 to 2 minutes followed by a tap water rinse) on the same day as staining (Cvitanich 1994). The use of a counterstain such as Evans blue (Cvitanich 1994), Eriochrome black T (Pascho et al. 1987), or rhodamine (Anderson 1990) can reduce background fluorescence or provide background contrast in tissue smears.

b. The ELISA also can be used to diagnose BKD. Fresh or frozen tissues or blood plasma from infected fish are used. Procedures for ELISAs using polyclonal antisera (e.g. Pascho and Mulcahy 1987; Pascho et al. 1987, 1991; Gudmundsdóttir et al. 1993; Meyers et al. 1993; Olea et al. 1993; Jansson et al. 1996) and monoclonal antibodies (e.g. Turaga et al. 1987; Rockey et al. 1991; Hsu et al. 1991) have been developed. Commercially prepared ELISA reagents are available for use in some ELISA procedures. A polyclonal antibody ELISA procedure for *R. salmoninarum* antigen detection is described in Appendix 1 to this chapter.

c. The kidney disease bacterium can be cultured from fresh tissues or body fluids and then shown to be identical to *R. salmoninarum* by biochemical (Austin et al. 1983; Goodfellow et al. 1985;

Bruno and Munro 1986), immunological or molecular testing. The culture medium for primary isolation of the bacterium should include cysteine and serum; the most commonly used media are variations of KDM2 medium (Evelyn 1977). Although it has been reported that charcoal can be substituted for the serum component in KDM2 medium (Daly and Stevenson 1985), this may reduce the effectiveness of the medium for *R. salmoninarum* isolation from fish kidney tissues (Gudmundsdóttir et al. 1991). Antimicrobial agents can be added to KDM2 medium to inhibit the growth of other organisms present in the samples (SKDM; Austin et al. 1983). The formulation of SKDM is described on Section 2, 2.3.A.2 "Selective Kidney Disease Medium-2 (SKDM-2) (Austin, et.al.1983)." The optimal incubation temperature is 15°C and extended incubations (up to 12 weeks or more) have been recommended (Benediktsdóttir et al. 1991). The use of a nurse culture (Evelyn et al., 1989) or a medium supplemented with spent growth medium (Evelyn et al. 1990) has been reported to accelerate the growth of *R. salmoninarum*. Metabolite(s) released into a medium by *R. salmoninarum* might provide growth initiating factor(s) (Evelyn et al. 1990) or detoxifying factors (Teska 1994). Homogenization of tissue prior to culture may facilitate release of *R. salmoninarum* from granulomatous lesions (Faisal et al. 2010).

d. Several polymerase chain reaction (PCR) procedures, which incorporate the enzymatic amplification of specific unique nucleic acid fragments, have been developed for *R. salmoninarum* diagnosis. Various non-quantitative PCR procedures have been described for the detection of *R. salmoninarum* in a variety of fish samples, including: kidney tissue (Léon et al. 1994; McIntosh et al. 1996; Miriam et al. 1997; Chase and Pascho 1998; Rhodes et al. 1998; Cook and Lynch 1999), ovarian fluid (Magnússon et al. 1994; Miriam et al. 1997; Pascho et al. 1998; Rhodes et al. 1998; Cook and Lynch 1999), blood (Rhodes et al. 1998), whole fry (Rhodes et al. 1998), and eggs (Brown et al. 1994; Rhodes et al. 1998). More recently, real-time quantitative PCR (qPCR) assays have been developed to enable both detection and quantification of *R. salmoninarum* DNA (Powell et al. 2005; Chase et al. 2006; Rhodes et al. 2006; Bruno et al. 2007; Jansson et al. 2008). Nested and quantitative PCR procedures for *R. salmoninarum* detection are described in Section 2, 3.5 "*Renibacterium salmoninarum* (Bacterial Kidney Disease, BKD)".

F. Procedures for Detecting Subclinical Infections

Monitoring of moribund fish in seemingly healthy stocks can be done by the FAT, by the ELISA, by culture, or by PCR. In critical situations, the use of more than one technique for *R. salmoninarum* detection is recommended. For standard methodology for fish health inspections, please refer to Chapter 3.5.

Some ELISAs have proven sensitive enough to detect the presence of low levels of *R. salmoninarum* antigens in asymptomatic fish in a population (Pascho et al. 1987; 1991) and are suitable for large-scale population screening. For detection of *R. salmoninarum* in fish kidney tissue, certain PCR procedures have been reported to have sensitivity equal to or greater than FAT or ELISA (Chase and Pascho 1998; Chase et al. 2006; Jansson et al. 2008), and sensitivity equal to or greater than culture (McIntosh et al. 1996; Miriam et al. 1997; Cook and Lynch 1999). The viability of *R. salmoninarum* can be determined by culture. Enhancement techniques such as the use of nurse cultures or supplementation of a medium with spent growth medium (*R. salmoninarum* metabolite) may be necessary to detect the bacterium in asymptomatic fish (Evelyn et al. 1989; 1990). Other methods to increase the sensitivity of *R. salmoninarum* culture include homogenization of tissue in saline diluent and use of a large inoculum on culture plates (Faisal et al. 2010), or centrifugation of homogenized and diluted tissue, followed by discard of the supernatant and resuspension of the tissue pellet in diluent for plate culture (Jansson et al. 1996; Elliott et al. 2013). The FAT, ELISA, and many PCR procedures cannot distinguish viable from non-

viable *R. salmoninarum*. However, non-quantitative and quantitative reverse transcription PCRs (RT-PCR) have been developed for the detection of viable or recently killed cells of *R. salmoninarum* (Magnússon et al. 1994; Cook and Lynch 1999; Suzuki and Sakai 2007; Halaihel 2009). These PCRs detect specific sequences of *R. salmoninarum* messenger RNA (mRNA), which has a very short half-life.

Examination of spawning fish is recommended. The bacterium can be transmitted vertically, and the expression of infections is often enhanced at spawning. The ELISA is a useful test for detecting and quantifying *R. salmoninarum* antigen in tissues such as the kidney and spleen and in the blood plasma of fish that cannot be sacrificed (Pascho et al. 1987). However, both polyclonal antibody ELISAs (Pascho et al. 1991; 1998) and monoclonal antibody ELISAs (Griffiths et al. 1996) may be unreliable for detecting antigens of the bacterium in ovarian fluid. In contrast, some PCR procedures have been reported to detect low numbers of *R. salmoninarum* in the ovarian fluid (Pascho et al. 2002); one such procedure is described in Section 2, 3.5.B.2 "Nested Polymerase Chain Reaction (PCR) for Confirmation of *R. salmoninarum* DNA." A drawback of many PCR procedures for *R. salmoninarum* detection is that they are not quantitative, but more recently developed real-time PCRs allow quantification of *R. salmoninarum* in tissues and ovarian fluid within the operating range of the assays (see e.g. Elliott et al. 2013). The membrane filtration-FAT (Elliott and Barila 1987; Elliott and McKibben 1997), in which bacteria are concentrated on 0.2 µm polycarbonate filters prior to FAT staining, is useful for enumeration of *R salmoninarum* in ovarian fluid. Although FAT staining of smears of material pelleted by centrifugation from ovarian fluid is frequently used instead of the MF-FAT for *R. salmoninarum* detection, the smear FAT (S-FAT) is not reliable for detection and quantification of *R. salmoninarum* in ovarian fluid when bacterial concentrations are low (Armstrong 1989; Elliott and McKibben 1997). Because of its greater sensitivity and quantitative ability, the MF-FAT is preferable to the S-FAT for use in critical situations requiring the detection of low numbers of bacteria ($<2.4 \times 10^3$ cells/mL; Elliott and McKibben 1997).

The nested and quantitative PCR tests described in Section 2, 3.5 have been tested for non-lethal detection of bacterial DNA in mucus, gill and fin tissue (Elliott et al. 2015). Quantitative PCR applied to mucus scrapes was the only assay / sample combination demonstrating estimates of both diagnostic sensitivity and specificity > 90% during testing.

G. Procedures for Determining Prior Exposure to the Etiological Agent

The antibody response of fish to *R. salmoninarum* infection has been reported to be variable (Bruno 1987). The measurement of antibody response is therefore not considered a reliable means of determining prior exposure to the pathogen. Conversely, the detection of *R. salmoninarum* antigen in fish by ELISA testing is not a reliable indication of an active infection, as antigens of killed *R. salmoninarum* can persist for more than three months in fish (Pascho et al. 1997).

H. Procedures for Transportation and Storage of Samples to Ensure Maximum Viability and Survival of the Etiological Agent

Samples that are to be cultured should be stored under refrigeration (10°C or lower, but not frozen) and inoculated into the appropriate culture medium within 72 hours. Samples for ELISA testing should be refrigerated if they are to be analyzed within 24 hours of collection, or frozen if longer storage is necessary. Freezing of ELISA samples at -20°C is acceptable but freezing at colder temperatures (-70°C) is desirable for retention of the antigens during prolonged storage. Samples for the FAT should be treated in the same manner as samples for the ELISA. If tissue smears are prepared on slides and the slides are not examined within one week, they should be stored under refrigeration until FAT staining is done.

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