An Overview on Bacterial Kidney Disease

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Abstract: Bacterial Kidney Disease (BKD) caused by a Gram-positive bacterium, *Renibacterium salmoninarum* (*R. salmoninarum*), is a systemic disease that threatens the expansion of both cultured and wild salmonids worldwide. BKD is virtually reported wherever salmonids are present, and continue to pose a threat to salmonids worldwide. Further, problems associated with BKD epizootics include high mortality rate, low growth rate, increased susceptibility to other diseases such as furunculosis and cold water disease (CWD) are another aspect of the problem. Moreover, despite the expanding risk of BKD, the pathogenesis of *R. salmoninarum* infection has only been partially elucidated, hindering the progress of competent preventive and control measures to efficiently combat this disease. For all the above mentioned reasons, scientific work and current research need to be continually updated to benefit the researchers, aquaculture sector and fisheries. The current review provides the most recent update of research work on BKD, discusses the agent and the disease it causes, with emphasis on the bacterium-host interactions in a trial for better understanding of the disease and its epizootiology. [Life Science Journal. 2006;3(3):58-76] (ISSN: 1097-8135).

Keywords: *Renibacterium salmoninarum*; bacterial kidney disease; salmon

Abbreviations: BKD: bacterial kidney disease; CWD: cold water disease; ECP: extracellular products; FAT: fluoresence antibody tests; KDM: kidney disease medium; MKDM: modified KDM; SKDM: selective KDM

1 Historical Perspectives

Bacterial Kidney Disease (BKD), caused by the Gram-positive bacterium *Renibacterium salmoninarum* (*R. salmoninarum*), is a systemic disease that afflicts salmonid fish populations worldwide. The condition was originally described as the Dee Disease because it was first observed among Atlantic salmon (*Salmo salar*) from Aberdeenshire Dee and the River Spey in Scotland in 1930 (Anonym, 1933; Smith, 1964). Other synonyms of the disease include Kidney Disease, Corynebacterial Kidney Disease and Salmonid Kidney Disease (Fryer and Sanders, 1981). Few years later, Belding and Merrill (1935) described a very similar infection that caused losses in brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*) and rainbow trout (*Oncorhynchus mykiss*) reared in a hatchery in Massachusetts, USA. By 1953, due to serious outbreaks, BKD had become a limiting factor in rearing brook trout, brown trout, rainbow trout, chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*Oncorhynchus kisutch*) and sockeye salmon (*Oncorhynchus nerka*) in many hatcheries in the State of Washington (Earp *et al*, 1953). In the following year, the disease was found in the feral salmon in the same state (Rucker *et al*, 1954). In 1955, BKD spread to the Great Lakes basin with the introduction of salmonines and their products from the Pacific Northwest (Allison, 1958). Reports from Canada linked the disease to mortalities in wild salmonines from Nova Scotia (Pippy, 1969; Paterson *et al*, 1979) to British Columbia (Evelyn *et al*, 1973; 1981). By 1988, the disease became widespread in Europe (England, France, Finland, Germany, Iceland, Italy, Spain, Turkey and Yugoslavia), North America (USA and Canada), and Japan (reviewed in Bullock and Herman, 1988; Fryer and Lannan, 1993). The disease continued its spread to Chile (Sanders and Barros, 1986) and there is a current consensus among fish health professionals that BKD is virtually prevalent in all parts of the world where wild or cultured salmonines exist (European Commission, 1999).

2 The Pathogen

2.1 Nomenclature and current classification of the etiological agent

Based on Gram stain properties, morphology, the causative bacterium was suggested to be a member of the genus *Corynebacterium* by Ordal and

Earp (1956) and subsequently by Smith (1964). Sanders and Fryer (1980) later refuted this classification based on the absence of mycolic acid, guanine plus cytosine (G + C) content of DNA, cell wall sugar and amino acid compositions of the peptidoglycan cell wall layer. The authors proposed that this bacterium formed a single species in a new genus *Renibacterium* and they identified the bacterium as *R. salmoninarum* (Sanders and Fryer, 1980). Sequencing of the 16S rRNA from *R. salmoninarum* (Gutenberger *et al*, 1991) and recent evaluation of G + C content (Banner *et al*, 1991) placed the organism in the Gram-positive eubacterial subdivision of actinomycetes. *Arthrobacter* and *Micrococcus* spp. are the closest relatives to *R. salmoninarum*.

2.2 Cell morphology

R. salmoninarum is a short rod (0.3 − 1.0 by 1 − 1.5 μm), Gram-positive, non-sporulated, non-capsulated, non-motile, and non acid-fast bacterium that is arranged in pairs (diplobacilli) and rarely as short chains (Sanders and Fryer, 1980). *R. salmoninarum* consists of two regions; a central region filled with lightly stained filaments (represent DNA) and a peripheral region filled with small, electron dense ribosomes (Young and Chapman, 1978).

2.3 Isolation, culture and cultural characteristics

R. salmoninarum is a slow growing organism (Sanders and Fryer, 1980). Earp *et al* (1953) cultured the bacterium on an artificial medium for the first time from infected kidney tissues on a medium that consisted of fish extract, glucose, yeast extract and meat infusion in agar. The authors achieved limited growth with first appearance of colonies after more than two weeks of incubation. When the same authors used minced chick embryo tissues embedded in 1% agar or Dorset's Egg medium, they achieved better growth. Addition of 0.05% to 0.1% L-cysteine to the Dorset's Egg medium has further enhanced the growth of *R. salmoninarum* upon primary isolation (Ordal and Earp, 1956). The authors noted that trypticase blood agar could be used for secondary cultures and bacterial maintenance. Based on years of research, Ordal and Earp (1956) formulated the Kidney Disease medium (KDM1) which consisted of: tryptose 1.0%, beef extract 0.3%, NaCl 0.5%, yeast extract 0.05%, cysteine-hydrochloride 0.1%, human blood 20 v/v and agar 1.5%. They designated this medium as "Cysteine Blood Agar Medium". While testing the *in vitro* sensitivity of *R. salmoninarum* to a large number of therapeutics, Wolf and Dunbar (1959), achieved fair growth on cysteine supplemented Mueller-Hinton medium (MH). This modified MH medium became the medium of choice for the growth of *R. salmoninarum* for several years (Bullock *et al*, 1974).

Evelyn (1977) modified Ordal and Earp's KDM1 by replacing human blood, tryptose and beef extract with 20% fetal bovine serum and peptone and designated the modified medium as KDM2. To reduce the time needed for primary isolation, Evelyn *et al* (1989) added 25 μl of heavy inoculum of *R. salmoninarum* culture (commonly known as a nurse culture) to the center of KDM2 plates. The authors reported that this modification has accelerated bacterial growth in primary cultures. Further, Evelyn *et al* (1990) were able to achieve more consistent growth of the primary culture by replacing the nurse culture with 25 μl of filter-sterilized *R. salmoninarum* spent medium. The major drawbacks of KDM2 medium, however, were the high cost and presence of serum proteins, which hampered the identification of proteins of bacterial origin.

A number of serum-free media for *R. salmoninarum* growth have also been formulated. For example, Embley *et al* (1982) described a serum-free, semi-defined growth medium that supported secondary, but not primary, growth of *R. salmoninarum*. Daly and Stevenson (1985) formulated the Charcoal Agar Medium in which they substituted activated charcoal for serum. Starliper *et al* (1998) compared the performance of 13 serum-free media and 1 serum-supplemented media for the growth of *R. salmoninarum* isolates and found that there were no significant differences among the 14 medium formulations used when mean cell counts were compared after 10, 20, 30 days incubation.

To control growth of other bacteria from fish lesions, Austin *et al* (1983) incorporated four antibiotics (Cycloheximide, D-cycloserine, Oxolinic acid and Polymyxin B) to the KDM2 medium and reduced the volume of serum from 20% to 10% (designated selective KDM or SKDM). By these modifications, the authors significantly reduced bacterial contaminants, a matter that facilitated the selected growth of *R. salmoninarum* from clinical and environmental samples. Our current lab experience (Eissa, 2005) suggested that the modification of SKDM by incorporating 1% Spent medium into the agar enhanced the growth of the *R. salmoninarum* colonies, shortened the period of incubation, and minimized the growth of contaminating bacteria.

R. salmoninarum colonies are creamy (non-

pigmented), shiny, smooth, round, raised, entire, and 1 – 2 mm in diameter on KDM2 after incubation at 15 °C for 20 days (Austin and Austin, 1999). On cysteine supplemented solid media, old colonies (i.e. 12 weeks) appeared extremely granular due to crystallization of cysteine, while in both culture media; some *R. salmoninarum* strains produced a uniform turbidity whereas others flocculated out of suspension (Austin and Austin, 1999). The organism grows slowly at 5 °C, 22 °C and optimally at 15 °C but there was no growth at 37 °C (Smith, 1964).

2.4 Preservation of cultures

Several methods have been used to preserve different species of actinomycetes including *Streptomyces*, *Actinomyces* and *Renibacterium* species. For long term preservation, methods such as lyophilization (Hopwood and Ferguson, 1969), storage under liquid nitrogen (Pridham and Hesseltine, 1975) were successfully used. Bacterial cells can also be preserved in diluted glycerol (10% – 20% v/v) and frozen at – 20 °C, but thawing, and freezing cycles can affect cell stability and viability (Wellington and Williams, 1979). To overcome this disadvantage, Feltham *et al* (1978) stored bacteria on glass beads in 10% (v/v) glycerol at – 76 ℃. The glass beads allowed removal of small samples without thawing the entire culture, which was advantageous for long-term preservation (Wellington and Williams, 1979). Preservation of small inocula of *R. salmoninarum* in KDM2 (Evelyn *et al*, 1977) or peptone saline (Starliper *et al*, 1997) and storage at – 80 °C were successfully used.

2.5 Biochemical characteristics

The organism is cytochrome oxidase negative, catalase positive, proteolytic and cysteine HCl is required for its growth (Ordal and Earp, 1956; Smith, 1964; Sanders and Fryer, 1980). Interestingly, *R. salmoninarum* isolates from different sources are identical in their biochemical characteristics (Austin *et al*, 1983; Goodfellow *et al*, 1985; Bruno and Munro, 1986a), but the result for a given test can vary depending upon the testing system used. Thus, the organism is positive for the gelatinase and DNase reactions by standard methods (Bruno and Munro, 1986a), but it was negative for these characters by the API-ZYM system (Goodfellow *et al*, 1985). The organism is β-hemolytic on media supplemented with blood (Bruno and Munro, 1986a). The organism can liquefy gelatin, degrade Tween (20 – 60), and hydrolyze casein. The bacterium is negative for esculin hydrolysis, DNase, urease, nitrate reduction, phosphatase, methyl red, indole test and carbohydrate utilization test (Table 1).

Table 1. Summary of the morphological and biochemical characteristics of *R. salmoninarum*

Test	Criteria	Notes
Gram stain	+	
PAS (Periodic Acid Schiff) stain	+	
Zeihl-Nielsen (Acid Fast) stain	–	Non acid fast
Arginine hydrolysis	–	
Bile solubility	–	
Agar hydrolysis	–	
Amylase	–	
Carbohydrate utilization	–	
Casein hydrolysis	+	
Catalase	+	
Cytochrome oxidase	–	
DNase	+	(–) By API – ZYM*
Esculin hydrolysis	–	
Esterase	–	
Gelatin liquefaction	+	(–) By API – ZYM*
Hemolytic activity	β hemolytic	Complete clearance zone around bacteria
Indole test	–	
Methyl Red	–	
Nitrate reduction	–	
Phosphatase	–	
Tween-20, 40 and 60 Hydrolysis	+	
Tween-80 hydrolysis	–	
Urease	–	

ABI – ZYM* is a bacterial enzymes based assay used for the specific identification of different bacteria.

2.6 Antibiotic susceptibility

R. salmoninarum isolates are sensitive to chloramphenicol, erythromycin, novobiocin, streptomycin, sulfamerazine, and tetracycline (Wolf and

Dunbar, 1959; Austin and Rodgers, 1980), carbenicillin, and cephaloridine (Goodfellow *et al*, 1985). *R. salmoninarum* is also sensitive to enrofloxacin (Hsu *et al*, 1994), tiamulin, cefazolin (Bandin *et al*, 1991) and azithromycin (Rathbone *et al*, 1999). Furthermore, the organism is resistant to D-cycloserine, oxolinic acid (4 μg/ml), polymyxin β and cycloheximide (Wolf and Dunbar, 1959; Goodfellow *et al*, 1985).

2.7 Antigenic characteristics and virulence factors

R. salmoninarum is an obligate intracellular pathogen that is able to invade all types of fish cells particularly phagocytic cells (Gutenberger *et al*, 1997; Ellis, 1999). The ability of *R. salmoninarum* to invade phagocytes or other cells depends upon certain virulence determinants (Gutenberger *et al*, 1997; Ellis 1999; Piganelli *et al*, 1999). It was demonstrated that *R. salmoninarum* secretes a number of extracellular products (ECP) that possess proteolytic, hemolytic and DNA degradation activities *in vitro* (Austin and Rodgers, 1980; Bruno and Munro, 1986a). When crude or precipitated culture supernatants were injected into Atlantic salmon fingerlings, 80% – 100% mortalities were reported (Shieh, 1988), but Bandin *et al* (1991) were unable to reproduce this finding using untreated culture supernatants. A 65-kDa *R. salmoninarum* zinc metalloprotease-like protein has been extracted from *R. salmoninarum* ECP that possesses hemolytic activities against a number of fish and mammalian erythrocytes. The encoding gene of the *R. salmoninarum* ECP with hemolytic activity was designated as hly (Grayson *et al*, 1995). *R. salmoninarum* secretes a water-soluble, heat stable, hydrophobic cell surface 57 kDa protein (p57) that is believed to be the major virulence determinant of this bacterium (Getchell *et al*, 1985). *In vitro*, purified p57 exhibited both hemolytic (Daly and Stevenson, 1990) and leucoagglutinating (Wiens and Kaattari, 1991) properties. Hamel (2001) reported that *R. salmoninarum* isolates differed in their pathogenicity to salmonids, a finding that correlated positively with the amount of surface associated p57.

Challenge of susceptible fish with non-auto-agglutinating strains of *R. salmoninarum* caused significantly lower mortality than auto-agglutinating strains (Daly and Stevenson, 1990). Soluble *R. salmoninarum* surface proteins possess immunosuppressive action against the salmonid specific antibody response (Turaga *et al*, 1987), which was attributed not only to the p57 protein, but also to a 22-kDa surface protein (Fredriksen *et al*, 1997). Starliper *et al* (1997) compared a number of strains of *R. salmoninarum* isolated from chinook and coho salmon from different regions in North America for virulence. The authors found that virulence differed among the used isolates and concluded that isolates retrieved from Michigan weirs in the late 1980s were the most virulent.

2.8 Molecular and genetic diversity

Although the biochemical uniformity (Bruno and Munro, 1986a) and phylogenetic homology of *R. salmoninarum* strains (Gutenberger *et al*, 1991), a minimal molecular diversity was detected among strains isolated from different parts in the world (Alexander *et al*, 2001). Alexander *et al* (2001) succeeded in differentiation between isolates of *R. salmoninarum* based on PCR amplification and length polymorphism in the tRNA intergenic spacer regions (tRNA -ILPs). Moreover, a genetic diversity was detected among 40 North American isolates by using the multilocus enzyme electrophoresis (MEE) assay with the highest genetic diversity detected in strains isolated from chinook and coho salmon spawners returning to the Little Manistee river weir in Michigan (Starliper, 1996). In particular, Michigan isolates showed a higher variation in succinate dehydrogenase and esterase loci.

3 The Disease

3.1 Disease course

Despite the fact that BKD develops slowly, progress of the disease depends on environmental factors such as water temperature (Sanders *et al*, 1978; Fryer and Sanders, 1981; Bullock and Herman, 1988), host factors (Evenden *et al*, 1993), and *R. salmoninarum* strain virulence (Starliper *et al*, 1997).

3.1.1 External signs

Affected fishes manifest a wide range of external lesions as well as behavioral changes that might vary according to the species, age of the fish affected and the virulence of the *R. salmoninarum* strain (Fryer and Sanders, 1981; Bullock and Herman, 1988; Evenden *et al*, 1993). Erratic swimming behavior, exophthalmia, superficial blebs of the skin, cavitations in muscles and deep abscesses all over the body surface have been reported in affected fish (Belding and Merrill, 1935; Smith, 1964; Fryer and Sanders, 1981; Bullock and Herman, 1988). The blebs and cavitations might contain a white to yellowish or hemorrhagic fluid (Bullock and Herman, 1988). Ascitis and peticheal hemorrhages in muscles and fins were also reported (Belding and Merrill, 1935; Earp *et al*, 1953; Evelyn, 1993). In very rare cases, the external

signs of the disease in chinook and coho salmon might only be manifested by exophthalmia with the accumulation of infective fluid containing large amount of the bacteria, pus and necrotic tissue in the enlarged eyes (Bullock and Herman, 1988).

3.1.2 Internal lesions

Kidneys of affected fishes are usually swollen and exhibit white foci that contain leucocytes, bacteria, and host cell debris (Figure 1) (Fryer and Sanders, 1981). In advanced cases the kidneys appear mostly grayish in color, the spleen may increase in size and the liver appears very pale in color (Woods and Yasutake, 1956; Fryer and Sanders, 1981). The most typical clinical lesions associated with BKD are the presence of scattered nodules of various sizes over the surface of the kidneys, spleen and liver (Belding and Merrill, 1935; Snieszko and Griffin, 1955; Klontz, 1983). In some cases, peticheal hemorrhages were noticed in the muscles lining the peritoneum with ascitic fluid accumulation (Ferguson, 1989). An opaque membrane (pseudomembrane) that covers internal organs was reported, especially in fish maintained at a temperature below 9 °C (Snieszko and Griffin, 1955; Bell, 1961; Fryer and Sanders, 1981). The pseudomembrane consists of fibrin and leucocytes (Smith, 1964). Similar membranes occur in trout at higher temperatures (12 – 13 °C) (Bullock and Herman, 1988). Hemorrhages with white or yellow viscous fluid in the hindgut and peticheal hemorrhages were often found in the peritoneum of infected Atlantic salmon (Smith, 1964).

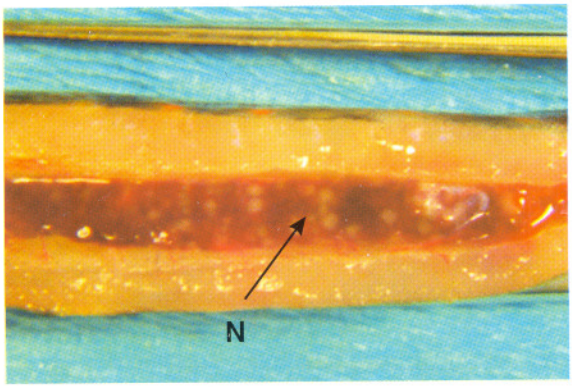

Figure 1. An Iron River brook trout fingerling with BKD. The kidney is swollen with multiple creamy-whitish nodules (N). The above case is from an outbreak of BKD that killed thousands of hatchery raised Iron River brook trout fingerlings in May 2003.

3.1.3 Histopathology

The initial histopathological description by Belding and Merrill (1935) indicated that the kidney as the major organ affected by the *R. salmoninarum* infection. All infected brook trout and brown trout exhibited microscopic lesions in the kidney, and to a lesser extent in the liver and spleen. Lesions were chronic in nature with multiple granulomas that resemble those noticed in mammalian tuberculosis (Figure 2) (Snieszko and Griffin, 1955; Wood and Yasutake, 1956). Fibrotic lesions were also noticed in kidneys, spleen, liver and intestines of the infected fish with proliferating fibroblasts forming distinct nodules that coalesced to form large masses of affected tissues (Woods and Yasutake, 1956). The granulomatous lesions apparently arose in the connective tissue stroma between the parenchymal cells of various organs (Woods and Yasutake, 1956; Jansson, 2002). It is believed that the granulomas are formed as a result of macrophages activation (Secombes, 1985) followed by its adherence to each other forming epithelioid appearance and then the fusion of a few number of these activated macrophages to form giant cells (Secombes, 1985). Both, giant cells and activated macrophages release large amounts of lytic enzymes into the surrounding tissues leading to necrosis at the central part of the granuloma (Bruno, 1986; Jansson, 2002). Interestingly, bacteria can occur intracellularly or extracellularly in the granulomas or necrotic foci (Bruno, 1986; Bullock and Herman, 1988). The hematopoietic tissue of the anterior kidney appeared to be affected firstly, followed by extensive damage to the excretory part of the kidneys (Woods and Yasutake, 1956; Jansson, 2002). Kidney pathology may contain eosinophilic granules in proximal tubules (Young and Chapman, 1978). Massive myocarditis (Wood and Yasutake, 1956), meningitis, and encephalitis (Speare, 1997) were recorded in some salmonids. In the liver, histopathological changes take the form of granulomatous nodules in the connective tissue stroma between the cords of the hepatic cells (Woods and Yasutake, 1956).

3.2 Susceptibility

There are a number of observations indicating that salmonid species and even different strain of the same species can differ in their susceptibility to BKD. For example, coho salmon of three different transferrin genotypes (AA, AC and CC) differed in resistance to experimental infection with *R. salmoninarum* (Suzumoto *et al*, 1977). Also, three populations of chinook salmon from different rivers, showed various mortality rates to experimental infection with *R. salmoninarum*. Winter *et al* (1980) reported similar results in coho salmon and steelhead trout (*Oncorhynchus mykiss*). Further, Belding and Merrill (1935) reported that brook trout was more susceptible to *R. salmoninarum* infection than the rainbow trout when ex-

perimentally infected. Mitchum and Sherman (1981) reported that brook trout were more susceptible to natural BKD infection than rainbow trout and brown trout. Eissa (2005) confirmed that the brook trout is the most susceptible species among all studied salmonids during a period of 4 years study. Moreover, he indicated that the Iron River strain of the brook trout species is more susceptible than the Assinica strain of the same species.

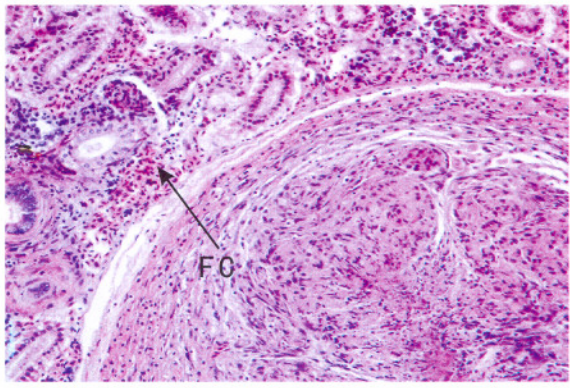

Figure 2. Hematoxylin and Eosin stained slide of kidney showing a severe granulomatous reaction that is replacing kidney tissues of a 3 years old Assinica brook trout. Notice the fibrous capsule (FC) surrounding the entire granuloma (× 100). The case is from an outbreak of BKD that killed captive 3 years old Assinica brook trout in mid September 2003.

3.3 Pathogenesis and immunity

3.3.1 Infection and pathogenesis

R. salmoninarum can induce uptake by non-phagocytic cells and can survive ingestion, which provides a means of entry into the host via the gills and the gastrointestinal tract (Evelyn, 1996; Flano *et al*, 1996; Balfry *et al*, 1996), however a study demonstrated that *R. salmoninarum* can not be internalized by healthy rainbow trout gills *in vitro* (McIntosh *et al*, 2000). *R. salmoninarum* uptake by eggs is another possibility that result in vertical transmission of the organism from parent to offspring (Evelyn *et al*, 1984; Evelyn *et al*, 1986a, 1986b; Bruno and Munro, 1986b).

R. salmoninarum is believed to spread through blood and also through intracellular habitation and replication in macrophages (Gutenberger *et al*, 1997; Ellis, 1999). Although *R. salmoninarum* is a slow growing organism, it can reach levels of 10^9 cells/g in spleen and kidney tissues before initiation of fish mortality (Evelyn, 1996).

Opsonization of the pathogen by antibody and /or complement increases the success of *R. salmoninarum* to survive and replicate within phagocytes more willingly than limit its activity as with most of other pathogens (Bandin *et al*, 1995). To survive and replicate, *R. salmoninarum* must acquire nutrients from the host. In the absence of iron, *R. salmoninarum* may produce iron reductase, which makes bound iron more available for bacterial uptake (Grayson *et al*, 1995).

R. salmoninarum produces large amounts of the p57 antigen (Wiens and Kaattari, 1989), both in serum and intracellularly. The quantity can neutralize the vast majority of antibodies that may be evoked in response to infection. These antibody-p57 complexes may remain in tissue and contribute to tissue destructive hypersensitivity resulting in granulomas (Bruno, 1986).

The p57 has immunosuppressive and tissue destructive properties. The p57 agglutinates salmon leukocytes (Wiens *et al*, 1991) and suppresses antibody production against unrelated antigens *in vitro* (Turaga *et al*, 1987). The p57 is a potent inhibitor of the phagocyte respiratory burst response (Campos-Perez *et al*, 1997) and could decrease the bactericidal activity of juvenile chinook macrophages against *Aeromonas salmonicida* (Siegel and Congleton, 1997).

Senson and Stevenson (1999) suggested that p57 and its breakdown products might form a protective layer around *R. salmoninarum* cells. Bacterial cells stripped of p57 induced stronger immune response than those not stripped of p57 (Wood and Kaattari, 1996). Cell surface associated p57 and its breakdown products may effectively block highly immunogenic areas of the bacterial cell surface from detection by host defenses (Wiens and Kaattari, 1999).

3.3.2 Effect of BKD on host immune response

Grayson *et al* (2002) studied the immunosuppressive effect of *R. salmoninarum in vitro* and *in vivo*. Within an *in vitro* assay, macrophages showed a rapid inflammatory response in which the expression of interleukin-1β, major histocompatibility complex class Ⅱ, inducible cyclooxygenase, and inducible nitric oxide synthase (iNOS) were enhanced, while tumor necrosis factor-α (TNF-α) expression was greatly reduced initially and then increased. *In vivo* study, intraperitoneal (*i. p.*) injection of *R. salmoninarum* DNA vaccine constructs (msa) reduced the expression of IL-1β, Cox-2, and MHC Ⅱ but stimulated TNF-α. In this study, the authors concluded that p57 suppresses the host immune response and hypothesized that the chronic granulomatous reaction is due to prolonged stimulation of TNF-α. The p57 possessed immunosuppressive action against salmonid specific antibody response (Turaga *et al*, 1987), tissue destructive properties (Bruno, 1986) and capable of

agglutinating salmon leukocytes (Wiens and Kaattari, 1999).

Aside from its opsonizing action, antibodies interact directly with free antigen (p57), creating immune complexes that aggregate within the tissue and cause hypersensitivity reactions, resulting in granulomas and tissue damage (Bruno, 1986). Macrophage activating factor (MAF)-activated macrophages can effectively kill *R. salmoninarum* cells (Hardie *et al*, 1996), but production of MAF in immature helper T-cells may be suppressed at low temperature (Siegel and Congleton, 1997). The proliferation and action of T cells in activating macrophages may be the primary successful immune response against *R. salmoninarum* (Secombes, 1985; Hardie *et al*, 1996).

3.3.3 Environmental factors

Effect of diet

Studies suggested that the prevalence and severity of BKD might be partly related to certain dietary and environmental factors. Diets formulated of gluten as opposed to cottonseed meal have resulted in higher BKD prevalence in several hatcheries in Washington (Wood, 1974). Wedemeyer and Ross (1973) demonstrated that BKD prevalence was similar in fish fed rations containing equivalent amounts of either gluten or cottonseed, but the non-specific stress of infection perhaps due to the increased ascorbate depletion was more severe in the corn gluten group. Sakai *et al* (1986) concluded that vitamins had no effect on BKD prevalence. Woodall and LaRoche (1964) suggested that iodine insufficiency was responsible for increased BKD incidence in juvenile chinook salmon. Paterson *et al* (1981) indicated that Vitamin A, zinc, and iron levels are significantly reduced in BKD-infected fish and subsequent feeding trials provided a lower incidence of BKD in fish fed diets high in trace elements (Fe, Cu, Mn, Co, I and F) or low in calcium (0.2%).

Effects of temperature

Several authors reported that BKD could occur over a wide range of water temperatures (Belding and Merrill, 1935; Earp *et al*, 1953; Fryer and Sanders, 1981; Bullock and Herman, 1988). For example, at 15 – 20 °C, experimentally infected juvenile salmon and trout died 21 – 34 days after inoculation, as opposed to 60 – 71 days post inoculation at 6.7 °C (Sanders *et al*, 1978). Also, Wood (1972, cited in Fryer and Sanders, 1981) reported that mortalities from BKD occurred after 30 – 35 days post exposure at temperatures above 11 °C and took 60 – 90 days at 7.2 – 10 °C. Sanders and Fryer (1981) indicated that most of epizootics occurred during the autumn and winter, under conditions of declining water temperatures; however the greatest mortality was associated with periods of highest water temperatures. Also, it was noted that during periods of low water temperatures the disease produced a slow steady death rate.

Water salinity

Despite the fact that BKD occurs mainly in freshwater, significant infections also occur in saltwater (Banner *et al*, 1983). Reports demonstrated that deaths continued in chinook, coho and pink salmon stocks after movement to salt water-rearing ponds (Earp *et al*, 1953; Bell, 1961). Frantsi *et al* (1975) reported that *R. salmoninarum* impaired the ability of Atlantic salmon smolts to acclimate to saltwater and caused a subsequent reduction in ocean survival. Ellis *et al* (1978) isolated the organism from juvenile chinook salmon that had spent two winters in the ocean. Fryer and Sanders (1981) indicated that BKD was thought to be the main cause of death among coho salmon smolts released from Siletz hatchery in Oregon. The authors reported that the majority of deaths occurred between two and four months after the fish entered saltwater. They also concluded that fish infected with BKD while in freshwater will continue to die from this disease, but at an accelerated rate, after migration to saltwater. BKD infection can impair acclimatization to seawater and cause death (Mesa *et al*, 1999). Further, Price and Schreck (2003) experimentally assessed the effect of BKD on saltwater preference of juvenile spring chinook salmon and concluded that there is a significant negative relationship between mean infection level and saltwater preference.

4 Epizootiology

4.1 Geographical distribution

BKD has been reported wherever susceptible salmonid populations are present (Fryer and Sanders, 1981; Klontz, 1983). The disease is commonly reported in cultured salmonid species from North America, Europe, Japan and South America (Fryer and Sanders, 1981; Bullock and Herman, 1988). BKD has also been observed in a wide range of wild (Pippy, 1969; Evelyn *et al*, 1973; Ellis *et al*, 1978; Paterson *et al*, 1979; Mitchum and Sherman, 1981) and feral salmonid populations from North America (Elliot and Pascho, 1991; Sanders *et al*, 1992; Holey *et al*, 1998; Jonas *et al*, 2002). The geographic range of BKD includes Canada, England, France, Finland, Germany, Iceland, Italy, Japan, Scotland, Spain, Turkey, United States, former Yugoslavia and Chile (Bullock and Herman, 1988). BKD was pre-

sumptively diagnosed and reported in Australian Victoria in the early 1970s in farmed chinook salmon however further work identified the syndrome to be nocardiosis (Humphrey *et al*, 1987). No evidence supported the presence of the disease in New Zealand, Russia. BKD was recently reported in Denmark (Lorenzen *et al*, 1997) and Norway (Jansson *et al*, 2002).

4.2 Host range

BKD has been reported in salmonids of the genera *Oncorhynchus*, *Salmo* and *Salvelinus* (Fryer and Sanders, 1981). *R. salmoninarum* has also been detected in chinook salmon (Holey *et al*, 1998), coho salmon (MacLean and Yoder, 1970), brown trout, brook trout, rainbow trout (Belding and Merrill, 1935; Mitchum *et al*, 1979), Pacific salmon, Atlantic salmon, lake trout (Bullock and Herman, 1988), pink salmon (Bell, 1961), Kokanee salmon (Awakura, 1978), Grayling (*Thymallus thymallus*) (Kettler *et al*, 1986), Lake Michigan whitefish (*Coregonus clupeformis*) and bloater (*Coregonus hoyi*) (Jonas *et al*, 2002) and whitefish (*Coregonus lavretus*) in Finland (Rimaila-Parnanen, 2002). The organism has also been detected in absence of disease in few non-salmonid species such as greenling (*Hexagrammos otaki*), flathead (*Platycephalus indicus*) and Pacific herring (*Glupea pallasi pallasi*) (Traxler and Bell, 1988). *R. salmoninarum* antigen has also been detected in Japanese sculpin (*Cottus Japonicus*) and Japanese scallops (*Patinopecten yessoensis*) (Sakai and Kobayashi, 1992). Recently, the organism has been isolated for the first time from clinically affected adult parasitic stage of Lake Ontario Sea Lamprey (*Petromyzon marinus*) (Eissa *et al*, 2006, In press).

4.3 Disease transmission

4.3.1 Source of infection

R. salmoninarum is excreted in the feces of clinically diseased trout and can survive for up to one week and two weeks in the feces and sterile seawater respectively (Balfry *et al*, 1996). The organism can also survive in non-sterile freshwater and pond sediments for up to 21 days (Austin and Rayment, 1985). Thus, the oro-fecal route of horizontal transmission may contribute significantly to the increasing prevalence of BKD in salmonids.

4.3.2 Horizontal transmission

R. salmoninarum possesses a powerful capability of inducing uptake by tissue cells including the epithelial lining of the gastro-intestinal tract (Bruno, 1986; Evelyn, 1996; Flaño *et al*, 1996). Infection is thereby likely to occur where sufficient numbers of bacteria are present within the immediate vicinity of aquatic environment. Oral-fecal route of infection can also, occur in net pens by ingestion of contaminated feces (with up to 10^7 bacteria/g of feces) during feeding (Balfry *et al*, 1996). Waterborne infection may occur through gills, eyes, lesions, wounds and ingestion (Evenden *et al*, 1993). The organism was also transmitted by feeding fish on infected or inefficiently pasteurized fish offales or fish flesh (Wood, 1974; Fryer and Sanders, 1981). Thus, uptake of *R. salmoninarum* through the intestinal wall is a likely pathway of infection (Jansson, 2002). Horizontal transmission can also occur between wild and stocked hatchery trout in natural systems (Mitchum and Sherman, 1981). Long-term exposure (180 days) of healthy fish to highly infected or dying salmon resulted in the infection and death of all exposed fish at an average water temperature of 10 ℃ (Murray *et al*, 1992).

4.3.3 Vertical transmission

Numerous studies have been conducted in the last two decades in order to study the possibility of vertical transmission of *R. salmoninarum* from mother to offspring via eggs. Allison (1958) was the first to report the development of BKD in offspring hatched from eggs transferred from a hatchery where the disease had been endemic for many years to another hatchery where it had never been detected. Bullock *et al* (1978) demonstrated transmission of *R. salmoninarum* from the broodstocks to their progeny via the eggs. Interestingly, the organism has been transmitted even after the surface disinfection of eggs which likely due to the fact that the pathogen was located within the perivitteline membrane of the egg away from the reach of the disinfectant (Evelyn, 1993). The intra-ovum route of transmission has now been firmly established (Evelyn *et al*, 1986a, 1986b) where the pathogen is located in the yolk and is protected from surface disinfectants (Evelyn *et al*, 1986a, 1986b; Bruno and Munro, 1986c). Infected coelomic fluid has been shown to be an important source of infection for the egg (Evelyn, 1993) where the organism found its way to the yolk via the micropyle due to high bacterial counts in coelomic fluid. There are some instances that intra-ovum infections can also occur prior to ovulation and directly from the ovarian tissue (Evelyn, 1993). The pathogen has been also detected in the semen (milt) of infected Brook trout brood stocks collected during spawning cycles in Michigan State hatcheries which suggests that male can play a possible role in transmission and spread of *R. salmoninarum* (Eissa, 2005).

4.3.4 Fish as possible vectors and carriers

Although there are enough satisfactory data indicating that *R. salmoninarum* is an obligate in-

tracellular pathogen of salmonid fishes and that the reservoir and carrier of infection are other infected salmonid (Woods and Yasutake, 1956; Fryer and Sanders, 1981; Klontz, 1983; Bullock and Herman, 1988), yet there are few existing data about the possibility that non-salmonids can act as a reservoir or vector for the organism. Few non-salmonid species were able to contract the infection naturally or experimentally and in turn they might become accidental carriers and play an important role in transmission of the disease to salmonid species by cohabitation. For example, Pacific herring (*Clupea harengus pallasi*) living in net pens with *R. salmoninarum* infected coho salmon have been reported as infected (Paclibare *et al*, 1988). Also, Pacific herring (Traxler and Bell, 1988), sablefish (*Anoplopoma fumbria*) (Bell *et al*, 1990), Common shiner (*Notropis cornutus*) (Hicks *et al*, 1986), and the fathead minnow (*Pimephales promelas*) (Hicks *et al*, 1986) were able to contract infection by *i. p.* injection of *R. salmoninarum*. The organism was also detected in moribund Pacific hakes (*Merluccius productus*) (Kent *et al*, 1998). In addition, Greenlings (*Hexagrammos otakii*) and flathead (*Platycephalus indicus*) were also reported as possible vectors for the disease (Sakai and Kobayashi, 1992).

4.3.5 Possible vectors other than fish

A limited number of studies have been conducted in the last two decades that have lead to the assumption that animals other than fish can act as possible vectors for the transmission of *R. salmoninarum* to salmonids. For example, the Japanese scallop (*Patinopecten yessoensis*) has been reported as a possible vector for *R. salmoninarum* transmission to coho salmon pen-raised in the neighboring seawater (Sakai and Kobayashi, 1992).

Some blood-sucking ectoparasites, like salmon lice (*Lepeophteirus salmonis*), can act as vectors for the pathogen. Although, salmon lice can occasionally harbor the pathogen, no record of active transmission of *R. salmoninarum* between sea lice infected and non-infected fish exists (Richards *et al*, 1985; Frerichs and Roberts, 1989).

4.3.6 Reservoirs

Clinically infected, subclinically infected or latent carrier salmonids are the main reservoir of infection (Klontz, 1983; Richards *et al*, 1985, Bullock and Herman, 1988). Bacterial laden-feces and *R. salmoninarum* rich pond sediment can also act as a reservoir of infection (Balfry *et al*, 1996; Austin and Rayment, 1985). In addition, inefficiently pasteurized infected salmon viscera are a confirmed reservoir of infection (Wood, 1974).

5 Diagnosis of BKD

5.1 Isolation and bacteriological identification of the agent

A number of culture media have been successfully used for the primary isolation of *R. salmoninarum* from clinically infected fish. Among these media cysteine blood agar (Ordal and Earp, 1956), KDM2 (Evelyn *et al*, 1977), SKDM (Austin *et al*, 1983) and charcoal agar medium (Daly and Stevenson, 1985) were used with varying degrees of success. The most common drawback of bacterial culture is the slow growing nature of *R. salmoninarum*, which requires up to 12 weeks to achieve bacterial growth. Most recently, Eissa (2005) has suggested a modified KDM medium (MKDM) which enhanced *R. salmoninarum* growth, minimized other bacterial contaminants and ultimately shortened the incubation time to 5–10 days.

The optimal incubation temperature for the isolation of *R. salmoninarum* on culture media is 15 °C (Sanders and Fryer, 1980). The organism is differentiated from other Gram-positive bacteria using the morpho-chemotaxonomic features described by Sanders and Fryer (1980).

5.2 Antigen-antibody reactions

5.2.1 Agglutination test

Although easy and rapid to perform, the test requires that bacteria are first cultured which conveys no advantage if compared with that of other diagnostic methods. Kimura and Yoshimizu (1981) used *Staphylococci* specifically sensitized with antibody against *R. salmoninarum* to develop a coagglutination test to detect *R. salmoninarum* in kidney tissues with limited success.

5.2.2 Immunofluorescence

Direct and indirect fluorescent antibody tests (FAT) have commonly been used to detect *R. salmoninarum* in infected tissues including fixed and paraffin embedded tissues. Bullock and Stuckey (1975) were first to describe the indirect fluorescent antibody technique (IFAT) to visualize the *R. salmoninarum* cells in tissues of infected fish. They concluded that IFAT is more sensitive than Gram stain and can detect the bacteria in subclinical infections. Several methods to quantify *R. salmoninarum* utilizing FAT have been used, including a subjective scoring of fluorescence intensity (1+ to 4+) in tissue smears (Bullock *et al*, 1980). In a later procedure, bacteria are immobilized on filter-paper grids and titers expressed as cells per unit of tissue or ovarian fluid (Elliot and Barila, 1987).

Elliot and McKibben (1997) compared two

fluorescent antibody techniques (FATs) (membrane filtration FAT or MF-FAT and Smear-FAT or S-FAT) for detection of *R. salmoninarum* in ovarian fluid from naturally infected chinook salmon. They reported greater sensitivity of MF-FAT compared to the S-FAT and concluded that MF-FAT was preferable for detection of low numbers of bacteria. Cross reactivity of other bacterial species with antisera prepared against *R. salmoninarum* have been reported (Bullock *et al*, 1980; Austin *et al*, 1985; Brown *et al*, 1995), thus the inclusion of any FAT of control material from *R. salmoninarum*-positive fish is necessary for comparison of cell morphology and staining properties of bacteria in test and control samples (Elliot and McKibben, 1997). Inter-laboratory comparisons revealed that FAT reproducibility is poor when used in detection of very low levels of infection (Armstrong *et al*, 1989).

5.2.3 Enzyme linked immunosorbent assay (ELISA)

Hsu *et al* (1991) developed an improved monoclonal antibody based ELISA for detection of the p57 protein of *R. salmoninarum*. The assay was both specific and sensitive for detection of soluble *R. salmoninarum* antigen at concentrations as low as 50 – 100 ng/ml. A double antibody sandwich ELISA, also known as quantitative ELISA (Q-ELISA), provides accurate indication about the real prevalence of BKD in the tested fish population because it determines both prevalence and intensity of the infection (Pascho *et al*, 1998). The procedures are fairly standardized by the studies of Pascho and Mulcahy (1987) and Pascho *et al* (1991). A positive threshold has been computed and proposed for Q-ELISA results interpretation (Meyers *et al*, 1993; Pascho *et al*, 1998). The positive-negative cutoff absorbance for the kidney homogenate was determined as 0.10. Pascho *et al* (1998) assigned the following antigen level categories for tested positive kidney samples: low (0.10 to 0.19), medium (0.20 – 0.99) and high (1.00 or more).

5.2.4 Immunohistochemistry (IHC)

Hoffmann *et al* (1989) compared various staining techniques (Gram, PAS, IFAT and indirect peroxidase procedures) for their ability to detect *R. salmoninarum* in the tissues of rainbow trout fixed by various methods (Fresh frozen tissue, frozen formalin-fixed tissue, formalin or Bouin's fixed paraffin-embedded tissue) and concluded that only the indirect peroxidase technique gave satisfactory results regardless of the fixation method used. IHC has the advantage of visualizing *R. salmoninarum* and the tissue alteration they cause simultaneously (Jansson *et al*, 1991; Evensen *et al*, 1994). IHC has been used to detect BKD natural and experimental infections. For example, using *in situ* IHC, Lorenzen *et al* (1997) reported the first demonstration of BKD in rainbow trout in Denmark. Evensen *et al* (1994) detected the organism *in situ* by using IHC in paraffin embedded tissue specimens from Atlantic salmon and they reported the use of monoclonal antibodies specific for the *R. salmoninarum* p57 protein. However, it has been reported that prolonged preservation of tissue samples in formalin has very deleterious effect on the antigen detection and retrieval in immunohistochemical assays (Evensen *et al*, 1994). A typical picture of how bacteria and tissue look like after IHC adopted on an infected kidney tissue is indicated in Figure 3 and Figure 4 (Eissa, 2005).

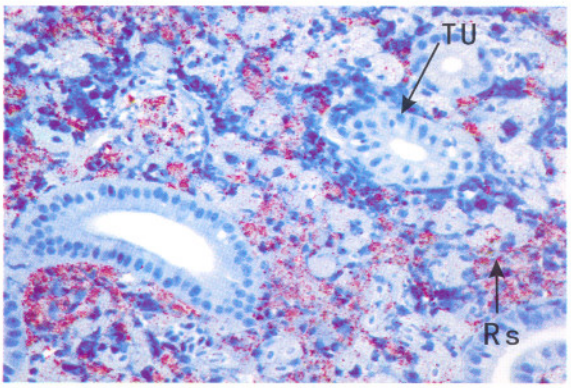

Figure 3. Kidney tissue of Iron River brook trout fingerling exhibiting heavy *R. salmoninarum* infection. Kidney section was stained using an anti-*R. salmoninarum* antibody based streptavidin-immunoperoxidase immmunolbeling (×400). Sections were counterstained with Mayer's hematoxylin (Blue background)
Rs: *R. salmoninarum* soluble antigens with the red staining affinity. Tu: Non-affected kidney tubules with blue counterstaining affinity.

5.3 Polymerase chain reaction (PCR)

PCR has been successfully used to detect *R. salmoninarum* DNA within individual chinook salmon eggs with a detection sensitivity of 2 bacterial cells/egg (Brown *et al*, 1994). A nested PCR (nPCR) has been developed by Chase and Pascho (1998) to amplify a 320 bp fragment of the gene encoding the p57 protein and they recorded no specific fragments amplification when other fish bacterial pathogens were used as templates for nPCR. The sensitivity of the method increased one hundred fold compared to a conventional PCR method (Pascho *et al*, 1998). The authors compared the sensitivities of nPCR, ELISA and FAT assays to detection *R. salmoninarum* in kidneys of infected chinook salmon and concluded that nPCR showed the highest sensitivity (61%), followed by ELISA

(47%) then FAT (43%). Pascho *et al* (1998) reported that nPCR detected *R. salmoninarum* in 100% of the tested ovarian fluid samples and thereby concluded that nPCR was the most accurate and sensitive method for detection of *R. salmoninarum*. Hong *et al* (2002) designed a pair of specific primer for nested amplification of 501 bp and 314 bp DNA fragments of the sequence coding p57 of *R. salmoninarum* respectively and they also recorded no specific fragments amplification when other principal fish bacterial pathogens were used as templates in PCR and nPCR tests. However, Miriam *et al* (1997) have cautioned that PCR positive samples may contain some proportion of dead *R. salmoninarum* with detectable level of DNA. This means that kidney tissues containing non-culturable *R. salmoninarum* can be falsely positive when tested with nPCR. In a recent study, Eissa (2005) used three diagnostic technique namely culture, Q-ELISA and nPCR to detect *R. salmoninarum* in kidney tissues of returning spawners salmon and concluded that nPCR is much more sensitive than the other two methods in discovering the very early infection.

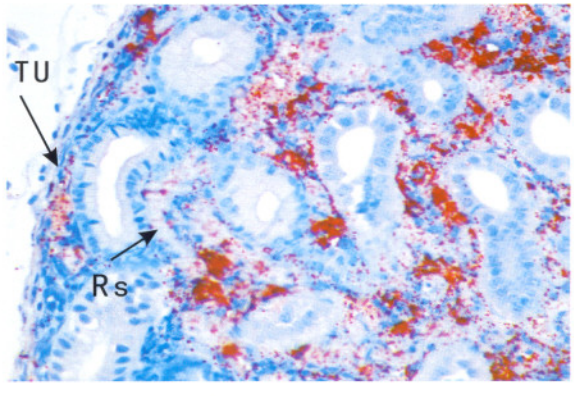

Figure 4. Kidney tissue of Iron River brook trout fingerling exhibiting heavy *R. salmoninarum* infection after enhanced antigen retrieval procedures using alkaline phosphatase red and goat anti-*R. salmoninarum* antibody. Sections were counterstained with Mayer's Hematoxylin (Blue background) (×400). This case is from an outbreak of BKD that killed thousands of hatchery raised Iron River brook trout fingerlings in May 2003.
Rs: *R. salmoninarum* soluble antigens with the red staining affinity. Tu: Non-affected kidney tubules with blue counterstaining affinity.

6 Differential Diagnosis

External manifestations of BKD are non-pathognomonic, but the course of the disease and the granulomatous nature of the kidney lesions may provide presumptive indications. The disease can be differentiated from other kidney diseases of chronic progression including pseudo-kidney disease (*Carnobacterium piscicola*) (Ross and Toth, 1974), nephrocalcinosis (calcium deposits) (Peddie, 2004) and proliferative kidney disease (lymphoid hyperplasia) in response to myxozoan parasite *Tetracapsula bryosalmonae*) (Clifton-Hadley *et al*, 1984). Differentiation is mainly based on observation and detection of the organism or its antigens using immunofluorescence, IHC, ELISA, PCR. In case of nephrocalcinosis, differentiation is mainly based on bacteriological assessment to rule out the presence of the bacterium, however on-farm examination of lesion consistency can help to discriminate between these conditions as BKD lesions are soft whilst those caused by nephrocalcinosis have a gritty texture (Peddie, 2004).

R. salmoninarum can be differentiated from coryneform group of bacteria, which includes the genera of *Listeria*, *Erysipelothrix*, *Corynebacterium*, *Actinomyces*, *Celullomonas*, *Curtobacterium*, *Arthrobacter* and *Brevibacterium* by cell wall composition and G+C contents of DNA (Stuart and Welshimer, 1974; Sanders and Fryer, 1980).

Although *R. salmoninarum* share certain characteristics with *Actinomyces pyogenes* (formerly *Corynebacterium pyogenes*), they differ in a number of other characteristics. *A. pyogenes* is facultatively anaerobic, catalase negative and produce acid from carbohydrates. The genus *Renibacterium* can be separated from pathogenic *Corynebacteria* and genus *Caseobacter* by the presence of lysine in the cell wall and the absence of mycolic acids. The genus *Caseobacter* is further differentiated by a mol % G + C of 60 – 67 (Crombach, 1978). Genus *Celullomonas* contains the diamino acid ornithine in its cell wall peptidoglycan and has a mol % G + C ranging from 65 – 72.

Interestingly some of the coryneform groups of bacteria have an overlapping characteristics and phylogenetic homology. Among this group of bacteria a cell wall peptidoglycan containing lysine occurs primarily in *Arthrobacter* and *Brevibacterium*. DNA homology studies showed close relationship between several species in these two genera. However, these bacteria have usually been isolated from the environment, are chemoorganotrophic, show a progression of morphological changes during the growth cycle and have a mol % G + C above 60. Interestingly, all these characteristics are distinctly different from that of *Renibacterium*.

7 Control

7.1 Chemotherapy

Since the early 1950s a relatively large number of chemotherapeutics have been intensively tested *in vivo* and *in vitro* for efficacy in treating BKD. Rucker *et al* (1951) was first to use antimicrobial agents against clinical BKD and their results showed a definite decrease in mortalities when sulfadiazine was incorporated into fish diets. Although treatment did not completely cure clinically sick fish, sulfamerazine reduced BKD mortalities alone and combined with sulfaguanidine and sulfadiazine (Allison, 1958). Wolf and Dunbar (1959) tested 34 therapeutic agents including erythromycin thiocyanate and sulfamerazine on 16 strains of *R. salmoninarum* using the disk method for drug sensitivity screening followed by *in vivo* feeding trials with experimentally infected fish. They concluded that erythromycin fed at the rate of 100 mg/kg of fish for 5 consecutive days gave the best results. Generally, due to the occurrence of the bacterium intracellularly as well as extracellularly, these treatments only suppressed the systemic spread of the organism and induced partial relief (Amos 1977). Intramuscular (*i.m.*) and *i.p.* administration of sulfonamide drugs significantly reduced prespawning mortality among chinook salmon broodstocks being hold prior to spawning (Amend and Fryer, 1968). However, sulfonamides administered by *i.m.* or *i.p.* routes often produced sterile abscesses at the injection site in adults and induced mortalities and teratogenicity with their progeny (Amos, 1977).

In an attempt to reduce or prevent vertical transmission of BKD, salmon eggs were water hardened for 1 hour in 2 ppm erythromycin (Amos, 1977). However, erythromycin was rapidly eliminated and dropped below detectable level within 24 hours after water hardening (Evelyn *et al*, 1986a). Monthly subcutaneous (*S.C.*) injection of adult female Pacific salmon with 11 mg/kg erythromycin reduced pre-spawning mortality due to BKD (Klontz, 1983). Interestingly, erythromycin remains in the eggs of injected females for up to 60 days before spawning (Evelyn *et al*, 1986; Moffitt, 1991). It is believed that erythromycin residues in the eggs assist in preventing vertical transmission of *R. salmoninarum* from parents to their offspring (Lee and Evelyn, 1994). Detectable amounts of erythromycin often remain in the perfused tissues of both juvenile and adult salmon long after they are no longer detected in the plasma and muscle (Moffitt, 1991; Haukenes and Moffitt, 1999) and this possibly contributes to the efficacy of erythromycin against the slow growing *R. salmoninarum*. Feeding erythromycin can efficiently reduce mortalities of infected hatchery raised salmonids (Wolf and Dunbar, 1959; Austin, 1985; Moffitt and Bjornn, 1989). A dose of 200 mg/kg body weight for 21 days was most effective (Moffitt, 1992). Erythromycin is only available as an Investigational New Animal Drug (INAD) through the Food and Drug Administration (FDA) (Moffitt, 1992).

Austin (1985) tested more than 70 antimicrobial compounds both *in vivo* and *in vitro* and found that clindamycin, erythromycin, kitasamycin, penicillin G and spiramycin were useful for combating early clinical BKD cases while cephradine, lincomycin and rifampicin were effective prophylactically but had limited use therapeutically. Hsu *et al* (1994) tested the efficacy of enrofloxacin in treating BKD *in vitro* and *in vivo* and they concluded that low minimal inhibition concentrations (MICs), high bioavailability and large volume distribution of the antibiotic make it good candidate for use as effective therapeutic against BKD.

7.2 Adult segregation

Broodstock segregation is a more practical method for reducing the prevalence and levels of *R. salmoninarum* in hatchery-reared salmon (Pascho *et al*, 1991) and for increasing survival during their downriver migration and entry into seawater (Pascho *et al*, 1993; Elliot *et al*, 1995). This procedure aims to interrupt vertical transmission of *R. salmoninarum* by isolating or destroying eggs from brood fish that exhibit clinical signs of BKD or test positive, with a high titer, against *R. salmoninarum* antigens. The method is used successfully in a number of U.S states and Canadian provinces such as Washington, Idaho, Michigan, Wisconsin, and Ontario.

7.3 Eradication

Due to the complicated nature of BKD and its obvious threats to fisheries, Hoskins *et al* (1976) recommended complete destruction of the infected stocks and disinfection of the holding facilities to achieve complete eradication of the disease. However, this procedure is considered by fisheries managers as impractical due to the widespread occurrence of *R. salmoninarum* (Sanders and Fryer, 1980).

Eradication can be of value in single fish farms or hatcheries that receive their water supply from specific pathogen free source (European Commission, 1999). Eradication procedures should be followed by standard, cleaning and disinfection procedures. Although some trials have been made to eradicate BKD from fish farmed in open waters (e.g. sea and lake cages) or from farms and hatcheries with water supply from rivers, results were very

discouraging.

After eradication procedures have been applied in the fish farm and hatcheries, restocking should only utilize certified BKD-free stocks. Restocking should be followed by two inspections and laboratory examinations per year for a total period of two years before the facility can be designated as "BKD-free" (European Commission, 1999).

7.4 Prophylaxis

7.4.1 Reducing the risk of BKD introduction

Special attention should be paid to prevent the introduction of infected fish or their gametes (Evelyn *et al*, 1984; Yoshimizu, 1996). This can only be achieved through prior examination and quarantine. Special requirements of water supply, wild birds and amphibians' control. In addition, restriction of movement of vehicle, visitors as well as utensils from infected into free areas is equally important. Repopulation must be accompanied with certificate issued by the competent authority certifying that the fish or eggs are specific pathogen free.

7.4.2 Vaccination

In the last two decades, vaccination against BKD has achieved different levels of success. Paterson *et al* (1981) reported that an inactivated suspension of *R. salmoninarum* mixed 1:1 with Freunds adjuvant (FCA) administered by *i. p.* injection, reduced the level of infection of *R. salmoninarum* in yearling salmon but, did not completely eliminate the infection. Sakai *et al* (1993; 1995) found that although vaccination evoked specific antibodies, these antibodies did not endow with a protection. Piganelli *et al* (1999) demonstrated that oral administration of *R. salmoninarum* expressing low levels of cell associated p57, resulted in an extension of the mean time to death after challenge and they concluded that the protection was not due to humoral antibody. This conclusion supported earlier histopathological indications of an involvement of the cell mediated immune response in recovery, due to intracellular survival and the composition of inflammatory cells in connection with signs of regression (Munro and Bruno, 1988). Rhodes *et al* (2004) presented DNA adjuvants and whole bacterial cell vaccines against *R. salmoninarum* that were tested in chinook salmon fingerlings. These authors concluded that whole cell vaccines of either a nonpathogenic *Arthrobacter* spp. or an attenuated *R. salmoninarum* strain produced limited protection against acute *i. p.* challenge with virulent *R. salmoninarum*. They also concluded that the addition of either synthetic oligodeoxynucleotides or purified *R. salmoninarum* genomic DNA as adjuvants did not increase protection, however a combination of both whole cell vaccines significantly increased survival among fish naturally infected with *R. salmoninarum*. Also, the surviving fish treated with the combination vaccine exhibited reduced levels of bacterial antigens in the kidney.

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